FOOD

RESEARCH

Optimization of enzymatic hydrolysis conditions on the degree of hydrolysis of edible bird's nest using Alcalase® and nutritional composition of the hydrolysate

*Amiza, M.A., Oon, X.X. and Norizah, M.S.

School of Food Science and Technology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia

Abstract

This study aimed to determine the optimum enzymatic hydrolysis conditions for edible bird's nest (EBN) using Alcalase® in order to achieve maximum degree of hydrolysis (DH). Four parameters namely temperature (45-65°C), pH (7.5-9.5), Alcalase® to substrate concentration (1-2%) and hydrolysis time (60-180 mins) were employed at three levels using a face-centered central composite design (CCD) by using RSM. The optimum conditions for hydrolysis of EBN were at the temperature of 64.99°C, Alcalase® concentration of 2%, hydrolysis time of 179.55 min and pH of 9.46. The suggested model was a 2-factor interaction (2FI) model. Under the optimum conditions, the predicted value for DH (37.90%) was close to the experimental data (37.92%). EBN hydrolysate comprised of 67.63±0.78% protein, 2.62±0.37% ash, 0.61±0.30% fat and 29.14±0.11 carbohydrate. EBN contained all essential amino acids except for tryptophan (which was not quantifiable using the method in this study), and their levels were higher than the suggested profile of essential amino acid requirements for adults, except for methionine. EBN hydrolysate also contained 34.04% hydrophobic amino acid.

1. Introduction

Edible bird's nest (EBN) is made from the saliva of swiftlet species, *Aerodromus fuciphagus*. Swiftlets may stay in natural caves or man-made swiftlet house. EBN has become an important cuisine and pharmacy since the beginning of Tang (618-907 AD) dynasty as it is high in medical values with properties of anti-aging, anti-cancer, immune-enhancing, dissolving phlegm, suppressing cough and others (Ma and Liu, 2012).

Traditionally in Chinese culture, EBN drink is a type of delicate food prepared by double boiling the EBN with rock sugar. However, double boiled EBN has low solubility. Thus, enzymatic protein hydrolysis may help to increase EBN solubility and thus maximize its functionality, digestibility and bioactivity. EBN hydrolysate may offer more convenient and marketable value-added ingredients for food and nutraceutical application. Nowadays, EBN is marketed as raw cleaned nest, ready to drink soup, health supplement and cosmeceutical products.

Protein hydrolysis is commonly used to modify physicochemical and bioactivity properties of EBN. Marcone (2005) reported that glycoprotein is the main component in EBN. Guo *et al.* (2006) have found that the enzymatic hydrolysis of EBN by Pancreatin F hydrolyzed glycoprotein to glycopeptides that have the ability to inhibit influenza virus. Matsukawa *et al.* (2011) reported that hydrolysis of EBN by Pancreatin F consisted of water-soluble compound like chondroitin glycosaminoglycans (GAG) which is the main component in the bone that can prevent osteoporosis. Epidermal growth factors are also present in EBN and EBN treated with HCl (Kong *et al.*, 1987; Rashed and Nazaimoon, 2010). Furthermore, hydrolysis of EBN using Alcalase® has resulted in angiotensin-I converting enzyme (ACE)-inhibitory activity (Amiza *et al.*, 2014; Babji *et al.*, 2018), while enzymatic hydrolysis of EBN has also resulted in antioxidative activity (Ghassem *et al.*, 2017; Babji *et al.*, 2018).

Enzymatic hydrolysis by commercial exogenous food-grade enzymes is more favourable due to better control in terms of properties of the hydrolysates such as the length of the peptide and resulted in desirable and consistent products. The four main factors that affect the enzymatic hydrolysis process are types of enzyme, pH, temperature and time (Bhaskar and Mahendrakar, 2008). In addition, pH and temperature are mainly depending on the type of enzyme as different enzymes have their own range of pH and temperature where they are active.

*Corresponding author.
Email: *ama@umt.edu.my*

According to Ovissipour, Abedian, Motamedzadegan *et al.* (2009), hydrolysis time and enzyme concentration will affect the degree of hydrolysis (DH) where higher DH will result in longer hydrolysis time. Thus, optimization of enzymatic hydrolysis of EBN protein is important to control the enzymatic hydrolysis process and as a basis to understand the structure-function relationship in EBN. Alcalase® is one of the most effective commercial enzymes to prepare food protein hydrolysis (Shahidi *et al.*, 1995). Previous study has been reported on the optimization of enzymatic hydrolysis of various foods including dark muscle tuna (Saidi *et al.*, 2013); palm kernel cake protein (Ng *et al.*, 2013); visceral waste proteins of *Catla catla* (Bhaskar *et al.*, 2008); pine seed protein (Wang *et al.*, 2011); and fish soluble concentrate (Nilsang *et al.*, 2005). It will be interesting to know how hydrolysis of glycoprotein in EBN differs from other food proteins.

Response Surface Methodology (RSM) is a collection of statistical and mathematical technique used in the study of optimization in order to solve multivariate problem, graphically represent as response surface (Raissi and Farsani, 2009). Based on the experimental data, RSM may be used to fit and predict a mathematical model to explain the relationship between the experimental variables and its response as well as to determine the optimum condition for a specific response.

Thus, this study aimed to optimize the enzymatic protein hydrolysis conditions on the DH of EBN using Alcalase®. Besides, the proximate analysis and amino acid composition of EBN hydrolysate prepared under optimum condition were determined as well.

2. Materials and methods

2.1 Raw material

EBN was purchased from a swiftlet farm in Gong Badak, Terengganu. The raw material purchased was in raw uncleaned form. The raw uncleaned EBN was stored in a plastic container at room temperature until further use. The food grade commercial enzyme used for in this study was Alcalase® 2.4 L in liquid form (2.4 AU/g), purchased from Novo Industry (Denmark). All other chemical reagents used were of analytical grades.

