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Optimization of enzymatic hydrolysis condition and functional properties of eel (*Monopterus* sp.) protein using response surface methodology (RSM)

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Abstract

The present study aims to optimize the enzymatic hydrolysis condition and determine the functional properties of eel *(Monopterus albus)* protein hydrolysate (EPH) at different hydrolysate concentrations (0.1%, 0.5%, 1.0%). The enzymatic hydrolysis (using alcalase) condition; namely, temperature (°C), enzyme to substrate concentration (%) and pH on both the yield and degree of hydrolysis (DH), as responses, was optimized using the response surface methodology (RSM) by employing three factors, 3-level, Central Composite Design (CCD). The optimum hydrolysis condition suggested was a temperature of 55.76 °C, enzyme concentration of 1.80% and pH of 9. The experimental result for yield (9.45%) was higher while the experimental result for DH (15.01%) was lower than the predicted values of the responses using the quadratic model, which were 5.67% and 16.73%, respectively. The findings for the functional properties showed that the Nitrogen Solubility Index (NSI) of EPH was 85%. The emulsion stability index (ESI) of EPH was shown to decrease with the increase hydrolysate concentration (0.1%, 0.5%, 1.0%) while the foam expansion of EPH showed an increase with the increase in concentration. High solubility and the ability of EPH to emulsify and form foam show its potential for use as a natural binding and emulsifying agent.

Introduction

Studies on fish protein hydrolysates have been widely conducted by researchers around the world in recent years (Benjakul and Morrisey, 1997; Abdul-hamid *et al*., 2002; Bhaskar and Mahendrakar, 2008; Betty *et al*., 2014). Numerous fish sources from freshwater and seawater, such as *Channa striatus* (Ghassem *et al*., 2011), *Oreochromis niloticus* (Raghavan and Kristinsson, 2009), *Merluccius productus* (Korzeniowska *et al*., 2013) and *Godus morrhua* (Farvin *et al*., 2014) have been used in producing hydrolysates to determine their physicochemical properties and biological activities. At the present time, the exploration of fish hydrolysates from other fish types including eels is still limited.

Enzymatic hydrolysis is one of the hydrolysis types to produce fish protein hydrolysates. Enzymatic hydrolysis is influenced by several factors, such as pH, temperature, time and enzyme concentration that cooperatively influence the enzyme activity thereby making the process more controllable (Viera *et al*., 1995; Liaset *et al*., 2000). The choice of substrate, protease employed and degree of hydrolysis generally affects the physicochemical properties of the resulting hydrolysates (Mullaly *et al*., 1995). Alcalase is an alkaline enzyme produced from *Bacillus licheniformis*. It has been reported to be one of the highly efficient bacterial proteases used to prepare functional fish and other protein hydrolysates (Adler-Nissen, 1986; Benjakul and Morrisey, 1997; Kristinsson and Rasco, 2000).

Generally, there are several controlled variables during the hydrolysis process, such as temperature, time, pH level and enzyme concentration (See *et al*., 2011; Prabha *et al*., 2013). In order to obtain the optimum hydrolysis conditions with the targeted responses, such as yield and degree of hydrolysis, optimization should be conducted. Optimization by response surface methodology (RSM) was mostly selected by researchers in the study of fish hydrolysates (Wasswa *et al*., 2008; Molla and Hovannisyan, 2011; Saidi *et al*., 2013; Thuy *et al*., 2014). One of the reasons for using RSM in the determination of hydrolysis conditions is that it generates a mathematical model that accurately describes the overall processes with significant estimation ability (Wasswa *et al*., 2007).

Functional properties, such as solubility, emulsifying properties, foaming properties, water holding capacity and fat binding capacity, are

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important characteristics to investigate the functional quality of the hydrolysates produced. According to Pacheco-Aguilar *et al*. (2008), a series of smaller polypeptides produced from the controlled enzymatic hydrolysis of protein can modify and improve the functional characteristics of the hydrolysates for different applications. Hence, the objectives of the study were to optimize the enzymatic hydrolysis condition and determine the functional properties of eel *(Monopterus albus)* protein hydrolysate (EPH) at different concentrations.

