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A response surface approach on hydrolysis condition of eel (*Monopterus* Sp.) protein hydrolysate with antioxidant activity

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Abstract

The study aims to determine the optimized condition of eel protein hydrolysate (EPH) produced using alcalase. The proximate compositions of eel flesh were determined as well. Enzymatic hydrolysis conditions were optimized using response surface methodology (RSM) by applying four factors, 3-levels Central Composite Design (CCD) with six centre points. The model equation was proposed with regards to the time (60min, 120min, 180min), temperature (40°C, 50°C, 60°C), pH (7, 8, 9) and enzyme concentration (1%, 2%, 3%). The optimum of hydrolysis condition that be suggested to obtain the optimum yield, degree of hydrolysis (DH) and antioxidant activity were 84.02 min, 50.18°C, pH 7.89 and 2.26% [enzyme]. The predicted response values using quadratic model were 10.03% for yield, 83.23% for DH and 89.24% for antioxidant activity. The chemical composition determination showed that the protein content increased by more than 5-fold (16.88% to 98.53%) while the fat content was decreased by 96.48% after hydrolysis. Hydrolysis process had significantly increased the amount of both hydrophilic (serine and threonine) and hydrophobic amino acids (valine, isoleucine, phenylalanine, methionine) which contributed to the antioxidant activity of hydrolyzed eel protein. The enzymatic hydrolysis of eel protein had improved the protein content of EPH with potential as new natural antioxidant.

Introduction

The Asian swamp eel, Monopterus albus is categorized under family Synbranchidae (Pederson *et al*., 2014). It has an elongated, snake-like body, with a blunt, rounded nose. Its slippery skin is commonly darker green or brown on the top and growing lighter towards its underbelly (Bricking, 2002). In contrast with American eel, the Asian swamp eel has no pectoral fins (Sterba, 1973). This type of eel has a V-shaped opening gill on underside below its head. It is able to grow to a length of one meter and weight up to 2 kg (Bricking, 2002). However, most of the eel grow between 25cm to 40cm (Smith, 1945). Eels are widely distributed in many countries from India to China, Japan, Malaysia, Indonesia, Bangladesh (Froese and Pauly, 2008), Thailand (Thongrod et al., 2004) and Vietnam. The eel, which is considered a nutritious and tasty species, is also a valued remedy in oriental medicine (Khanh and Ngan, 2010). Therefore, eel can be a potential source in producing hydrolysate with bioactive properties as additional food ingredient.

Hydrolysate is a biologically active protein produced from hydrolysis of protein using proteolytic enzymes such as alcalase, papain, pepsin, trypsin, α-chymotrypsin, pancreatin, flavourzyme, pronase, neutrase, protamex, bromelain, cryotin F, protease N, protease A, orientase, thermolysin, and validase (Chalamaiah *et al*., 2012). The food-derived bioactive peptides which undergo hydrolysis process can be from plant and animal sources (Hartmann and Meisel, 2007). Among various sources, hydrolysates produced from fish and fish by-products are one of the major concerns to be studied because the fish processing industries produce a high amount (60% from the whole fish) of fish by-products which are potential protein sources to obtain bioactive compounds instead of removing them without any attempts (Dekkers *et al*., 2011). Recently, there are many research conducted on fish protein hydrolysates and their potential activities against oxidation, hypertension and proliferation (Je *et al.*, 2009; Batista *et al*., 2010; Vignesh *et al.*, 2011; Suarez-Jimenez *et al*., 2012; Li *et al.*, 2013; Betty *et al*., 2014).

In order to obtain hydrolysis condition suitable for the production of hydrolysate with specified responses such as yield, degree of hydrolysis and antioxidant activity, optimization need to be conducted by controlling various hydrolysis conditions, for example, time, temperature, enzyme concentration and pH of the mixture (Nurdiyana

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and Siti Mazlina, 2008; Shun *et al.*, 2013; Roslan *et al*., 2014). Optimization by using Response Surface Methodology (RSM) is the most commonly used technique by many researchers in producing fish protein hydrolysates (Bhaskar and Mahendrakar, 2008; Prabha *et al*., 2013; Thuy *et al*., 2014). Hence, the objectives of the study were to optimize the enzymatic hydrolysis of eel (*Monopterus* sp.) protein on yield, degree of hydrolysis and antioxidant activity by response surface methodology (RSM) and to characterize the hydrolysate in terms of its chemical and amino acid compositions.