2.2 Preparation of raw material

Prior to protein hydrolysis, the raw uncleaned bird's nest was cleaned by soaking them in water until the nest cement is softened and the tightly bound laminae partially loosen. After that, it was rinsed many times with water to remove its contaminants (guano, cracked eggs, dust) and feathers. Next, the small feather and fine plumage were removed manually by using fine tips

tweezers and with the aid of magnifying lens. The cleaned bird's nest was then kept in a container and stored in freezer (-20°C) until further use.

2.3 Determination of proximate analysis of EBN sample

The raw cleaned EBN and the lyophilized EBN hydrolysate prepared under optimum condition were subjected to proximate analysis according to AOAC method (AOAC, 2000). Prior to optimization study, protein content of raw cleaned EBN is needed to calculate the mass of EBN, water and enzyme needed for each run of enzymatic hydrolysis (Kristinsson and Rasco, 2000).

2.4 Optimization study of EBN hydrolysis

2.4.1 Experimental design for optimization study

There are four independent variables involved in this optimization study. The independent variables are temperature (°C), enzyme to substrate concentration (E/S, %v/w), hydrolysis time (mins) and pH was employed at equidistant level (-1, 0 and +1) as shown in Table 1. The ranges of the independent variables used were chosen based on the previous study on optimization of enzymatic hydrolysis from silver catfish (*Pangasius* sp.) frame by using Alcalase® (Amiza *et al.*, 2011). Degree of hydrolysis (DH) was set as a dependent factor or response variable in this study.

The experimental design used in this study was a face-centered central composite design. A total of 30 runs of protein hydrolysis of EBN using various combinations of independent variables were automatically generated by Design Expert software (Version 6.0.10) (Stat Ease Inc.). There were 6 center points set in this experimental design. After each run, the resulting hydrolysate was freeze-dried. Then, the lyophilized powder was subjected to determination of DH. The data on DH was further analyzed using Design Expert software (Version 6.0) (Stat Ease Inc.).

2.4.2 Enzymatic hydrolysis of EBN

Firstly, the frozen cleaned raw EBN was thawed. Then, the mass of the raw material and water was calculated as described by Kristinsson and Rasco (2000). Enzymatic hydrolysis of EBN was employed as described by Amiza *et al.* (2014). For each run, the EBN sample was added to distilled water and homogenized using homogenizer (IKA® T18 basic, IKA Works Inc, USA) at 10,000 rpm for 2 mins. Prior to enzymatic hydrolysis, the homogenized EBN was boiled for half an hour in a water bath (TE-10D, Techne®, UK) and allowed to cool to room temperature afterward. After cooling, the temperature and the pH of the solution were adjusted (amount of pH added included in the mass of

Table 1. Experimental range and values of the independent variables used in the optimization of protein enzymatic hydrolysis conditions on the degree of hydrolysis of edible bird's nest

Source	Sum of Squares	df	Mean of Square	F value	p-value Prob>F
Model	853.53	5	170.71	21.56	<0.0001 significant
A - Temperature	216.67	1	216.67	27.36	<0.0001
B - Enzyme concentration	113.65	1	113.65	14.35	0.0009
C - pH	124.29	1	124.29	15.70	0.0006
D- Hydrolysis time	361.98	1	361.98	45.71	<0.0001
AD	36.94	1	36.94	4.66	0.0410
Residual	190.04	24	7.92		
Lack of Fit	160.24	19	8.43	1.42	0.3744 not significant
Pure Error	29.80	5	5.96		
Cor Total	1043.58	29			

water needed for hydrolysis), before the addition of enzyme. After that, an Alcalase® enzyme solution was added to the homogenize EBN and the hydrolysis process was carried out using water bath shaker (Shaker bath-903, Protech®, UK) at the specific temperature and time as designated by Response Surface Methodology. After the hydrolysis process was completed, the enzyme was inactivated at 85°C for 20 mins in the water bath. The resultant mixture was freeze-dried, and the lyophilized hydrolysate sample was crushed into powder and kept in an airtight container until further analysis.

2.4.3 Determination of degree of hydrolysis

Determination of DH was carried out using trichloroacetic acid (TCA) method (Hoyle and Merritt, 1994). Total nitrogen content was determined by analyzing 0.5 g of freeze-dried EBN powder using Kjeldahl method. As for the determination of 10% TCA soluble nitrogen, 0.5 g of freeze-dried EBN powder was added to 10 mL of distilled water. Then, 10 mL of 20% (w/v) TCA was mixed with the sample. The sample was left to stand for 30 mins for precipitation. Later, the sample was centrifuged (High speed centrifuges model 1580R, Gyrozen Co., Ltd, Korea) at 4000 rpm for 15 mins. The resulting supernatant was filtered and analyzed using Kjeldahl method (AOAC, 2000). DH is defined as the percentage ratio between the number of peptide bonds cleaved and the total number of peptide bonds in the sample (Adler-Nissen, 1979). DH was computed as follows:

$$\% DH = \frac{10\% \, TCA \, soluble \, N \, in \, the \, sample}{Total \, N \, in \, the \, sample} \times 100$$

2.4.4 Verification for optimum condition

After optimization, the suggested solution for the optimum condition was obtained. From the optimum condition, enzymatic hydrolysis of the suggested condition was carried out in quadruplicate. Then, the degree of hydrolysis of lyophilized hydrolysate resulted from the hydrolysis were determined. The predicted

value given by Response Surface Methodology (RSM) was then compared with the experimental value obtained using one sample t-test.

2.5 Determination of amino acid composition

Amino acid composition of the lyophilized EBN hydrolysate was analyzed using amino acid analyzer (L-8800 Hitachi) (Guo *et al.*, 2007). Two types of hydrolysis were carried out i.e. 6N HCl and performic acid. Only 17 types of amino acids are quantifiable using this method, excluding tryptophan. There is a limitation of the acid hydrolysis method, whereby cysteine, methionine and tryptophan could not be accurately quantified due to partial oxidation of cysteine and methionine, and complete destruction of tryptophan. Apart from that, deamidation of asparagine and glutamine to aspartic acid and glutamic acid, respectively, will lead to a mixture quantification for asparagine with aspartic acid and glutamine with glutamic acid. To quantify tryptophan, alkaline hydrolysis is needed, and it is not carried out in this study.