Materials and Methods

Materials

Eels *(Monopterus albus)* with an average weight of 125 g and length of 51 cm were purchased from the local market in Kuala Terengganu, Malaysia. The eels were beheaded, eviscerated, filleted and de-skinned in order to obtain the flesh, which was further frozen at -40°C until further use. The protease employed for the optimization studies was alcalase 2.4 L (2.4 AU/g) purchased from Sigma-Aldrich, USA. All the chemicals used were of analytical grade.

Sample preparation

Frozen eel flesh was thawed in a chiller at 4°C overnight. The thawed eel flesh was rinsed to remove the water-soluble compounds, minerals, enzymes and pigments. After cleaning, the flesh was chopped into small pieces before being homogenized by using a Waring blender (model HGB2WTS3, Connecticut, USA) at high speed for 60 sec. The minced flesh was sealed in plastic packs and stored in a freezer at -40°C until further use.

Preparation of the eel protein hydrolysate (EPH)

The preparation of eel protein hydrolysate (EPH) was conducted according to the method by Klompong *et al*. (2007) with modification. The raw eel flesh and distilled water (3:5) (w/w) was used for the homogenization process (2 min) using a Waring blender. The minced eel was heated at 85°C for 20 min in the autotitrator vessel (Metrohm model 799 GPT Titrino) in order to inactivate the endogenous enzyme and stirred continuously using a magnetic stirrer. After cooling down at a specified temperature (40°C, 50°C, 60°C), 20 g of alcalase (1%, 2%, 3%) (enzyme was diluted to a final volume of 20 g with distilled water) was added to the mixture and the hydrolysis process was continued for 2 hours with a constant desired pH value (pH 7, pH 8, pH 9) that was adjusted using 1N NaOH. The hydrolysis was stopped by heating the mixture at 85°C for 20 min to stop the alcalase activity. The hydrolysate was then cooled and centrifuged at 4000 rpm for 30 min. The supernatant of hydrolysate was collected and freeze dried.

Optimization of enzymatic hydrolysis conditions of eel protein hydrolysate (EPH) by response surface methodology (RSM)

Response surface methodology (RSM) was used to predict the optimal hydrolysis conditions of eel protein hydrolysate (EPH) using alcalase for two responses – yield and degree of hydrolysis (DH). The optimized hydrolysis condition employing RSM, as previously conducted by Klompong *et al.* (2007), was used with some modifications. Twenty hydrolysis trials were randomly run per Central Composite Design (CCD) with independent variables including temperature (A: 40, 50, 60°C); enzyme concentration (B: 1, 2, 3%) and pH (C: 7, 8, 9) were employed at three equidistant levels (−1, 0, +1).

Degree of hydrolysis (DH) of eel protein hydrolysate (EPH)

The degree hydrolysis of EPH was determined using the trichloroacetic acid (TCA) method with slight modification (Adler-Nissen, 1986; Klompong *et al*., 2007). After the hydrolysis process, 1 g of EPH was mixed with 10 ml of distilled water. About 5 ml of 10% (w/v) TCA was added to the EPH mixture. It was then left to stand for 30 min to allow precipitation and centrifuged (GYROZEN 1580R, Korea) at 4000 rpm for 15 min. The supernatant was filtered and analysed for protein content using the Kjedahl method (AOAC, 2002). The degree of hydrolysis of the EPH was determined using the following formula:

$$\text{Degree hydrolysis (\%)} = \frac{\text{Soluble N in 10\% TCA (w/v)}}{\text{Total N in the sample}} \times 100$$

Solubility of eel protein hydrolysate (EPH)