Materials and Methods

Materials

Eels were purchased in Kuala Terengganu, Malaysia. The eels were beheaded, eviscerated, filleted and de-skinned in order to obtain the flesh. The flesh was frozen until further use at -40°C. Liquid Alcalase® 2.4 L (2.4 AU/g) was purchased from Novo Industry (Denmark). All chemicals used were of analytical grade.

Sample preparation

The frozen flesh of eels was thawed in a chiller at 4°C for 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 and homogenized by using a Waring blender (model HGB2WTS3, Connecticut, USA) at high speed for 60 sec. Minced flesh was sealed in plastic packs and stored in freezer at -40°C until further use.

Preparation of eel protein hydrolysate (EPH)

Several calculations were carried out prior to hydrolysis to determine the mass of raw material, distilled water and enzyme solution which was used during hydrolysis (Kristinsson and Rasco, 2000). The calculation is necessary because the mass of raw material depends on its protein content. The substrate was prepared by homogenizing eel flesh in distilled water at a protein substrate concentration of 7.68% (55 g per reaction).

The hydrolysis was performed according to the procedure of Klompong *et al.* (2007) with slight modification. For each batch, about 55 g eel flesh was added with 33g of distilled water. It then was heated at 85ºC for 20min before hydrolysis for inactivation of endogenous enzyme. After cooling, 20 g of alcalase enzyme solution (prepared by diluting the required enzyme mass to a final weight of 20 g with distilled water) was mixed into the eel flesh and the hydrolysis was initiated immediately. Parameters of hydrolysis were based on the temperature (40ºC, 50ºC and 60ºC) time (60min, 120min and 180min), alcalase to protein ratio (1%, 2% and 3%) and pH (pH 7, pH 8 and pH 9). The hydrolysis process was carried out using water bath shaker with manual pH adjustment using 1N NaOH.

After hydrolysis was completed, the process was terminated by heating the hydrolysate samples at 85°C for 20min to inactivate the alcalase activity. Next, the hydrolysate was centrifuged for 20min at 6000 x *g* in order to remove the insoluble particles and oil layer. The supernatants were then freeze-dried.The eel protein hydrolysate was stored in a container at – 40°C prior to analysis.

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

Response surface methodology (RSM) was used to predict optimal hydrolysis condition for eel protein hydrolysate (EPH) using alcalase. Optimized hydrolysis condition was previously accomplished by employing RSM by Klompong et al. (2007) with some modifications. Thirty hydrolysis trials were randomly run per Central Composite Design (CCD). Four independent variables including temperature (A: °C); time (B: min); enzyme to substrate concentration (C: % v/w); and pH (D) were employed at three equidistant levels (−1, 0, +1).

Chemical composition of eel flesh and eel protein hydrolysate (EPH)

The moisture, protein, fat and ash content of eel flesh and EPH were determined according to AOAC (2002). The yield of EPH was calculated by using the following formula:

$$\text{Yield (\%)} = \frac{\text{Mass of dried EPH (g)}}{\text{Mass of eel used for 1 hydrolysis run (g)}} \times 100$$

Degree of hydrolysis of eel protein hydrolysate (EPH)

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

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

1, 1- diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of eel protein hydrolysate (EPH)

With some modification, DPPH radical-scavenging activity for all 30 hydrolysate runs was measured according to the method by Hamid *et al.* (2015). 4 ml (20 mg/ml) of each sample solution was vigorously mixed with 1.0 ml of 0.2 mM DPPH. After 30min of incubation, the absorbance of sample was measured at 517 nm by using a spectrophotometer. A control sample was similarly processed with distilled water. DPPH radical scavenging activity was calculated according to the calculation given below:

$$\text{DPPH radical scavenging activity (\%)} = 1 - \frac{A_{517} \text{ of sample}}{A_{517} \text{ of control}} \times 100$$

Amino acid composition of eel flesh and eel protein hydrolysate (EPH)

The eel flesh and EPH were hydrolysed and derivatized prior to the injection for analysis. The hydrolysis was carried out by mixing 0.2 g sample with 5 ml 6N HCl at 110°C for 24h. Upon completion, 4 ml of the internal standard (AA) was added to the residues and then made up to 100 ml by deionised water. The determination of the resulting amino acids was carried out by Waters-Pico Tag Amino Acid Analyzer High Performance Liquid Chromatography (Waters 2690/5, Waters Co., Milford, USA) system (Shamloo *et al*., 2012).