2.6 Data analysis

All data was stated as mean ± standard deviation. For optimization study, the experimental run and data analysis were carried out by using the Design Expert software Version 6.0.10 (Stat Ease Inc., USA). Analysis of variance of the RSM model was carried out using Design Expert software (version 10.0.7) (StatEase Inc., USA). The experimental data from the experiments were fitted into an empirical second-order polynomial using steepest accent method according to the following equation:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i 2 + \sum \beta_{ij} X_i X_j$$

Here, Y is the response variable. β_0 is the offset term. β_i , β_{ii}, and β_{ij} are the linear, quadratic, and interaction regression coefficient variables, respectively. While X_i

and Xj are experimental factors. For the models calculated from the linear regression, analysis of variance (ANOVA) was performed and the R^2 value, residual error, pure error and lack of fit were calculated. Optimization was carried out as described in Design Expert tutorial (https://www.statease.com/docs/v11/tutorials/multifactor-rsm.html#response-surface-design-and-analysis). Mean values were accepted as significantly different at 95% level (p<0.05). Model reduction was carried out by eliminating model terms with p value>0.05. Mean values were accepted as significantly different at 95% level (p< 0.05). The mean of experimental DH and predicted DH under the optimum condition were compared using one-sample t-test (IBM SPSS v. 20).

3. Results and discussion

3.1 Optimization of enzymatic protein hydrolysis of EBN using Alcalase®

3.1.1 Experimental data for optimization study

Response surface methodology was used to optimize the protein hydrolysis of EBN using Alcalase®. The independent variables used in this study were temperature, pH, hydrolysis time and Alcalase® concentration. Degree of hydrolysis (DH) was set as an independent variable in order to measure the cleavage of the peptide bond in the EBN. DH of EBN ranged from 14.27-37.89%. The highest DH (37.89%) was obtained at the temperature of 65°C, pH of 9.5, hydrolysis time of 60 mins and enzyme concentration of 2%. Meanwhile, the lowest DH (14.27%) was obtained at 45°C, pH of 7.5, hydrolysis time of 60 mins and enzyme concentration of 1%

The range of DH obtained in this study is in similar range to those of EBN hydrolysis using Protamex® (13.79- 33.26%) (Amiza *et al*., 2019) and visceral waste protein of beluga sturgeon (*Huso huso*) hydrolysis using Alcalase® (11.28-30.87%) (Ovissopour *et al*., 2009b). However, it was higher compared to those of Grass carp skin hydrolysis which ranged from 1-15.2% (Wasswa *et al*., 2008) and lower compared to salmon skin hydrolysate which ranged from 43.98-74.54% (See *et al*., 2011). The difference in DH obtained between these studies could be due to the difference in protein source used, the type of enzyme used as well as the different range of parameters applied during enzymatic hydrolysis that may promote proteolysis. Different protein source will have different types of protein and amino acid composition which may lead to different proteolytic susceptibility, thus resulting in different DH. Different enzymes will work differently during proteolysis depending on their preferred amino acid to be cleaved during proteolysis. For example, okara protein

hydrolysates produced by Alcalase® and Flavourzyme™ gave DH values of 33.6% and 5.8%, respectively, indicating that Alcalase® cleaved more peptide bonds than Flavourzyme™ (Sbroggio *et al*., 2016). However, Bamdad *et al*. (2011) reported that Flavourzyme hydrolysis (100 LAPU/g protein) of hordein was relatively more extensive than the hydrolysis by Alcalase (0.24 AU/g protein), which resulted in lower DH values. Thus, in order to maximize the DH, we need to optimize the enzymatic hydrolysis condition for a particular protein source.

3.1.2 Analysis of variance (ANOVA) for degree of hydrolysis

ANOVA table is used to summarize the test for significance of regression models, test for significance for individual model coefficient and test for lack of fit (Kohli and Singh, 2011). In this study, Design Expert software has suggested two-factor interaction (2FI) model for the EBN hydrolysis using Alcalase®. Then, the model reduction was performed to further improve the model. The model reduction was carried out by excluding all insignificant model terms (i.e. model terms with p value>0.05). Table 2 shows the ANOVA table of Response Surface Reduced 2FI model for degree hydrolysis. It is important to examine the fitted model to ensure adequate approximation is provided to the true system. Table 2 shows the model F-value of 21.56, which implies that the model is significant. There is only a 0.01% chance that a 'Model F-Value' could occur due to noise. The "p-value" for "Lack of Fit" of 1.42 implies that the "Lack of Fit" is not significant relative to the pure error. There is a 37.44 % chance that a "Lack of Fit-F value" could occur due to noise. The non-significant "Lack of Fit" is good in optimization study because it shows how well the model fits well with the experimental data. The coefficient of determination (R^2) of 0.8179 was in good agreement with the adjusted R^2 of 0.7800. The ANOVA table shows that all linear model terms (pH, temperature, enzyme concentration, hydrolysis time) and one interaction model term (AD, which was interaction between temperature and pH) affected the DH significantly.

Table 2. ANOVA table of Response Surface Reduced 2FI model for degree of hydrolysis

Independent variables	Symbol	Range and levels		
		-1	0	1
Temperature (°C)	A	45	55	65
Enzyme concentration (E/S, %v/w)	B	1.0	1.5	2.0
Hydrolysis time (min)	C	60	120	180
pH	D	7.5	8.5	9.5

R^2 =0.8179; Adjusted R^2 =0.7800

Based on the sequential model sum of squares, the appropriate models were selected based on the highest-order polynomial where the additional terms were significant. The experimental result in this study was evaluated and the model equation for DH of EBN as a function of four independent variables was generated.

The final equation in terms of coded factors given by the Design Expert software was:

Degree of Hydrolysis = + 23.54 + 3.47 * A + 2.51* B + 2.63* C + 4.48* D + 1.52* A*D

Where, A = Temperature; B = Enzyme Concentration; C = Hydrolysis time; and D = pH.