The solubility test was conducted by dispersing 200 mg of protein hydrolysate sample in 20 ml of deionized water (Sathe and Salunkhe, 1981). The mixture was stirred at room temperature for 30 min and centrifuged at 7500 x *g* for 15 min. Next, the nitrogen content in the supernatant was determined by using the Kjedahl method. The protein solubility was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{Protein content in supernatant}}{\text{Protein content in sample}} \times 100$$

Emulsifying properties of eel protein hydrolysate (EPH)

The emulsifying properties of EPH were determined according to the method as described by

Pearce and Kinsella (1978) with some modification. About 500 mg of hydrolysate was added with 50 ml 0.1 M NaCl. About 50 ml of soybean oil was added. The mixture was then homogenized for 2 min at 100% output at 120 V to make an emulsion. About 25 ml aliquot was immediately taken from the emulsion and transferred to a 25 ml graduated cylinder. The emulsion was allowed to stand for 30 min at room temperature. The aqueous volumes were read and the emulsification stability was calculated:

$$\text{Emulsification stability (\%)} = \frac{V_{total} - V_{aqueous}}{V_{total}} \times 100$$

Foaming properties of eel protein hydrolysate (EPH)

The whipping ability of hydrolysate was determined according to the method of Shahidi *et al*. (1995) with slight modification. A mass of 0.25 g of protein hydrolysate was dispersed in 25 ml of distilled water. The mixture was adjusted to pH 4, 6, or 7 with 2 M HCl and then homogenized for 2 min at 16000 rpm at room temperature for air incorporation. The whipped sample was immediately transferred into a 25 ml cylinder and the total volume was read after 30 min. The foaming capacity was calculated according to the following equation (Sathe and Salunkhe, 1981):

$$\text{Foam expansion (\%)} = \frac{A - B}{B} \times 100$$

Where,
A = volume after whipping (ml)
B = volume before whipping (ml)

Statistical analysis

To optimize the enzymatic hydrolysis conditions, the RSM Design-Expert 6.0.10 software (Stat-Ease 2003) was used. The results were expressed as a mean (±SD) for each analysis. The comparative statistical analysis between means with ANOVA was calculated using Minitab 14.0 to assess the significant differences between treatments.

Results and Discussion

Optimization of enzymatic hydrolysis conditions on yield and degree of hydrolysis (DH) by response surface methodology (RSM)

The response surface methodology (RSM) was used to optimize the enzymatic hydrolysis conditions of eel (*Monopterus* sp.) protein. The data of 20 experimental runs using central composite design (CCD) with three independent factors, namely, temperature (°C, A), enzyme concentration (%, B), pH (C) and two responses; yield (%) and degree of hydrolysis (DH, %) were obtained (Table 1).

The yield of EPH obtained from these 20 runs ranged from 1.94% - 7.65%, which was quite low for typical fish hydrolysate with a reported yield range of 10% - 15% (Quaglia and Orban, 1990). Meanwhile, the degree of hydrolysis (DH) of EPH ranged from 1.28% - 20.86%. The observed DH from the study was similar to the DH of fish hydrolysates from tuna dark muscle by-product (10.22%) (Saidi *et al*., 2013) and grass carp skin (16.11%) (Wasswa *et al*., 2007). The difference in the yield and DH of fish hydrolysates might be due to the difference in fish species, fish parts, types of enzyme used and hydrolysis conditions applied. However, to date, no study has discussed the relationship between the yield and degree of hydrolysis of the hydrolysates produced.

Analysis for yield of eel protein hydrolysate (EPH)

Model of summary statistics for eel protein hydrolysate (EPH) on yield

The quadratic model is the model summary suggested for the EPH yield, which was in agreement with the model reported by Nurdiyana and Siti Mazlina (2009) on the optimization of sardine waste hydrolysate.