Statistical analysis

The chemical composition determination of eel flesh and EPH were conducted in triplicate. The data was presented as mean±SD. Meanwhile, for optimization of EPH, response surface methodology (RSM) was statistically analyzed by using Design-Expert, 6.0.10 software (Stat-ease Inc., Minneapolis, Minn., USA). Statistical analysis of the model was performed using the software to evaluate the analysis of variance (ANOVA).

Results and Discussion

Chemical composition of eel flesh and eel protein hydrolysate (EPH)

The eel flesh moisture, protein, fat and ash content was 83.87%, 16.88%, 3.41% and 0.89%, respectively. Meanwhile, the moisture, protein, fat and ash content of the freeze-dried eel protein hydrolysate (EPH) was 2.64%, 98.53%, 0.12% and 17.28%, respectively. The high protein content of EPH is due to solubilisation of proteins during hydrolysis and removal of insoluble solid matter by centrifugation. Thus, it demonstrates the hydrolysate potential as protein supplements for human nutrition (Chalamaiah *et al*., 2012).

The fat content of EPH was similar to the fat content of hydrolysate produced from capelin *(Mallotus villosus)* (Shahidi *et al*., 1995), round scad *(Decapterus maruadsi)* (Thiansilakul *et al*., 2007), Pacific whiting *(Merluccius productus)* (Pacheco-Aguilar *et al*., 2008) and Persian sturgeon *(Acipenser persicus)* (Ovissipour *et al*., 2009) which was ranged from 0.1% – 0.18%. According to Chalamaiah *et al.* (2012), the low fat content is caused by the removal of lipids with insoluble protein fractions during centrifugation. The decreasing of fat content might significantly increase stability of material towards lipid oxidation, thus, it will enhance product stability (Ovissipour *et al*., 2009).

Many studies on fish hydrolysate showed low moisture content of hydrolysates produced and it was due to the evaporation during drying process (Nilsang *et al*., 2005; Wasswa *et al.*, 2007; Bhaskar *et al.,* 2008; Abdul-Hamid *et al.*, 2002). However, the ash content of EPH is higher compared to the ash content of eel flesh. The high content of ash is due to the addition of acid (hydrochloric acid) or base (sodium hydroxide) for pH adjustment during hydrolysis process (Pacheco-Aguilar *et al.,* 2008; Gbogouri *et al.*, 2004).

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

The response surface methodology (RSM) was used to optimize enzymatic hydrolysis condition of eel (*Monopterus* sp.) protein. Six central point values ranged between 10.18% - 11.45% for yield, 78.61% - 86.74% for degree of hydrolysis and 88.19% - 89.63% for antioxidant activity. The yield of EPH obtained for all 30 runs ranged from 4.18% - 11.82% which was quite similar with the range of typical fish hydrolysate yields, 10% - 15% as reported by Quaglia and Orban (1990). The percentage ratio of the number of broken down peptide bonds to the number of peptide bonds in protein mass is called degree of hydrolysis (DH) (Souissi *et al.*, 2007). The DH for all 30 runs was ranged from 63.77% - 90.59%. The mechanism of enzymatic hydrolysis on protein had been described by Adler-Nissen (1976). Generally, the native globular proteins are rarely broken down by proteolytic enzymes due to their tertiary structure which protects most of the peptide bonds. However, the enzymatic cleavage could be occurred from the exposure of more peptides bonds in the denatured

Table 1. Analysis of variance (ANOVA) after choosing significant model for EPH yield

Source	Sum of Squares	DF	Mean square	F Value	Prob > F	
Model	72.96	6	12.16	16.07	< 0.0001	Significant
A	11.17	1	11.17	14.76	0.0008	
B	3.88	1	3.88	5.13	0.0332	
C	4.80	1	4.80	6.35	0.0191	
D	17.66	1	17.66	23.34	< 0.0001	
D^2	22.89	1	22.89	30.25	< 0.0001	
BC	12.55	1	12.55	16.58	0.0005	
Residual	17.41	23	0.76			
Lack of Fit	16.29	18	0.90	4.04	0.0644	not significant
Pure Error	1.12	5	0.22			
Cor Total	90.37	29				

R^2 = 0.8074, A = time (min), B = temperature (°C), C = pH, D = enzyme concentration (%)

form of protein. The breaking of more peptide bonds had destabilized the protein molecule and hence, the polypeptide chains were extensively degraded into smaller peptide units. In most cases, the difference in rate of the peptide cleavage was depended on the parameter of hydrolysis used such as time, temperature, pH and enzyme concentration (See *et al*., 2011; Saidi *et al*., 2013).