For the final equation in terms of coded factors, the higher the coefficient of estimation for a particular term, the stronger the effect of the term towards DH. The positive sign of the coefficient of estimation for all factors (A, B, C and D) shows that each individual factor gave linear effect which resulted in increased DH. For linear effect, DH was most affected by pH (D)> temperature (A) > hydrolysis time (C) > enzyme concentration (B). While for AD, it also gave an increasing effect on DH.

In this study, the model suggested for enzymatic protein hydrolysis of EBN is a two-factor interaction (2FI) model. However, most of the previous studies suggested quadratic models to predict the DH of an enzymatic protein hydrolysis. For example, optimization of DH from EBN using Protamex® (Amiza *et al.*, 2019), visceral waste protein of beluga sturgeon *Huso huso* (Ovissipour, Taghiof, Motamedzadegan *et al.*, 2009), grass carp (*Ctenopharyngodon idella*) skin (Wasswa *et al.*, 2007), silver catfish (*Pangasius* sp.) frame (Amiza *et al.*, 2011), Salmon (*Salmo salar*) skin (See *et al.*, 2011), tuna dark muscle by-product (Saidi *et al.*, 2013) and threadfin bream (*Nemipterus japonicus*) (Normah *et al.*, 2005). The difference in the model between this study and the above-mentioned protein sources could be due to the same reasons as mentioned previously for differences in DH.

3.1.3 Response surface plot and effect of factor for degree of hydrolysis

Figure 1 shows the contour plots that represented a function of two factors which are temperature and pH and the other factors such as enzyme concentration and time was hold at optimum level. The figure shows that the DH increased with an increased in temperature and pH. In this study, pH and temperature were maximum at its maximum points. This study was in an agreement with the hydrolysis of Grass carp skin using Alcalase® (Wasswa *et al.*, 2008) where an increase of DH during

was achieved by increasing the temperature to 60°C and pH 9. Besides that, enzymatic hydrolysis of silver catfish (*Pangasius sp.*) frame (Amiza *et al.*, 2011) also found that DH was increased with pH. The optimum pH of silver catfish (*Pangasius* sp.) frame was pH 9.5.
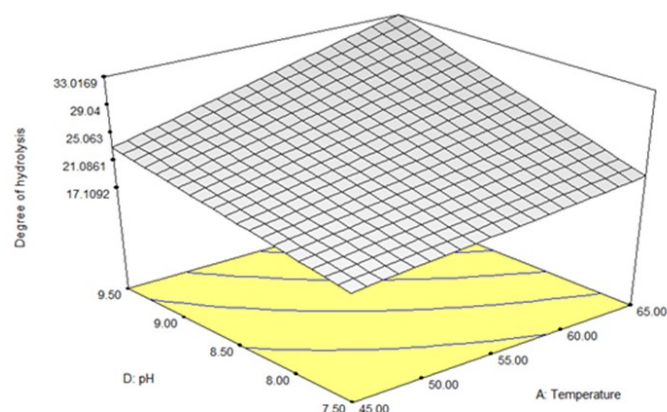

Figure 1. Response surface plot of degree of hydrolysis (%) of EBN hydrolysate as a function of temperature (°C) and pH during enzymatic hydrolysis of EBN using Alcalase®

In this study, the optimum condition for EBN in terms of DH was highest at pH at 9.5. This is because Alcalase® showed a broad range of activity in alkaline pH. The optimum pH range for Alcalase® to function is ranged from pH 6 to pH 10 (Aspmo *et al.*, 2005). pH can affect both the substrate and enzyme by changing the distribution and confirmation of the molecules at very acidic or alkaline condition. Enzyme tends to undergo irreversible denaturation and loss of stability (Benjakul and Morrisey, 1997). A sharp decrease in Alcalase® activity at pH 11.5 was reported by Benjakul and Morrisey (1997).

Besides that, the protein present in EBN was glycoprotein that is a combination of carbohydrate and protein. According to Roussel *et al.* (1988), glycoprotein found in EBN contain hundreds of carbohydrate chains attached to the peptide by O-glycosidic linkages between N-acetylgalactosamine and a hydroxylated amino acid made from serine and threonine. The O-glycosidic linkages are alkaline labile (Shylaja and Seshadri, 1989). This may explain why in alkaline condition (pH 9.5), EBN gave the highest DH. The breakage of O-glycosidic linkages enhanced the protein hydrolysis by Alcalase® enzyme.

Furthermore, the optimum condition for EBN in terms of DH was highest at the temperature of 65°C. Benjakul and Morrisey (1997) have found that above 70° C, enzyme activity decrease due to thermal irreversible denaturation of enzyme. This finding is in an agreement with Baek and Cadwallader (1995) whereby they also reported that the optimum temperature of Alcalase® on crayfish processing by-product was at the temperature of

70°C. Thus, the range of temperature and pH should be increased in order to get the curvature ideally within the midpoint of the two factors.

3.1.4 Optimization of degree of hydrolysis

For optimization purposes, the importance of all four independent variables in this study was set at 3 or (+ + +). While the response variable of this study (DH) was considered the most importance by setting the goal to the maximum and the "Importance" has been set as 5 or (+ + + + +).

After this limitation was set, the optimized test was evaluated by Design Expert Software version 6.0 and the 10 best solutions of optimization conditions for further evaluation were shown in Table 3. The desirability of the 10 optimization conditions was closed to 1. The desirability function brings the meaning of "goodness" of a specific setting. If the desirability is close to 1, this indicates a desirable set of various response (Vining and Kowalski, 2011). Solution No. 1 was selected, giving the optimum condition at the temperature of 64.99°C, Alcalase® concentration of 2%, hydrolysis time of 179.55 min and pH of 9.46. The optimum conditions obtained in this study is in agreement with that of protein enzymatic hydrolysis of blood cockles whereby the optimum conditions were at temperature of 65°C, enzyme concentration of Alcalase® at 2%, hydrolysis time at 180 mins and pH at 9.5 (Amiza and Masitah, 2012). However, it is quite different compared to that of EBN hydrolysis using Protamex® (Amiza *et al.*, 2019) which were at the temperature of 59.9°C, pH of 6.3, enzyme concentration of 2% and hydrolysis time of 5.4 hr (or 324 mins). This could be due to the different enzymes used to hydrolyze EBN, whereby Alcalase is active in alkaline region (6.5-8.5) and Protamex® is active in neutral region (pH 5.5-7.5). The only similarity between the optimum condition in these two EBN studies is the optimum enzyme concentration at 2%. The optimum condition for EBN hydrolysis using Alcalase® is at a shorter time, but higher pH and temperature as compared to Protamex®.

