Research Article

Fish Protein Hydrolysate Production by Acid and Enzymatic Hydrolysis

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Received: 14 June 2016; Accepted: 12 July 2016; Published online: 11 November 2016
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Abstract
Fish protein hydrolysates were produced from minced by-catch fish using hydrochloric acid and two protease enzymes of Papain and Alcalase. In order to optimize conditions for production, Taguchi design and Central Composite Design (CCD) were applied for experimental purposes to evaluate the degree of hydrolysis. Response surface methodology was performed in order to determine the optimal production conditions. The optimal condition for acid hydrolysis was 4 mol/L hydrochloric acid at 100°C for 90 minutes, which yielded 50.70% degree of hydrolysis. For enzymatic hydrolysis, Alcalase is more suitable protease enzyme for fish protein hydrolysate production. The optimal condition was 6% (w/w) Alcalase concentration at a temperature of 61.23°C and a reaction time of 27.36 minutes, resulting in 88.90% degree of hydrolysis. Amino acid profiles for fish protein hydrolysates hydrolyzed under optimal conditions were analyzed by HPLC, with the results showing that fish protein hydrolyzed by Papain had the most suitable nutritional properties. Glutamic acid had the highest percentage (16.35%), followed by aspartic acid (10.41%) and lysine (8.48%).

Keywords: Fish protein hydrolysate, Enzymatic hydrolysis, Papain, Alcalase

1 Introduction

During processing of fish products, solid waste is generated from the viscera, head, skin, bones and some muscle tissue, which can account for as much as 70% of the original raw material [1]. Solid waste is not the only concern, as 38.5 million tons of species are discarded globally as by-catch fish due to their low economic value [2], despite the fact that these by-catch fish are considered valuable sources of essential protein. In order to convert low-valued waste into a beneficial and nutritive product, multiple processes have been used to hydrolyze fish protein to protein hydrolysate. A conventional method for hydrolyzing fish protein is to use strong chemicals and solvents. In this process, fish protein is hydrolyzed into peptides of variable molecular weights by using a commonly known hydrochloric acid. The reaction takes place under high temperature (121°C) and high pressure (100 kPa) [3]. Due to the harsh conditions used for acid hydrolysis, reaction times are usually reduced in order to save production cost. Gao *et al*. reported that the most efficient process for fish protein hydrolysate production involves using 6M hydrochloric acid at 121°C for 20 minutes [4].

The method is not absent of disadvantages, which include poor functionality, off-flavour, and high traces of the solvent in the final product, factors making it commercially unsuccessful [5]. Hydrolysis by proteolytic enzymes is a method that uses mild

Please cite this article as: N. Wisuthiphaet, S. Klinchan, and S. Kongruang, "Fish protein hydrolysate production by acid and enzymatic hydrolysis," *KMUTNB Int J Appl Sci Technol*, vol. 9, no. 4, pp. 261–270, Oct.–Dec. 2016.

conditions and increases utilization of fish protein hydrolysis due to the better nutritional properties of the final product [6]. Even though enzymatic hydrolysis needs longer reaction time and requires higher production cost, the fish protein hydrolysate obtained would be more marketable and valuable [7]–[12]. There were several protease enzymes used to hydrolyze fish protein. Further, protease enzymes from plants and microorganisms are considered more suitable for protein hydrolysates production [1], [12]–[14]. Among these enzymes, there are two well-known commercially hydrolyzing proteases. One is Alcalase, an alkaline bacterial protease enzyme produced from *Bacillus licheniformis*, which is widely used in fish protein hydrolysate production [1], [8], [13]–[15]. Bhaskar *et al*. optimized production conditions for protein hydrolysate from the visceral waste proteins of fish by Alcalase enzyme using a Response Surface Methodology (RSM) with a factorial design. The results indicated that an enzyme to substrate level of 1.5% (v/w), pH 8.5, temperature of 50°C and hydrolysis time of 135 minutes were found to be the optimal conditions for obtaining a degree of hydrolysis close to 50% [13]. Another well-known commercial hydrolyzing protease is Papain, a thiol protease extracted from the latex of *Carica papaya*. It has been widely used in the food industry [16], beer clarification [17], meat tenderizing, preparation of protein hydrolysate and other uses [18]. Abdulazeez *et al*. studied the production of protein hydrolysate from king fish by Papain enzyme. The results showed that DH was observable at 24.7% for enzyme substrate ratio of 4:100 at 37°C for 6 hours. [19]. Based on previous studies, DH obtained from enzymatic hydrolysis was quite low. Consequently, extending the reaction time could lead to higher DH.