The observed DH from the study was higher as compared to DH of fish hydrolysates from different sources such as herring muscle (40.20%) (Prabha *et al*., 2013), beluga viscera (30.00%) (Molla and Hovannisyan, 2011), tuna dark muscle by-product (10.22%) (Saidi *et al*., 2013), catla viscera (48.56%) (Bhaskar and Mahendrakar, 2008), grass carp skin (16.11%) (Wasswa *et al.*, 2007) and channel catfish skin (35.12%) (Shun *et al*., 2013). However, the DH of EPH obtained was similar to the DH of salmon skin hydrolysate (77.03%) (See *et al*., 2011).

The antioxidant activity of EPH for all 30 hydrolysis run ranged from 85.32% - 90.85%. The result obtained was higher than DPPH radical scavenging activity of yellow stripe trevally protein hydrolysate which was below 80.00% (Klompong *et al.*, 2008). Hydrolysis of eel using alcalase had allowed the exposure of hydrophobic group peptides which potentially influenced the antioxidant activity of EPH (Song *et al*., 2011). The difference in yield, DH and antioxidant activity of fish hydrolysates might be due to the different in fish species, fish parts, types of enzymes used and hydrolysis conditions applied, which correspond to the hydrolysis parameters used.

Analysis for yield of eel protein hydrolysate (EPH)

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

The quadratic model is the model summary suggested by RSM for EPH yield (Table 1). Quadratic model were usually generated by the design for the hydrolysis of fish proteins in which it has equation consist of regression coefficient of intercept, linear, quadratic and interaction coefficients (Molla and Hovannisyan, 2011; Prabha *et al*., 2013; Bas and Boyaci, 2007). The model suggested was in the same agreement with the model reported by Nurdiyana and Siti Mazlina (2008) on the optimization of sardine waste hydrolysate.

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

The analysis of variance (ANOVA) of the Response Surface Quadratic model for EPH yield after model reduction is shown in Table 1. According to Bas and Boyaci (2007), the overall predictive capability of the model could be explained by the coefficient of determination (R^2) which is calculated from prediction error sum of squares (PRESS) residuals generated by RSM. Based on the result presented, the R^2 value (0.8074) for EPH yield was significant ($p<0.05$). In order to obtain a good fit model, R^2 should be greater than 0.80 which indicate the data variation is adequately explained (Roslan *et al*., 2014). The ANOVA results demonstrated that the linear model terms of A, B, C and D had significantly ($p<0.05$) affect the yield of EPH with the most significant was enzyme concentration (D) followed by time (A), pH (C) and temperature (B).

Meanwhile, most quadratic coefficients (A^2, B^2 and C^2) did not have significant ($p>0.05$) effect on EPH yield and therefore, the coefficients were not included in Table 1. In contrast, D2 showed significant ($p<0.05$) effect on yield of EPH (<0.0001). In terms of interaction coefficient, only temperature-pH (BC) interaction had significant effect ($p<0.05$) on the yield of EPH. The lack of fit test was used to determine the fitness of the model. The result obtained shows that the p-value (0.0644) for the lack of fit test was not significant ($p>0.05$), thus, indicate that the model was fitted for predicting enzymatic hydrolysis condition