3.1.5 Verification of optimum conditions

By using the optimum condition at the temperature of 64.99°C, Alcalase® concentration of 2%, hydrolysis time of 179.55 min and pH of 9.46., the predicted value of the response variable (DH) was 37.90%. To validate the model, four replicates of EBN hydrolysis were carried out under optimum condition as suggested by this study. A one sample t-test showed that there was no significant difference between the experimental value (37.92%) and the predicted value (37.90%) ($p < 0.05$). This shows that the equation fitted well with the experimental data in this study.

3.2 Proximate analysis of EBN and protein hydrolysate

Proximate analysis was carried out on soaked cleaned raw EBN and lyophilized EBN hydrolysate prepared under optimum condition. The EBN hydrolysate was prepared using the hydrolysis conditions that gave the highest DH based on experimental data at the temperature 64.99°C, enzyme concentration of Alcalase® at 2%, hydrolysis time at 179.55 min and pH at 9.46. Table 4 shows the proximate analysis of the soaked cleaned raw EBN and freeze-dried EBN hydrolysate (dry basis).

The lyophilized EBN hydrolysate has lower moisture content (3.85%) as compared to the moisture contained in soaked raw EBN (91.26%). The huge differences in moisture content are due to uncleaned raw EBN was soaked in water up to 12 hours to expand so that the nest cement is softened, and tightly bound laminae were partially loosen. During soaking, the raw EBN absorbed water and result in high moisture content. According to Severin and Xia (2005), the differences in moisture content may arise due to the varying efficiency of freeze-drying and storage conditions.

For a fair comparison, both EBN samples should be converted to dry basis (Table 4). In dry basis, protein gave the highest composition in both cleaned raw EBN (70.55%) and lyophilized hydrolysate powder (67.63%). According to Ma and Liu (2012) and Marcone (2005), the protein content of dry EBN (in wet basis) was in the range of 42% to 63%. This shows that the protein content of EBN samples in this study was slightly higher compared to the previous study by Ma and Liu (2012) and Marcone (2005). The difference in protein content could be due to differences in the source of EBN, cleaning extent and moisture content between the samples.

It was found that the ash content in the cleaned EBN was higher than EBN hydrolysate which was 11.59% and 2.52%, respectively. The ash content in EBN hydrolysate was lower than raw EBN. The ash content in EBN hydrolysate was in reasonable agreement with Ma and Liu (2012) that was in the range of 2.1% to 7.3%, but it was a bit higher for raw EBN. The pH of cooked EBN was around pH 7. Thus, NaOH was added to adjust the pH to alkaline range prior to enzymatic hydrolysis. Severin and Xia (2005) and Benjakul and Morrissey (1997) have reported that the addition of NaOH in conjunction with the adjustment of pH before enzymatic hydrolysis caused an increased in ash content. However, in this study, the increase in ash content due to NaOH addition was not observed. This loss of mineral/ash in

Table 3. Solutions for optimization conditions of EBN hydrolysis to obtain maximum degree of hydrolysis

No.	Temperature	Enzyme Concentration	Hydrolysis Time	pH	Desirability	
1	64.99	2.00	179.55	9.46	1.000	Selected
2	64.99	2.00	179.04	9.47	1.000	
3	65.00	2.00	174.57	9.50	1.000	
4	65.00	1.92	180.00	9.50	0.993	
5	65.00	2.00	173.64	9.46	0.988	
6	63.38	2.00	180.00	9.50	0.976	
7	57.68	1.99	180.00	9.50	0.854	
8	62.91	2.00	103.32	9.50	0.825	
9	64.44	1.07	180.00	9.50	0.802	
10	64.62	1.03	180.00	9.50	0.797	

Table 4. Proximate analysis of soaked raw edible bird's nest and lyophilized edible bird's nest hydrolysate prepared at optimum condition

	Moisture content (%)	Ash (%)	Protein (%)	Fat (%)	Carbohydrate (%)
Soaked raw edible bird's nest (wet basis)	91.26±0.22	1.02±0.00	6.19±0.27	0.48±0.08	1.25±0.28
Edible bird's nest hydrolysate powder (wet basis)	3.85±0.06	2.53±0.36	65.03±0.71	0.59±0.30	28.39±0.64
Soaked raw edible bird's nest (dry basis)	-	11.59±0.33	70.55±1.33	5.43±1.02	12.43±0.31
Edible bird's nest hydrolysate powder (dry basis)	-	2.62±0.37	67.63±0.78	0.61±0.30	29.14±0.11

Mean ±SD (n=2)

raw materials could be due to leaching of mineral from soaked cleaned raw EBN during rinsing and storage.

The fat content (dry basis) in soaked clean raw EBN (5.43%) was higher compared to EBN hydrolysate (0.62%). Previous studies on EBN has reported that the fat content in EBN was in the range of 0.14-1.28% (Marcone, 2005; Ma and Liu, 2012). This shows that the fat content in cleaned raw EBN was quite high compared to the previous study. However, the fat content in the EBN hydrolysate is consistent with the previous study. This discrepancy could be due to loss of some fat from the raw EBN during storage, heat treatment, hydrolysis and freeze-drying process. Heating treatment carried out before enzymatic hydrolysis (cooking for half an hour) and after enzymatic hydrolysis (85°C for 15 mins) may help to remove fat from raw materials (Bhaskar *et al.*, 2008). Low-fat content is desirable because it will increase the stability of the hydrolysate towards lipid oxidation (Ovissipour, Taghiof, Motamedzadegan *et al.*, 2009).