Analysis of variance (ANOVA) for yield of eel protein hydrolysate (EPH)

The linear (A, B, C), quadratic (A^2, B^2, C^2) and interaction terms (AB, AC, BC) of the effects of variables for the yield of EPH were evaluated in terms of their adequacy, fitness and significance by analysis of variance (ANOVA). In order to improve the model, reduction was done by removing the insignificant model terms. The ANOVA of the Response Surface Quadratic model for the EPH yield after model reduction is shown in Table 2. The statistical significance of the proposed model could be obtained using the Fisher's test (F-test) (Maache-Rezzoug *et al.*, 2011). Table 2 shows the F-value (3.00), which indicates a significant model. There is only a 4.81% chance that a "Model F-value" this large could occur due to noise. The lack of fit test was used to predict the fitness of the model. In terms of the lack of fit value, Karki *et al*. (2011) suggested a non-significant value, which means the model experienced a non-significant lack of fit, which occurred due to noise. In the model of EPH yield, it was found that the p-value for the lack of fit value was not significant ($p>0.05$) (0.6914). Thus, the model was fit to determine the optimum hydrolysis condition of EPH.

Based on the result presented, the model for

Table 1. Optimization of eel protein hydrolysate (EPH)

Run	Temperature (A; °C)	Enzyme concentration (B; %)	pH (C)	Degree of hydrolysis (%)	Yield (%)
1	40	2.5	7	3.19	3.12
2	55	1.5	8	14.18	7.34
3	40	0.5	9	6.78	4.50
4	55	2.5	8	12.26	3.83
5	70	0.5	7	2.74	2.14
6	70	0.5	9	11.46	2.54
7	55	1.5	8	15.51	4.68
8	40	0.5	7	1.28	1.94
9	70	1.5	8	11.23	3.00
10	55	1.5	8	11.75	5.00
11	55	1.5	7	4.29	4.32
12	55	1.5	8	6.53	4.46
13	40	1.5	8	4.49	2.30
14	70	2.5	9	11.51	4.40
15	40	2.5	9	11.97	4.00
16	55	1.5	9	14.06	4.46
17	55	0.5	8	5.43	2.25
18	55	1.5	8	20.34	7.65
19	70	2.5	7	2.61	3.28
20	55	1.5	8	20.86	5.18

Table 2. Analysis of variance (ANOVA) after choosing significant model for EPH yield

Source	Sum of Squares	DF	Mean square	F Value	Prob > F	
Model	23.97	5	4.79	3.00	0.0481	Significant
A	0.025	1	0.025	0.016	0.9023	
B	2.77	1	2.77	1.73	0.2096	
C	2.60	1	2.60	1.63	0.2231	
A^2	5.15	1	5.15	3.22	0.0945	
B^2	2.47	1	2.47	1.54	0.2347	
Residual	22.40	14	1.60			
Lack of Fit	12.57	9	1.40	0.71	0.6914	not significant
Pure Error	9.83	5	1.97			
Cor Total	46.37	19				

R^2 = 0.5169, A = temperature (°C), B = enzyme concentration (%), C = pH

EPH yield had a significant ($p<0.05$) R^2 value, which indicates that 51.69% of the behaviour variation could be explained by the fitted model. The "Pred R-Squared" of 0.0814 was not as close to the "Adj R-Squared" of 0.3444. This might be due to the fluffy and light weight of EPH powder which resulted some of the powder stick on the wall of freeze drier. Generally, a ratio of more than 4 for the "Adeq Precision", which measures the signal to noise ratio, is desirable. In this model, the ratio was 5.402 indicating an adequate signal. Therefore, this model can be used to navigate the design space. The ANOVA results demonstrated that the linear model terms of A, B and C had no significant ($p>0.05$) effect on the yield of EPH. In addition, the quadratic coefficients (A^2, B^2) did not have a significant ($p>0.05$) effect on the EPH yield. Although the value of R2 is low, however, due to the insignificant of lack of fit ($p<0.05$), therefore, the suggested model can be used to predict the hydrolysis condition of EPH. The insignificant lack of fit could be used to determine the acceptance or rejection of the model used in the determination of hydrolysis condition.