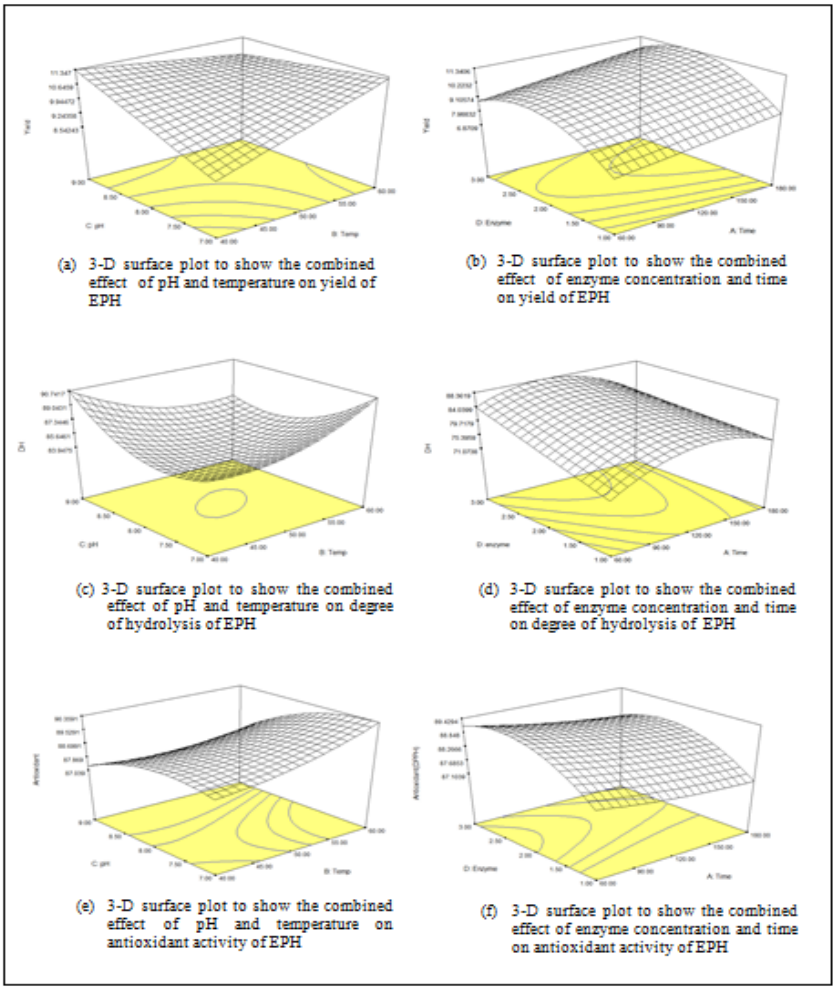

Figure 1. Response surface plot of the combined effect of two independent variables (time, temperature, pH, enzyme concentration) on responses for EPH

of eel protein.

Model graph for yield of eel protein hydrolysate (EPH)

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

$$Y = +10.41 + 0.79\,A + 0.46\,B + 0.52\,C + 0.99\,D - 1.78\,D2 - 0.89\,BC$$

Effect of temperature and pH on yield of eel protein hydrolysate (EPH)

A 3-dimensional (3D) response was developed to study the effect between the two independent factors (pH and temperature) on yield of EPH when enzyme concentration and time were fixed at 2% and 120 min, respectively, as suggested by the design. The interaction between temperature (B) and pH (C) has been presented in Table 1 in which the F value for BC was 0.0005. Figure 1(a) shows the 3D response surface graph of the regression coefficient which presents the effect of these factors on EPH yield. Results indicated that the EPH yield increases with the increase of pH value and temperature. According to Roslan *et al.* (2014), alcalase is active at the pH range from 6 – 10 and temperature range from 50°C – 70°C. Based on the graph, it can be seen that the breakdown of eel protein into smaller peptides was actively occurred from pH 7 to pH 9, hence, increase the yield of hydrolysate. The same trend was found with the increasing of hydrolysis temperature. Studies conducted by Saidi *et al.* (2013), See *et al*. (2011), Bhaskar *et al.* (2008) and Thuy *et al.* (2014) showed the optimum pH temperature values were varies within the range depending on the substrate used.

Effect of enzyme concentration and time on yield of eel protein hydrolysate (EPH)

The effect of enzyme concentration and time on yield of EPH at pH 8 and temperature of 50°C

Table 2. 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	643.57	5	128.71	8.05	0.0001	Significant
D	275.73	1	275.73	17.25	0.0004	
A^2	153.93	1	153.93	9.63	0.0049	
B^2	29.47	1	29.47	1.84	0.1872	
AD	102.87	1	102.87	6.43	0.0181	
BC	84.96	1	84.96	5.31	0.0301	
Residual	383.69	24	15.99			
Lack of Fit	324.90	19	17.10	1.45	0.3613	not significant
Pure Error	58.79	5	11.76			
Cor Total	1027.26	29				