Several factors including the type of acid and enzyme concentration, reaction time, temperature and pH influence the efficiency of hydrolysis reaction. In order to obtain the maximum DH, Response Surface Methodology (RSM) was used to determine the ideal production conditions. RSM is one of the most effective tools for optimization of the process when there are many factors and interactions that affect the response [20]. The main advantage of RSM is that it reduces the number of experimental trials necessary to evaluate multiple parameters and their interactions [21], [22]. It is usually used in combination with an experimental design such as a Central Composite Design (CCD) to fit a first- or second- order polynomial by least significance technique. The contour plots can be applied to study the response surfaces and determine the optimal point. Another experimental design used in this study is the Taguchi method, which is a statistical method initially developed by Genichi Taguchi to improve the quality of products manufacturing and eventually applied for use in engineering [23] and biotechnology research as well [24], [25].

The objectives of this study were to determine the optimal conditions for the hydrolysate production process of fish protein by acid Hydrolysis (hydrochloric acid), Papain and Alcalase using RSM in combination with the Taguchi method and Central Composite Design experimentation, as well as to evaluate the amino acid composition of fish protein hydrolysate produced under such conditions.

2 Materials and Methods

2.1 *Materials and enzymes*

Low-valued marine fish including Ponyfish (*Eubleekeria splendens*), Yellow-striped Trevally (*Selaroides leptolipis*) and Mackerel (*Decapterus maruadsi*) were purchased at a local fish market in Samut Sakhorn, Thailand. Each kind of fish was washed and mixed in equal proportion, then minced completely into a homogeneous paste. The resulting raw material was packed in plastic bags for immediate storage at –20°C until use. The composition of the raw material derived from fish is shown in Table 1. The two endoprotease enzymes used in this study were Papain, which is derived from papaya (*Carica papaya*), and Alcalase, which is a bacterial enzyme from *Bacillus licheniformis*. Both were purchased from EMD Millipore, USA and stored at 4°C until used. The acid used in this study was Hydrochloric acid (HCl), purchased from QRëCTM, New Zealand.

Table 1: The composition of the raw material derived from fish

Composition	g/100g sample
Moisture	79.67
Protein	15.69
Lipid	2.56
Ash	3.51

2.2 *Experimental design and statistical analysis*

In order to study the efficiency of the acid hydrolysis reaction, acid concentration and reaction temperature were varied into three levels, as shown in Table 2. For enzymatic hydrolysis, three factors that influence the efficiency of the hydrolysis reaction, including temperature, enzyme concentration, and reaction period were studied by varying them into three levels, as shown in Table 3. Before any experimentation was conducted, design of the experiments was performed according to the Taguchi method and Central Composite Design (CCD). Response surface methodology was analyzed statistically. Degree of hydrolysis as a response of the factors can be explained by the following quadratic equation:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n-1} \sum_{j=2}^{n} \beta i_j X_i X_j + \sum_{i=1}^{n} \beta i_i X_i^2$$

Where Y is a response (degree of hydrolysis), X_i, X_j are levels of factors, β_0 is a constant, β_i, β_{ii} and β_{ij} are coefficients obtained through multiple regression analysis. The responses were analyzed statistically by analysis of variance (ANOVA).

Experimental design and statistical analysis in this study were completed by Design-Expert software Version 9 (STAT-EASE Inc., Minneapolis, MN, USA).