Lastly, the carbohydrate content of raw EBN (12.43%) was lower compared to EBN hydrolysate (29.14%). The values are consistent with the previous study by Ma and Liu (2012) and Marcone (2005), whereby the carbohydrate content of EBN ranged from 10.63% to 27.26%. Since carbohydrate content is calculated by the difference method, it is directly affected by other proximate composition of the EBN.

3.3 Amino acid composition in lyophilized EBN hydrolysate

Table 5 shows the amino acid composition of lyophilized EBN hydrolysate (stated in percentage). The total amount of amino acid i.e. 60.17±1.03% was quite close to the protein content of EBN hydrolysate of 65.03% (wet basis). Table 5 shows that there were 16 types of amino acids found in EBN hydrolysate using 6N HCl hydrolysis and performic acid oxidation. Thus, tryptophan is expected to be absent in the amino acid composition in this study. Marcone (2005) reported 15 types of amino acids present in EBN while Babji *et al.* (2018) reported 18 amino acids in EBN and EBN hydrolysate. The difference in the EBN amino acid composition in these studies is mainly due to the difference in preparation methods prior to HPLC analysis. Acid hydrolysis with HCl will result in 15 types of amino acids to be quantifiable by HPLC, because cysteine, methionine and tryptophan will be destroyed in the acid hydrolysis. Further treatment with performic acid oxidation will enable another 2 amino acids to be quantifiable i.e. cysteine and methionine. To quantify tryptophan, alkaline hydrolysis has to be carried out as well. Marcone (2005) only reported 15 types of amino acids because he only performs acid hydrolysis, while this study reported 16 types of amino acid because we performed acid hydrolysis and performic acid oxidation (cysteine supposed to be quantifiable, but not detected in our EBN hydrolysate sample). Meanwhile, Babji *et al.* (2018) reported 18 types of amino acid because they performed acid hydrolysis, performic acid oxidation and alkaline hydrolysis (cysteine and tryptophan were present in their EBN sample). Furthermore, the difference in amino acid composition between these studies could be due to differences in the composition of EBN obtained from different locations and in the degree of cleanliness of EBN from contaminants (such as fine

Table 5. Amino acid composition of lyophilized edible bird's nest hydrolysate prepared at optimum condition as compared to those of other study

[a]Amino acid	*Content (g/100g)			
	Edible bird's nest hydrolysate (this study)	Edible bird's nest (Babji *et al.,* 2018)	Edible bird's nest hydrolysate (Babji *et al.,* 2018)	FAO/WHO[b]
Essential amino acid				
Threonine	8.18±0.18	4.70±0.254	6.93±0.017	0.7
Valine	9.11±0.03	5.29±0.002	7.81±0.108	1
Methionine	0.95±0.16	0.63±0.081	1.18±0.256	1.3
Isoleucine	3.49±0.02	5.52±0.288	3.17±0.056	1
Leucine	7.45±0.02	8.47±0.086	7.00±0.021	1.4
Phenylalanine	5.61±0.02	4.34±0.314	6.54±0.010	1.4
Lysine	4.19±0.09	9.11±0.546	5.21±0.677	1.2
Histidine	3.41±0.02	2.25±0.073	3.43±0.494	1.2
Tryptophan	Not quantifiable	1.23±0.009	1.43±0.067	
Non-essential amino acid				
Serine	12.19±0.29	3.84±0.008	9.27±0.030	
Glutamic acid	6.57±0.06	15.70±0.946	7.09±0.026	
Glycine	6.86±0.01	4.13±0.774	4.34±0.494	
Alanine	3.85±0.15	6.34±0.071	2.92±0.546	
Tyrosine	4.53±0.40	4.56±0.347	6.66±0.167	
Aspartic acid	9.71±0.06	10.39±0.110	10.07±0.001	
Arginine	5.17±0.09	6.15±0.347	6.29±0.305	
Proline	8.74±0.08	4.75±0.378	7.43±0.163	
Cysteine	-	2.60±0.148	3.23±0.157	

a Values are % of amino acids expressed as mean±SD (n=2); b Suggested profile of essential amino acid requirements for adults (FAO/WHO, 2007); *Tryptophan was not analyzed in this study

plumage, guano and eggshell). The habitat of swiftlets will affect the types of feed that they consume and may result in different protein content, which may indirectly affect the types of amino acid present on it (Norhayati *et al.*, 2010).

Table 4 shows that the amino acid content in EBN hydrolysate in this study has a similar trend with that of Babji *et al.* (2018). This could be due to both hydrolysates were hydrolyzed using Alcalase®. As shown in Table 4, EBN hydrolysate contained 8 types of essential amino acids needed for adults except for tryptophan (which was not quantifiable using the method in this study). In fact, except for methionine, the levels of all other essential amino acid presented in EBN hydrolysate were higher than the suggested profile of essential amino acid requirements for adults (FAO/WHO, 2007). Thus, the essential amino acid in EBN fulfilled this requirement and it can be suggested as a good source essential amino acid for nutritional value (Vidotti *et al.*, 2003). The highest composition of essential amino acid in EBN was valine (9.11%) while the highest composition of non-essential amino acid in EBN was serine (12.19%) which is similar to the previous study reported by Marcone (2005) and Babji *et al.* (2018).In EBN hydrolysate, the hydrophobic amino acids are valine (9.11%), leucine (7.45%), isoleucine (3.49%), phenylalanine (5.61%), alanine (3.85%) and tyrosine (4.53%), accounted for 34.04%. Since

hydrophobic amino acid is always associated with the presence of antioxidative and ACE inhibitory peptides, this indicates that EBN hydrolysate is a potential source of antioxidant and ACE inhibitory peptides to be explored.