R^2 = 0.6265, A = time (min), B = temperature (°C), C = pH, D = enzyme concentration (%)

was illustrated in Figure 1(b). In terms of enzyme concentration, it can be seen that the yield of EPH increase with the increase of enzyme concentration. However, at the concentration of 2.5% to 3.0% the yield of EPH was slowly decreased. This result indicates that there will be more chances for yield to increase until it achieves enzyme concentration at 2.5%. According to Thuy *et al*. (2014), the enzyme breaks down protein peptide up to a certain limit where it become "saturated" and the hydrolysis remains constant. This might be the reason of visible gradual decrease on yield of EPH. In contrast, the yield of EPH was increased with the increase of hydrolysis time. This result explains that the breakdown of peptide was continuously occurred with the increasing time. However, the findings cannot conclude that the yield increase from the initial hydrolysis time. A study conducted by Nurdiyana *et al.* (2008) showed that the amount of extracted protein was decreasing at hydrolysis time of 30 min to about 50 min and started to increase after 50 min. From the result obtained and findings from other study, it is possibly explain that the yield of fish hydrolysate might decrease at hydrolysis time below 50 min and will increase beyond 50 min of hydrolysis time.

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

Model of summary statistics for degree of hydrolysis of eel protein hydrolysate (EPH)

The suggested model summary for the degree of hydrolysis (DH) of EPH by RSM was quadratic model (Table 2). The same model was reported in the study 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, respectively.

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

The analysis of variance (ANOVA) of the Response Surface Quadratic model for DH of EPH after model reduction is shown in Table 2. Based on the result presented, the model for DH of EPH had significant ($p<0.05$) R^2 value (0.6265). The ANOVA results demonstrated that only linear model term of enzyme concentration (D) had significant ($p<0.05$) effect on the DH of EPH (0.0001).

In terms of quadratic coefficients, A^2, B^2 had significantly ($p<0.05$) affected the DH of EPH. Meanwhile, the interaction coefficient of time-enzyme concentration (AD) and temperature-pH (BC) had significant ($p<0.05$) effect on the DH of EPH. The lack of fit test was used to determine the fitness of the model. The result obtained shows that the p-value (0.3613) for the lack of fit test was not significant ($p>0.05$). Therefore, the model fitted the experimental data and was chosen for predicting enzymatic hydrolysis condition of eel protein.

Model graph for degree of hydrolysis (DH) of eel protein hydrolysate (EPH)

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

$$Y = + 84.21 + 3.91\ D + 6.68\ A2 + 2.93\ B2 - 2.54\ AD - 2.30\ BC$$

Effect of temperature and pH on degree of hydrolysis (DH) of eel protein hydrolysate (EPH)

The 3D response surface plot of the effect of temperature and pH on DH of EPH is shown in Figure 1(c). The enzyme concentration and hydrolysis time were kept constant as suggested by RSM at 2% and 120min, respectively. Based on the result,

Table 3. Analysis of variance (ANOVA) after choosing significant model for EPH antioxidant activity

Source	Sum of Squares	DF	Mean square	F Value	Prob > F	
Model	643.57	5	128.71	8.05	0.0001	Significant
A	2.77	1	2.77	10.04	0.0064	
B	5.71	1	5.71	20.71	0.0004	
C	16.63	1	16.63	60.28	< 0.0001	
D	6.89	1	6.89	24.99	0.0002	
A^2	0.039	1	0.039	0.14	0.7107	
B^2	0.34	1	0.34	1.24	0.2830	
C^2	1.52	1	1.52	5.52	0.0329	
D^2	1.90	1	1.90	6.89	0.0191	
AB	2.09	1	2.09	7.57	0.0148	
AC	3.08	1	3.08	11.17	0.0045	
AD	0.090	1	0.090	0.33	0.5763	
BC	3.40	1	3.40	12.34	0.0031	
BD	6.400E-003	1	6.400E-003	0.023	0.8810	
CD	0.71	1	0.71	2.56	0.1306	
Residual	4.14	15	0.28			
Lack of Fit	2.25	10	0.23	0.60	0.7718	not significant
Pure Error	1.88	5	0.38			
Cor Total	55.17	29				

R^2 = 0.9250, A = time (min), B = temperature (°C), C = pH, D = enzyme concentration (%)

the DH of EPH was increased with the increase of both temperature and pH of hydrolysis mixture. As mentioned before, alcalase activity was high at the pH range from 6 – 10 and temperature range from 50°C – 70°C (Roslan *et al*., 2014). The result shows that alcalase was actively broken down the peptides of eel within the pH 7 to pH 9 and temperature of 40°C to 60°C. The same trend was seen in the study by Wasswa *et al.* (2008). However, the study conducted by Saidi *et al.* (2013) which was also using alcalase found a little different in which the DH increased at pH level up to 8.5 and temperature of 55°C and started to decrease beyond the stated pH and temperature.