Response surface plots and effects of factors for eel protein hydrolysate (EPH) on yield

The model equation for yield and the response variable (Y) of EPH obtained was derived using the regression coefficient of linear and quadratic terms to fit a full response surface model. According to the model's regression analysis, the best explanatory model equation of EPH yield was given as follows:

$$Y = +5.09 - 0.05\,A + 0.53\,B + 0.51\,C - 1.27\,A^2 - 0.88\,B^2$$

A 3-dimensional (3D) response was developed to study the effect between the two independent factors (enzyme and temperature) on the yield of EPH. Figure 1 shows the 3D response surface graph of the regression coefficient, which represents the effect of these factors on EPH yield. Based on the figure, it shows that the optimum hydrolysate yield was 9%. As the enzyme concentration increased the hydrolysis became more rapid. Once the active enzymes reached their optimum level at 1.50% they gradually decreased thereafter, hence, leading to a low hydrolysate yield. Shahidi *et al.* (1995) reported

Table 3. Analysis of variance (ANOVA) after choosing significant model for EPH degree of hydrolysis

Source	Sum of Squares	DF	Mean square	F Value	Prob > F	
Model	355.64	3	118.55	6.66	0.0040	Significant
A	14.02	1	14.02	0.79	0.3881	
C	173.89	1	173.89	9.76	0.0065	
A^2	167.74	1	167.74	9.42	0.0073	
Residual	284.98	16	17.81			
Lack of Fit	139.01	11	12.64	0.43	0.8852	not significant
Pure Error	145.98	5	29.20			
Cor Total	640.63	19				

R^2 = 0.5552, A = temperature (°C), C = pH

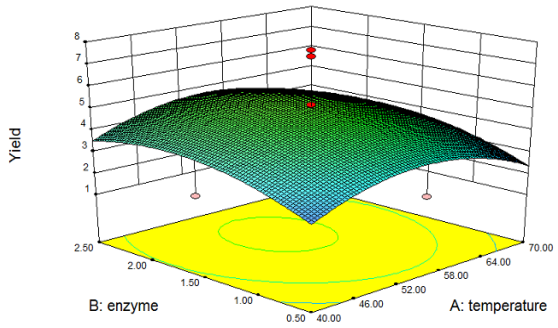

Figure 1. Response surface graph for yield (%) as function of enzyme concentration and temperature

that considerable soluble protein was released during the initial phase and no increase in soluble hydrolysate was observed when additional enzyme was added during the stationary phase of hydrolysis. However, the result presented contradicted the study conducted by Nordiyana and Siti Mazlina (2008), which showed an increase in the hydrolysate yield of sardine fish waste with an increase in the enzyme ratio.

Figure 1 also shows that the hydrolysate yield increased at a temperature of around 60°C and decreased thereafter. The hydrolysate yield was low at the higher temperature because of the denaturation of protein, while the low yield obtained at low temperature may be due to incomplete enzymatic hydrolysis reaction as alcalase activity is in a temperature range of 55-70°C with an optimum temperature at 60°C (Roslan *et al*., 2014). In addition, the study by Slizyte *et al*. (2005) showed that the yield of heated cod by-product hydrolysate was higher than the unheated sample. Hence, this finding showed the importance of temperature as a factor of the hydrolysate yield of fish protein hydrolysate.

Analysis for the degree of hydrolysis (DH) of eel protein hydrolysate (EPH)

Model summary statistics for the DH of eel protein hydrolysate (EPH)

The suggested model summary for the degree of hydrolysis (DH) of EPH was the quadratic model. The same model was reported in the studies by Prabha *et al*. (2013), See *et al.* (2011), Molla and Hovannisyan (2011), and Wasswa *et al*. (2007) from the enzymatic hydrolysis of different fish species, such as herring, salmon, beluga, silver catfish and grass carp.