4. Conclusion

In conclusion, the optimum condition for enzymatic protein hydrolysis of EBN was at the temperature of 64.99°C, Alcalase® concentration of 2%, pH of 9.46 and hydrolysis time of 179.55 mins. The experimental DH obtained under this optimum condition (37.92%) was close to the predicted value (37.90%). The suggested model for enzymatic protein hydrolysis of EBN was two-factor interaction (2FI) model. The proximate composition of lyophilized EBN hydrolysate in dry basis consisted of 67.63±0.78% protein, 2.62±0.37% ash, 0.61±0.30% fat and 29.14±0.11 carbohydrate. The EBN hydrolysate contained all essential amino acids except tryptophan (which was not quantifiable using the method in this study) and their levels were higher than the suggested profile of essential amino acid requirements for adults (FAO/WHO, 2007), except for methionine. EBN hydrolysate contained 34.04% hydrophobic amino acid.

Conflict of Interest

Acknowledgements

References

Amiza, M.A. and Masitah, M. (2012). Optimization of enzymatic hydrolysis of blood cockle (*Anadara granosa*) using Alcalase®. *Borneo Science*, 31, 1-8.

Amiza, M.A., Khuzma, D. and Kee, C.H. (2019). Optimization of enzymatic hydrolysis condition of edible bird's nest using Protamex to obtain maximum degree of hydrolysis. *Asian Journal of Agriculture and Biology*, 7(1), 1-9

Amiza, M.A., Nurul Ashikin, S. and Faazaz, A.L. (2011). Optimization of enzymatic protein hydrolysis from silver catfish (*Pangasius* sp.) frame. *International Food Research Journal,* 18, 775-781.

Amiza, M.A., Sai, J.Y. and Sarbon, N.M. (2014). Optimization of enzymatic hydrolysis conditions on angiotensin converting enzyme (ACE) inhibitory activity from edible bird's nest. Proceedings of International Conference on Food Innovation 2014 (INNOVA2014). Penang. 27-28 August 2014. Malaysia: Unversiti Sains Malaysia

AOAC. (2000). Official methods of analysis. 17th ed. Washington: Association of Official Analytical Chemist.

Aspmo, S.I., Horn, S.J. and Eijsink, V.G.H. (2005). Hydrolysates from Atlantic cod (*Gadus morhua L.)* viscera as components of microbial growth media. *Process Biochemistry*, 40(12), 3714-3722. https://doi.org/10.1016/j.procbio.2005.05.004

Babji A.S., Etty Syarmila, I.K., NurAliah, D., Nurul Nadia, M., Hadi Akbar, D., Norrakiah, A.S., Ghasssem, M., Najafian, L. and Salma, M.Y. (2018). Assessment of bioactive components of hydrolysed edible bird nest. *International Food Research Journal,* 25(5), 1936-1941.

Baek, H.H. and Cadwallader, K.R. (1995). Enzymatic hydrolysis of crayfish processing by-product. *Journal of Food Science,* 60(5), 929-935. https://doi.org/10.1111/j.1365-2621.1995.tb06264.x

Bamdad, F., Wu, J. and Chen, L. (2011). Effects of enzymatic hydrolysis on molecular structure and antioxidant activity of barley hordein. *Journal of Cereal Science,* 53(1), 20-28. https://doi.org/10.1016/j.jcs.2011.01.006

Benjakul, S. and Morrissey, M.T. (1997). Protein hydrolysates from Pacific whiting solid wastes. *Journal of Agricultural and Food Chemistry*, 61 (1/2), 131-138. https://doi.org/10.1021/jf970294g

Bhaskar, N., Benila, T., Radha, C. and Lalitha, R.G. (2008). Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (*Catla catla*) for preparing protein hydrolysate using a commercial protease. *Bioresource Technology*, 99(2), 335-343. https://doi.org/10.1016/j.biortech.2006.12.015

FAO/WHO. (2007). Protein and amino acid requirements in human nutrition. Report of a joint WHO/FAO/UNU Expert Consultation. Switzerland: World Health Organization.

Ghassem, M., Arihara, K., Mohammadi. S., Sani, N.A. and Babji, A.S. (2017). Identification of two novel antioxidant peptides from edible bird nest (*Aerodramus fuciphagus*) protein hydrolysate. *Food and Function*, 8(5), 2046-2052. https://doi.org/10.1039/C6FO01615D

Guo, C-T., Takahashi, T., Bukawa, W., Takahashi, N., Yagi, H. Kato, K., Hidari, K.I.P.J., Miyamoto, D., Suzuki, T. and Suzuki, Y. (2006). Edible bird's nest extract inhibits influenza virus infection. *Antiviral Research,* 70(3), 140-146. https://doi.org/10.1016/j.antiviral.2006.02.005

Guo, L-Q., Lin, J-Y. and Lin, J-F. (2007). Non-volatile components of several novel species of edible fungi in China. *Food Chemistry,* 100(2), 643-649. https://doi.org/10.1016/j.foodchem.2005.09.087

Hoyle, N. and Merritt, J.H. (1994). Quality of fish protein hydrolysates from herring. *Journal of Food Science*, 59(1), 76-79. https://doi.org/10.1111/j.1365-2621.1994.tb06901.x

Kohli, A, and Singh, H. (2011). Optimization of processing in induction hardening using response surface methodology. *Indian Academy of Sciences*, 36(2), 141-152. https://doi.org/10.1007/s12046-011-0020-x

Kong, Y.C., Keung, W.M., Yip, T.T., Ko, K.M., Tsao, S.W.T. and Ng, M.H. (1987). Evidence that epidermal growth factor is present in swiftlet's (*Collocalia*) nest. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 87 (2), 221-226. https://doi.org/10.1016/0305-0491(87) 90133-7

Kristinsson, H.G. and Rasco, B.A. (2000). Kinetics of the hydrolysis of Atlantic salmon (*Salmo salar*) muscle proteins by alkaline proteases and a visceral serine protease mixture. *Process Biochemistry*, 36(1-2), 131-139. https://doi.org/10.1016/S0032-9592(00)

00195-3

Ma, F. and Liu, D. (2012). Sketch of the edible bird's nest and its importance bioactivities. *Food Research International*, 48(2), 559-567. https://doi.org/10.1016/j.foodres.2012.06.001