Table 2: Levels for each factor that affects the efficiency of fish protein hydrolysate production by acid hydrolysis

Factor	Level		
	1	2	3
A: Concentration (M)	4	6	8
B: Temperature (°C)	80	100	120

Table 3: Levels for each factor that affects the efficiency of fish protein hydrolysate production by protease enzymes

Factor	Level		
	1	2	3
A: Concentration (%w/w)	2	4	6
B: Temperature (˚C)	40	60	80
C: Time (hour)	5	10	15

2.3 *Preparation of fish protein hydrolysate*

The raw material from fish was thawed at room temperature, after which 20 g of raw material was adjusted to pH 7 in 250 mL Erlenmeyer flask. Papain and Alcalase were added at 2%, 4% and 6% (w/w). The temperature was studied at 40°C, 60°C and 80°C. Flasks were shaken at 200 rpm for 5, 10 and 15 hours. In order to end the reaction, the mixture was heated at 90°C for 15 minutes. For acid hydrolysis, HCl with concentrations of 2, 4 and 6 M were used to hydrolyze the raw material. The temperature was set at 80, 100 and 120°C using an autoclave. The reactions were terminated by adjusting pH value to 5 using 6 M NaOH. Fish protein hydrolysate was filtered to remove any solid residue and then centrifuged. The clear solution was collected and stored at –20°C for further analysis. All experiments were carried out in duplicate.

2.4 *Degree of Hydrolysis (DH)*

DH of the fish protein hydrolysate was analyzed according to the trinitro-benzene-sulfonic acid (TNBS) method [26], Two mL of 0.2125 M phosphate buffer pH 8.2 and 2 mL of 0.1% TNBS solution were added to 0.25 mL of fish protein hydrolysate then incubated at 50°C for 1 hour. After that, 5 mL of 0.1 M HCl was added to terminate the reaction. The mixture was analyzed by a spectrophotometric method measuring absorbance at a wavelength of 340 nm. Free α-amino acid was obtained using the standard curve of leucine and the DH was calculated using Equation (1):

$$DH = [(L_t - L_0) / (L_{max} - L_0)] \times 100 \quad (1)$$

Where, L_t is the amount of α-amino acid of fish protein hydrolysates hydrolyzed for t hours, L_0 is the amount of α-amino acid of the raw material fish and L_{max} is the amount of α-amino acid of fish protein hydrolysate completely hydrolyzed by 8 M HCl at 100°C for 24 hours.

2.5 *Amino acid composition and chemical score*

The amino acid (AA) components were evaluated by High-Performance Liquid Chromatography according to previously described methods [27]. Chemical score is a value used to evaluate the nutritional properties of protein and can be calculated by using the following formula [28].

$$Chemical\ score = \frac{EAA\ in\ sample}{EAA\ in\ standard\ protein}$$

Where EEA in a sample is the essential amino acid (g/100g

sample) and EEA in the reference protein is the essential amino acid in standard protein (g/100g sample) [12].

3 Results and Discussion

3.1 *Optimization for fish protein hydrolysate production conditions*

According to the factors and levels identified in Tables 2 and 3, Design Expert program generated all experimental conditions based on Taguchi method, which resulted in 9 experimental conditions for both acid and enzymatic hydrolysis. Fish protein hydrolysates were produced under these conditions and DH was measured according to TNBS method. The DH for fish protein hydrolysates is shown in Tables 4–6.

From the results, ANOVA method was used to determine the factors that significantly affected DH, with temperature being the only factor that affected DH when hydrolyzed by acid ($p < 0.05$).

Table 4: DH of fish protein hydrolysate when hydrolyzed by hydrochloric acid

No.	Factors		DH (%)
	Acid Concentration (M)	Temperature (°C)	
1	6	100	45.65
2	4	100	50.70
3	6	120	33.12
4	8	120	45.77
5	8	100	37.51
6	8	80	14.60
7	6	80	15.15
8	4	80	9.68
9	4	120	36.24

Table 5: DH of fish protein hydrolysate when hydrolyzed by Papain

No.	Factor			DH (%)
	Concentration (% w/w)	Temperature (°C)	Time (hour)	
1	4	60	15	66.21
2	4	40	10	34.33
3	6	40	15	85.09
4	6	80	10	88.53
5	4	80	5	55.49
6	2	40	5	29.20
7	6	60	5	87.15
8	2	60	10	56.09
9	2	80	15	48.56