Analysis of variance (ANOVA) on the DH of EPH

The linear (A, B, C), quadratic (A^2, B^2, C^2) and interaction terms (AB, AC, BC) of the effects of variables on the DH of EPH were evaluated in terms of their adequacy, fitness and significance by analysis of variance (ANOVA). The model reduction was done in order to eliminate insignificant models. The ANOVA of the Response Surface Quadratic model for the DH of EPH after model reduction is shown in Table 3. The "Model F-value" of 6.66 implied that the model was significant. Meanwhile, the lack of fit value of 0.43 had an 88.52% chance of occurring due to noise. An insignificant lack of fit was desirable in the determination of the optimum EPH hydrolysis condition (Karki *et al*., 2011). The lack of fit for the reduced DH of EPH model was not significant ($p>0.05$) (0.8852). Hence, the model was fit to determine the optimum hydrolysis condition of EPH.

Based on the result presented, the model for DH of EPH had a significant ($p<0.05$) coefficient variation (R^2) value (0.5552). An R^2 value greater than 0.80 is desirable in order to obtain a good fit model (Joglekar and May, 1987). The low value of the R^2 may due to the fluctuation of the required pH occurred as NaOH was added for adjustment during the hydrolysis process. When NaOH was dropped by autrotitrator into the hydrolysis mixture, the pH might increase above the required value. In the meantime, the "Pred R-Squared" of 0.4073 was in reasonable agreement with the "Adj R-Squared" of 0.4717, while the "Adeq Precision" ratio of the model was 8.115, which indicated adequate signals. Therefore, this model can be used to navigate the design space for the determination of hydrolysis condition of EPH. In addition, the ANOVA results after model reduction

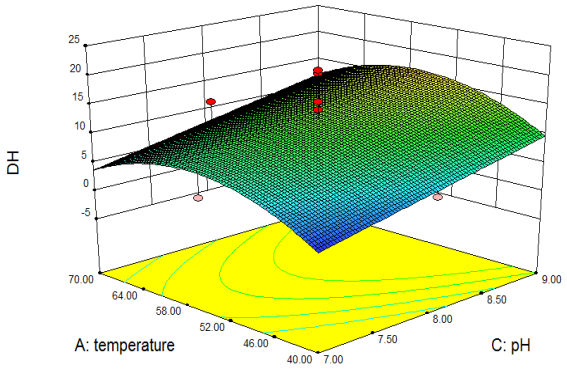

Figure 2. Response surface graph for degree of hydrolysis (DH) (%) as function of temperature and pH

demonstrated that only the linear model term of pH (C) had a significant ($p<0.05$) effect on the DH of EPH (0.0065). In terms of quadratic coefficients, A^2 significantly ($p<0.05$) affected the DH of EPH (0.0073). Although the value of R^2 is low, however, due to the insignificant of lack of fit ($p<0.05$), therefore, the suggested model can be used to predict the hydrolysis condition of EPH. The insignificant lack of fit could be used to determine the acceptance or rejection of the model used in the determination of hydrolysis condition.

Response surface plots and the effects of factors on the DH of eel protein hydrolysate (EPH)

The model equation for the DH and the response variable (Y) of EPH obtained was derived using the linear and quadratic regression coefficient to fit a full response surface model. According to the model's regression analysis, the best explanatory model for the DH of EPH equation was given as follows:

$$Y = + 12.52 + 1.18\ A + 4.17\ C - 2.54\ A^2$$

Figure 2 shows the 3D response surface plot of the effect of temperature and pH on DH of EPH. Based on the figure, the DH of EPH was constant from pH 7 to pH 9. According to Roslan *et al*. (2014), alcalase is active at a pH range from 6 – 10. Therefore, it shows that the alcalase constantly broke down the protein into smaller peptides from pH 7 to 9. The pH value also had a significant effect on tuna dark muscle by-product hydrolysate, as the DH increased from pH 7 to 8.5 (Saidi *et al.*, 2013).