Marcone, M.F. (2005). Characterization of the edible bird's nest the "Caviar of the East". *Food Research International*, 38(10), 1125-1134. https://doi.org/10.1016/j.foodres.2005.02.008

Matsukawa, N., Matsumoto, M., Bukawa, W., Chiji, H., Nakayama, K., Hara, H. and Tsukahara, T. (2011). Improvement of bone strength and dermal thickness due to dietary edible bird's nest extract in ovariectomized rats. *Bioscience Biotechnology and Biochemistry*, 73(3), 590-592. https://doi.org/10.1271/bbb.100705

Ng, K.L., Ayob, M.K., Said, M., Osman, M.A. and Ismail, A. (2013). Optimization of enzymatic hydrolysis of palm kernel protein (PKCP) for producing hydrolysates with antiradical capacity. *Industrial Crops and Products*, 43, 725-731. https://doi.org/10.1016/j.indcrop.2012.08.017

Nilsang, S, Lertsiri, S., Suphantharika, M. and Assavanig, A. (2005). Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial protease. *Journal of Food Engineering*, 70(4), 571-578. https://doi.org/10.1016/j.jfoodeng.2004.10.011

Norhayati, M.K., Azman, O. and Wan Nazaimoon W.M. (2010). Preliminary study of the nutritional content of Malaysian edible bird's nest. *Malaysian Journal of Nutrition*, 16(3), 389-396.

Normah, I., Jamilah, B., Saari, N. and Yaakob, B.C.M. (2005). Optimization of hydrolysis conditions for the production of threadfin bream (*Nemipterus japonicas*) hydrolysate by Alcalase. *Journal of Muscle Foods*, 16(2), 87-102. https://doi.org/10.1111/j.1745-4573.2005.07404.x

Ovissipour, M., Abedian, A., Motamedzadegan, A., Rasco, B., Safari, R. and Shahiri, H. (2009). The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from Persian sturgeon (*Acipenser persicus*) viscera. *Food Chemistry*, 115(1), 238-242. https://doi.org/10.1016/j.foodchem.2008.12.013

Ovissipour, M., Taghiof, M., Motamedzadegan, A., Rasco, B. and Molla, A.E. (2009). Optimization of enzymatic hydrolysis of visceral waste proteins of beluga sturgeon *Huso huso* using Alcalase. *International Aquatic Research*, 1, 31-38.

Raissi, S. and Farsani, R-E. (2009). Statistical process optimization through multi-response surface methodology. *International Journal of Mathematical and Computational Sciences*, 3(3), 197-201.

Rashed, A.A. and Nazaimoon, W.M. (2010). Effect of edible bird's nest on Caco-2 cell proliferation. *Journal of Food Technology*, 8(3), 126-130. https://doi.org/10.3923/jftech.2010.126.130

Roussel, P., Lamblin, G., Lhermitte, M., Houdret, N., Lafitte, J-J., Perini, J-M., Klein, A. and Scharfman, A. (1988). The complexity of mucins. *Biochimie*, 70 (11), 1471-1482. https://doi.org/10.1016/0300-9084(88)90284-2

Saidi, S., Belleville, M-P., Deratani, A. and Amar, R.B. (2013). Optimization of peptide production by enzymatic hydrolysis of tuna dark muscle by-product using commercial proteases. *African Journal of Biotechnology*, 12(13), 1533-1547.

Sbroggio, M.F., Montilha, M.S., Figueiredo, V.R.G., Georgetti, S.R. and Kurozawa, L.E. (2016). Influence of the degree of hydrolysis and type of enzyme on antioxidant activity of okara protein hydrolysates. *Food Science and Technology*, 36(2), 375-381. https://doi.org/10.1590/1678-457X.000216

See, S.F., Hoo, L.L. and Babji, A.S. (2011). Optimization of enzymatic hydrolysis of Salmon (*Salmosalar*) skin by Alcalase. *International Food Research Journal*, 18(4), 1359-1365.

Severin, S. and Xia, W.S. (2006). Enzymatic hydrolysis of whey proteins by two different proteases and their effect on the functional properties of resulting protein hydrolysates. *Journal of Food Biochemistry*, 30(1), 77-97. https://doi.org/10.1111/j.1745-4514.2005.00048.x

Shahidi, F., Han, X.Q. and Synowiecki, J. (1995). Production and characteristics of protein hydrolysates from capelin. *Food Chemistry*, 53, 285-293. https://doi.org/10.1016/0308-8146(95)93934-J

Shylaja, M. and Seshadri, H.S. (1989). Glycoproteins: an overview. *Biochemical Education*, 17(4), 170-178. https://doi.org/10.1016/0307-4412(89)90136-2

Vidotti, R.M., Viegas, E.M.M. and Careiro, D.J. (2003). Amino acid composition of processed fish silage using different raw materials. *Animal Feed Science and Technology*, 105, 199-204. https://doi.org/10.1016/S0377-8401(03)00056-7

Vining, G.G. and Kowalski, S.M. (2011). Statistical Methods for Engineers, p. 555. Cengage Learning: United States of America.

Wang, S., Jiang, L., Li, Y., Li, D. and Sui, X. (2011). Optimization on aqueous enzymatic extraction conditions of pine seed protein by using response surface method. *Procedia Engineering*, 15(2011), 4956-4966. https://doi.org/10.1016/j.proeng.2011.08.922

FULL PAPER

Wasswa, J., Tang, J., Gu, X-H. and Yuan, X-Q. (2007). Influence of the extent of enzymatic hydrolysis on the functional properties of protein hydrolysate from grass carp (*Ctenopharyngodon idella*) skin. *Food Chemistry*, 104(4), 1698-1704. https://doi.org/10.1016/j.foodchem.2007.03.044

Wasswa, J., Tang. J. and Gu, X-H. (2008). Optimization of the production of hydrolysates from grass carp (*Ctenopharyngodon idella*) skin using Alcalase. *Journal of Food Biochemistry*, 32(4), 460-473. https://doi.org/10.1111/j.1745-4514.2008.00176.x