Table 6: DH of fish protein hydrolysate when hydrolyzed by Alcalase

No.	Factor			DH (%)
	Concentration (% w/w)	Temperature (°C)	Time (hour)	
1	6	40	15	81.69
2	2	40	5	34.95
3	2	80	15	36.43
4	4	40	10	40.90
5	6	60	5	81.98
6	4	80	5	43.42
7	6	80	10	72.78
8	4	60	15	41.81
9	2	60	10	35.58

For enzymatic hydrolysis, concentration was the only factor that exhibited significant effect on DH ($p < 0.05$). Reduced quadratic models were created as Equation 2 for acid hydrolysis, Equation (3) for enzymatic hydrolysis by Papain and Equation (4) for enzymatic hydrolysis by Alcalase. Only the affected factors appeared in the equations.

$$DH = -31.37 + (0.63*Temperature) \quad : R^2 = 0.9126 \tag{2}$$

$$DH = -8.88 + (10.58*Concentration) : R^2 = 0.7882 \tag{3}$$

$$DH = 9.01 + (10.79*Concentration) \quad : R^2 = 0.8442 \tag{4}$$

The regression coefficient (R^2) indicated that the model was suitable for representation of the relationship between variable and response. Nilsang *et al*. optimized the conditions for production of fish protein hydrolysate from fish-soluble concentrate using two commercial protease enzymes, Flavozyme and Kojizyme. The models obtained fit the experimental data with an acceptable determination coefficient (Flavourzyme; $R^2 = 0.8316$ and Kojizyme; $R^2 = 0.8079$) [7]. Kangrang *et al*. optimized the conditions for biogas production using response surface methodology. The model was obtained with a regression coefficient (R^2) of 0.7661, which indicated that it was suitable for representing the relationship among the studied variables [29].

Response surface models were developed in order to illustrate the trend of DH affected by various factors. Figure 1 shows the effects of acid concentration

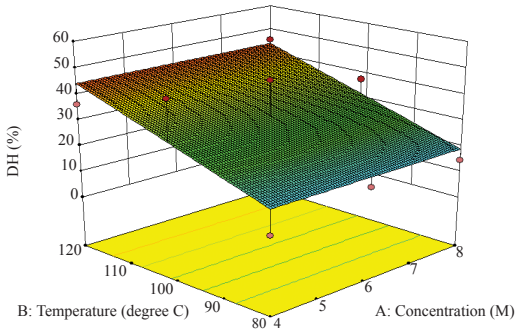

Figure 1: Response surface for DH obtained by acid hydrolysis as a function of different concentration and temperature.

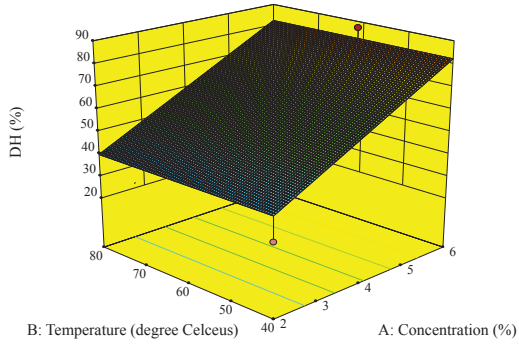

Figure 2: Response surface for DH obtained by Papain as a function of different concentration and temperature.

and temperature on DH of fish protein hydrolysate hydrolyzed by acid. The results showed that temperature was the only factor that affected DH ($p = 0.0008$), with an increase in temperature resulting in increased DH. The highest value for DH was in the range of 40–50% when the temperature was 120°C, while an increase in acid concentration made no change in DH. Figures 2–3 illustrate the response surface that shows the effects of temperature and enzyme concentrations on DH of fish protein hydrolysates produced by enzymes. For both Papain and Alcalase, the only factor that significantly affected DH was the concentration of enzyme, with the p-value of 0.0066 and 0.0002, respectively. The results showed that DH increased as the enzyme concentration increased, while changes in temperature and reaction time had no significant effect on DH.