Meanwhile, the surface plot shows that the degree of hydrolysis increased as the temperature increased until it reached an optimum temperature at 55.5°C. This was consistent with the optimal temperature of alcalase enzyme, which was 55°C (Roslan *et al*., 2014). However, the DH exhibited a decreasing trend from the temperature of 55.5°C to 70°C. The same trend was found in the study by See *et al.* (2011) on salmon skin hydrolysate. This is because alcalase started to denature and inactivate its activity above the optimum temperature, hence, leading to a reduction in the hydrolysis of eel protein (See *et al.*, 2011).

Optimization of eel protein hydrolysate (EPH) yield and DH

Optimal response conditions

The suggested hydrolysis conditions for EPH were temperature of 55.76°C, enzyme concentration of 1.80% and pH of 9. The optimum conditions suggested by RSM were within the range of optimized conditions on the hydrolysis of fish by alcalase, which were temperature of 35.00°C to 64.00°C, pH value of 8.24 to 9.45 and enzyme concentration of 0.20% to 2.50% (See *et al*., 2011; Nurdiyana and Siti Mazlina, 2009; Saidi *et al*., 2013).

Validation test

The degree of hydrolysis (DH) is one of the important factors that affect the physicochemical properties of the hydrolysate produced (Bhaskar and Mahendrakar, 2008). Therefore, validation of the hydrolysis responses should be done in order to approve the predicted responses generated by RSM. The yield and DH predicted from the optimum conditions of the hydrolysis of EPH were 5.67% and 16.73%, respectively. In order to validate the predicted responses, an additional experiment was conducted with three replicates for each response. The yield obtained was 9.45%, which was higher than the predicted value generated by RSM. However, the DH of EPH (15.01%) was lower than the predicted value. Previous studies showed a lower (See *et al*., 2011), similar (Roslan *et al*., 2014) and higher (Wasswa *et al*., 2008) value for the experimental responses compared to the predicted value. Based on the experimental DH value obtained, the suggested optimum condition by RSM in this study is suitable for the preparation enzymatic hydrolysate of eel protein, as the DH of fish protein hydrolysates from previous studies ranged from 10.22% to 77.03% (Bhaskar and Mahendrakar, 2008; Saidi *et al*., 2013; See *et al*., 2011).

Nitrogen Solubility Index (NSI) of EPH

The EPH produced from the optimized condition showed good solubility. The Nitrogen Solubility Index (NSI) of EPH obtained in the study was 85%. According to Pacheco-Aguilar *et al.* (2008), a solubility in a broad pH range is desired, which will

derive the emulsifying and foaming properties in a food system. The nitrogen solubility in this study was similar to the hydrolysate of yellow stripe trevally (hydrolysed at pH 8.5 and temperature of 60°C) (Klompong *et al*., 2007) and herring (hydrolysed at pH 8.0 and temperature of 50°C) (Sathivel *et al*., 2003), lower than hydrolysed ornate threadfin bream (hydrolysed at pH 2.0 and temperature of 50°C) (Nalinanon *et al.*, 2011) but higher than bluewing searobin hydrolysate (hydrolysed at pH 7.5 and temperature of 70°C) (dos Santos *et al*., 2011).

The high NSI is due to the removal of insoluble protein fractions during centrifugation before the freeze-drying process of the hydrolysate. In addition, peptides with low molecular weight, which are expected to have more polar residues than intact proteins, are able to form more hydrogen bonds with water and increase solubility as found in the fish hydrolysate studied by Chi *et al.* (2014) (more than 95.0% solubility at 5 kDa and 89.7% at 48 kDa). According to Tanuja *et al.* (2012), the high nitrogen solubility will impart an attractive appearance and a 'smooth' feel to the mouth when eating food that incorporates protein hydrolysates. Therefore, hydrolysates with high NSI are suitable for use as food ingredients in the production of human and animal food.