For acid hydrolysis, the optimal conditions needed to hydrolyze fish protein with 4 M hydrochloric acid

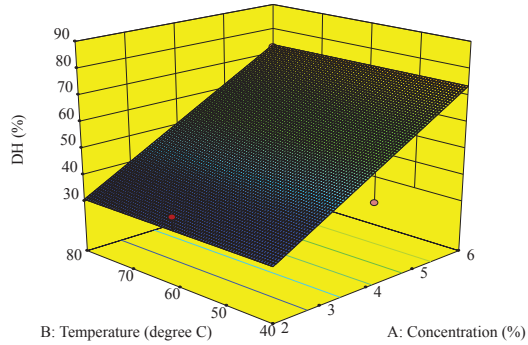

Figure 3: Response surface for DH obtained by Alcalase as a function of different concentration and temperature.

included a temperature of 100°C and a reaction time of 90 minutes. Under these conditions, predicted DH was 43.59%. For enzymatic hydrolysis, the optimal conditions were 6% w/w of enzyme at 40°C for 5 hours. Using Papain resulted in 86.92% DH, while predicted DH value when using Alcalase was 78.82%. Fish protein hydrolysates were reproduced under these optimal conditions in order to validate the predicted values.

Validated DH value for fish protein hydrolysate are shown in Table 7. Fish protein hydrolysate produced by 4M concentration of hydrochloric acid at 100°C for 90 minutes was 50.70% (16.31% error).

Table 7: Optimal conditions, predicted DH value and validated DH value of fish protein hydrolysate

Predicted Optimal Conditions	DH (%)		% Error
	Predicted Value	Validated Value	
4M hydrochloric acid at 100°C for 90 minutes	43.59	50.70	16.31
6% (w/w) of papain at 40°C for 5 hours	86.92	88.53	1.85
6% (w/w) of alcalase at 40°C for 5 hours	78.82	81.98	3.89

From the validated value, acid hydrolysis resulted in high percentage error because the chemical reaction is difficult to control for product quality due to its harsh reaction and non-specific peptide bonds cleaving [30]. For enzymatic hydrolysis, using Papain under the selected optimal conditions, including 6%w/w of Papain at 40°C for 5 hours, gave a DH value of 88.53% (1.82% error), while Alcalase gave a DH value of 81.98% (3.89% error). However,

enzymatic hydrolysis seems to be more suitable for fish protein hydrolysis since acid hydrolysis has several drawbacks that make it inappropriate from an industrial aspect. Acid hydrolysis has been found to cause racemization, which converts L-form amino acid to D-form amino acid and cannot be utilized by humans or animals [31]. Further, some essential amino acids such as tryptophan and cysteine will be diminished during the reaction [32]. Moreover, neutralized fish protein hydrolysate from acid hydrolysis generates a high amount of salt in a downstream process, which could affect the nutritional properties of the products. Therefore, enzymatic hydrolysis is considered to be a more appropriate process for the production of fish protein hydrolysate. The results shown in Table 7 indicate that Papain gave higher DH value. However, the purpose of this research was not limited to determining the optimal conditions, but included considering the possibility of applying the conditions of the process in the aquaculture feed industry.

Table 8: Comparison of the enzyme cost for 1kg of fish protein hydrolysate production with 6%w/w of enzyme

Enzyme	Retail Price ($/L)	Retail Price ($/1000U)	Unit Activity of Enzyme Used in 1 kg FPH	Enzyme Cost ($) for 1 kg FPH
Papain (EMD Millipore, USA, 30000 U/mg)	2,739	9.13×10^{-5}	1.80×10^{9}	164.34
Alcalase (EMD Millipore, USA, 2.590 U/ml, Density = 1.166g/ml)	272	105.02	133.26	13.99

Therefore, the cost of the enzymes is an important factor that requires further evaluation. Table 8 shows the cost for both Papain and Alcalase enzymes required to produce 1 L of fish protein hydrolysate. Even though the Papain used in this study has higher activity, 6% w/w of Papain (30,000U/mg) has the ability to hydrolyze fish protein to a similar level of 6%w/w of Alcalase (2.590 U/ml); Papain gave an estimated 10% higher DH, but the cost of Papain for 1L of fish protein hydrolysate production is 10 times more expensive than for Alcalase. Among commercial protease enzymes used at the industrial level, Alcalase tends to exhibit lower cost per unit for enzyme activity compared to other enzymes [30]. Moreover, Alcalase offers several advantages, including a wide variety of available catalytic activity [33]. Fish protein hydrolysate from Alcalase also has less bitter components than those of Papain [15]. Therefore, Alcalase was used in further experiments with optimization for fish protein hydrolysates production carried out by Central Composite Design, which is an efficient technique for experimentally exploring the relationships between investigated factors and system response [34]. It demands a smaller number of experiments while providing comparable results [35]. According to the preliminary experiments, the concentration of enzymes was kept constant at 6% w/w. The reaction time was reduced in order to lessen the production cost due to its insignificant effect on the degree of hydrolysis when using hour-long reaction times. However, not only do concentration, time and temperature potentially affect the degree of hydrolysis, environmental factors such as pH can also greatly affect the enzyme reaction kinetics. The effect of these factors is different for each enzyme [30].

In subsequent study, the effect of pH, temperature and time were varied in 3 levels. Table 9 shows the selected upper and lower limits: pH range from 6 to 10, temperature from 50 to 70 °C and time from 10 to 30 minutes.

Table 9: Levels of factors that affect the efficiency of fish protein hydrolysis by Alcalase enzyme

Factor	Level		
	1	2	3
A: pH	6	8	10
B: Temperature (°C)	50	60	70
C: Time (minute)	10	20	30

According to ANOVA analysis, reaction time, temperature and pH had a significant effect on DH ($p < 0.05$). Moreover, the interaction between the different factors significantly influenced DH ($p < 0.05$). The following quadratic model explains the effects of the factors on DH:

$$DH = -840.52 + (3.79*Time) + (20.97*Temp) + (56.70*pH) + (0.017*Time*Temp) - (0.047*Time*pH) - (0.25*Temp*pH) - (0.25*Temp*pH) - (0.082*Time2) - (0.16*Temp^2) - (2.36*pH^2) : R^2 = 0.8465$$

Figure 4 shows the effect of temperature and reaction time on DH. The results showed that DH increased as time and temperature increased. However, a decreasing trend was observed at temperatures above 61°C. Higher temperatures tend to deactivate the enzymes used, resulting in lower DH being achieved [36].

This result was supported by a study on threadfin bream protein hydrolysate production, in which 60°C was determined as the optimal temperature [39]. The results of this study were slightly higher than those in a study on the hydrolysis of Catla visceral waste protein, where the optimal temperature obtained was 55°C [13]. DH also increased as reaction time increased. DH reached its maximum value (88.90%) when the optimal temperature was reached and began to decrease after 27 minutes of reaction. Therefore, the optimal condition for hydrolyzing fish protein was using 6% w/w of Alcalase enzyme at 61.23°C, pH 8 for 27.36 minutes.

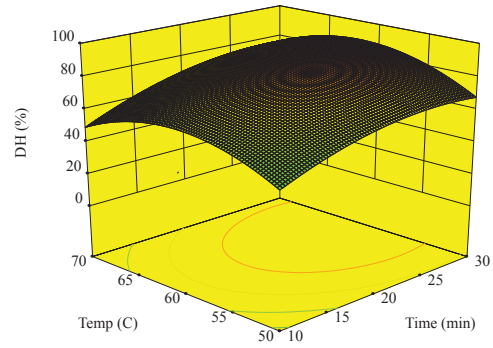

Figure 4: Response surface for degree of hydrolysis obtained by Alcalase as a function of different concentrations and temperatures.