Emulsifying stability index (ESI) of EPH

The emulsifying properties of enzymatically hydrolysate compounds are directly connected to the effectiveness of the compounds in reducing the interfacial tension between hydrophobic and hydrolytic components in food products (dos Santos *et al.*, 2011). The emulsion stability index (ESI) of EPH at different all hydrolysate concentrations (0.1, 0.5, and 1.0%) was not significant ($p>0.05$) with the value of 22.58±2.76 min, 15.66±0.57 min and 10.39±0.06 min, respectively. Based on the results, the ESI of EPH decreased as the concentration increased, which was in line with the ESI of the round scad protein hydrolysate studied by Thiansilakul *et al.* (2007). However, a study by Nalinanon *et al.* (2011) on ornate threadfin bream hydrolysate showed an increase of ESI with an increase in concentration (0.10%, 0.25% and 0.50%) at DH 20. Based on the results, the difference in ESI obtained in this study and other studies might be because of the high concentration range.

According to Kristinsson and Rasco (2000), protein hydrolysates are surface-active materials and promote an oil-in-water emulsion because of the presence of hydrophobic, hydrophilic groups and their charge. The composition of hydrophobic amino acids, such as alanine, isoleucine, leucine and hydrophilic amino acids, such as glutamine, histidine and serine contained in hydrolysate contributes to the emulsifying property of the hydrolysate produced (Klompong *et al*., 2007; Cho *et al.*, 2008). Kinsella (1976) explained the emulsifying activity of different concentrations based on adsorption kinetics. The protein adsorption at the oil-water interface is diffusion-controlled at a low hydrolysate concentration. Meanwhile, at a high concentration, the activation energy barrier prevents protein migration from taking place in a diffusion-dependent manner, which leads to the accumulation of proteins in the aqueous phase, hence reducing the emulsion stability of the mixture (Thiansilakul *et al*., 2007). The statement supported the ESI of EPH obtained in the study, which decreased when the concentration increased.

Foaming properties of EPH

The foam expansion of eel protein hydrolysate at all different concentrations (0.1%, 0.5%, and 1%) was significant ($p<0.05$) with the value of 23.39±0.09%, 34.60±1.28% and 64.73±1.10%, respectively. The result showed an increase in foaming properties with the increase of concentration. A similar observation was obtained from the study by Thiansilakul *et al.* (2007) in which the resulting foam expansion was 23.33%, 43.00% and 47.00% at the respective concentrations of 0.1%, 0.5%, and 1%. In the present study, it can be said that higher diffusion of protein hydrolysate occurred at the air-water interface at a higher concentration. The transportation, penetration and rearrangement of molecules at the air-water interface influence foaming properties of protein hydrolysates (Elavarasan *et al.*, 2014). The stability of foam is the result of the well-ordered orientation of the molecules at the interface, where the hydrophilic head is located in the aqueous phase and the hydrophobic tail faces non-polar components (Thiansilakul *et al*. 2007).

Conclusion

In conclusion, the EPH yield and degree of hydrolysis (DH) was significantly affected by the hydrolysis condition including temperature, enzyme concentration and pH. Based on the model, the optimum conditions were temperature of 55.76°C, enzyme concentration of 1.80% and pH of 9.0. The corresponding responses were 16.73% of DH and 9.45% of hydrolysate yield. Meanwhile, the result of the functional properties showed that the Nitrogen Solubility Index (NSI) of EPH was

85%. The emulsion stability index (ESI) of EPH at different concentrations (0.1, 0.5, and 1.0%) was 22.58±1.95 min, 15.66±0.570 min and 10.39±0.06 min, respectively. The foam expansion of EPH at different concentrations (0.1%, 0.5%, and 1%) was 23.39±0.06%, 34.6±0.90% and 64.7±0.70%, respectively. High solubility and the ability of EPH to emulsify and form foam show its potential for use as a natural binding and emulsifying agent.

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