3.2 *Amino acids composition and chemical score*

The amino acids composition and chemical score for fish protein hydrolysate were analyzed by HPLC. The results are provided in Table 10. From the results,

Table 10: Amino acids composition of fish protein hydrolysate and its chemical score in comparison with FAO/WHO and NRC reference proteins

	Quantity (g/100g sample)					Chemical Score					
	Amino Acid Composition of Fish Protein Hydrolysate			Reference Protein 1[a]	Reference Protein 2[b]	Acid		Papain		Alcalase	
	Acid	Papain	Alcalase			RP-1	RP-2	RP-1	RP-2	RP-1	RP-2
Essential amino acid											
Histidine	0.111	0.284	0.162	2.00	2.10	0.06	0.05	0.14	0.14	0.08	0.08
Isoleucine	0.170	0.584	0.408	4.00	2.50	0.04	0.07	0.15	0.23	0.10	0.16
Leucine	0.380	0.861	0.693	7.00	3.30	0.05	0.12	0.12	0.26	0.10	0.21
Lysine	0.409	0.892	0.466	5.50	5.70	0.07	0.07	0.16	0.16	0.08	0.08
Methionine	0.186	0.419	0.316	3.50	3.10	0.05	0.06	0.12	0.14	0.09	0.10
Phenylalanine	0.238	0.621	0.474	4.29	6.50	0.06	0.04	0.15	0.10	0.11	0.07
Tyrosine	0.138	0.193	0.099	-	-	-	-	-	-	-	-
Threonine	0.202	0.555	0.156	4.00	3.90	0.05	0.05	0.14	0.14	0.04	0.04
Tryptophan	0	0.154	0	1.21	0.80	0.00	0.00	0.13	0.19	0.00	0.00
Arginine	0.254	0.241	0.147	5.00	1.31	0.05	0.19	0.05	0.18	0.03	0.11
Valine	0.243	0.857	0.458	5.42	3.60	0.04	0.07	0.16	0.24	0.08	0.13
Non-essential amino acid											
Alanine	0.493	0.674	0.756								
Aspartic acid	0.497	1.095	0.841								
Cystine	0.163	0.211	0.193								
Glycine	0.435	0.539	0.555								
Glutamic acid	0.781	1.72	1.336								
Proline	0.404	0.364	0.458								
Serine	0.168	0.258	0.09								

[a] Reference protein 1: Essential amino acid of reference protein according to FAO/WHO (1985) [37]
[b] Reference protein 2: Essential amino acid requirement of common carp according to NRC (1993) [38]

glutamic acid was the most abundant amino acid present in all the samples, which was similar to several previous studies showing that, among all amino acids, the levels of aspartic acid and glutamic acid were found to be higher in most of the reported fish protein hydrolysates [1], [13], [40]–[42]. Fish protein hydrolysates produced by Papain had the highest amino acid content. Tryptophan was absent in fish protein hydrolysate produced by acid and Alcalase, but present in that of Papain. Bhaskar *et al*. studied the amino acid composition of protein hydrolysate prepared from the visceral waste proteins of Catla. Their results indicated that methionine was the most limiting amino acid [13]. In order to evaluate the nutritional properties of protein hydrolysate, the chemical score was calculated based on two standard proteins. The results indicated that the nutritive value of fish protein hydrolysates are less than both standard proteins and cannot fulfill the minimum requirement of 30% for common carp diets. However, fish protein hydrolysate for industrial use will be evaporated in order to concentrate the protein. Therefore, concentrated protein hydrolysates could lead to higher amino acid content and offer higher nutritive value.

4 Conclusions

Alcalase was found to be the efficient enzyme for production of fish protein hydrolysates. The optimal conditions were determined to be 6% w/w of alcalase enzyme at 61.23°C, pH 8 for 27.36 minutes. Fish protein hydrolysis produced by enzymatic hydrolysis affords better nutritional properties than those produced by acid hydrolysis. With further research, the production of fish protein hydrolysates by Alcalase enzymes could be economically suitable for industrial application.

Acknowledgments

The author would like to thank the Science and Technology Research Institute, King Mongkut's University of Technology North Bangkok for financial support of this research project (KMUTNB-NEW-59-10) and Inteqc Feed Co., Ltd. for the raw material.

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