Effect of enzyme concentration and time on degree of hydrolysis (DH) of eel protein hydrolysate (EPH)

Figure 1(d) shows the 3D response surface plot of the effect of enzyme concentration and time on DH of EPH with the fixed pH (pH 8) and temperature (50°C). The DH of EPH was significantly increased with the increase of enzyme concentration. However, different trend was found on hydrolysis time towards DH of EPH. Based on the graph, the DH of EPH was increased from 60 min to 150 min and started to decrease beyond 150 min of hydrolysis time. As mentioned before, the denaturation of parent protein allows the breakdown of polypeptide chains with the presence of proteolytic enzymes such as alcalase (Adler-Nissen, 1976). Based on the statement, it can be explained that the polypeptide chains of eel protein were highly available to be cleaved at hydrolysis time 60 min to 150 min. However, the availability of peptide bonds decreased beyond 150min resulting in the decrease of its DH.

In terms of enzyme concentration, the higher concentration indicates the higher enzyme molecules available to breakdown protein into smaller peptide units, hence, increase the degree of hydrolysis. Therefore, linear relationship was found between enzyme concentration and DH of EPH in this study. Previous studies showed the optimum alcalase concentration was ranged from 0.2% - 2.5% depending on the fish species and their parts (See *et al*., 2011; Saidi *et al.*, 2013; Nurdiyana and Siti Mazlina, 2008; Wasswa *et al*., 2007; Thuy *et al*., 2014).

Analysis for antioxidant activity of eel protein hydrolysate (EPH)

Model of summary statistic for antioxidant activity of eel protein hydrolysate (EPH)

The model summary suggested by RSM for the antioxidant activity of EPH was quadratic model (Table 3) which was in the same agreement with the previous study conducted by Kuo *et al*. (2009) on tilapia skin gelatin hydrolysate.

Analysis of variance (ANOVA) for antioxidant activity of eel protein hdyrolysate (EPH)

Table 3 shows the analysis of variance (ANOVA) of the Response Surface Quadratic model for antioxidant activity of EPH after model reduction. The model for EPH antioxidant activity had a high and significant ($p<0.05$) R^2 value (0.9250). Based

on the results obtained, it can be seen that the linear model terms of A, B, C and D had significantly ($p<0.05$) affect the antioxidant activity of EPH with the most significant was pH (C) followed by enzyme concentration (D), temperature (B) and time (A).

The same trend was found for quadratic coefficients (A^2, B^2, C^2 and D^2) which had significant effect ($p<0.05$) on EPH antioxidant activity. In terms of interaction coefficient, AB, AC and BC had significant effect ($p<0.05$) on the antioxidant activity of EPH. The lack of fit test was used to determine the fitness of the model. The result obtained shows that the p-value for the lack of fit test (0.7718) was not significant ($p>0.05$). Hence, it indicates that the model was fitted for predicting enzymatic hydrolysis condition of eel protein.

Model graph of antioxidant activity of eel protein hydrolysate (EPH)

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

$$Y = + 88.77 + 0.39\ A + 0.56\ B + 0.96\ C + 0.62\ D + 0.12\ A^2 + 0.36\ B^2 - 0.77\ C^2 - 0.86\ D^2 + 0.36\ AB - 0.44\ AC - 0.075\ AD - 0.46\ BC - 0.020\ BD - 0.21\ CD$$

Effect of temperature and pH on antioxidant activity of eel protein hydrolysate (EPH)

Figure 1(e) shows the 3D response surface graph of the regression coefficient which represents the effect of temperature and pH of hydrolysis on antioxidant activity of EPH at enzyme concentration of 2% and hydrolysis time of 120 min. During hydrolysis, the breakdown of peptide bond allows the release of hydrophobic peptides which capable *to inhibit free radicals and lipid peroxidation (Song et al.*, 2011). The higher hydrolysis temperature leads to higher peptides breakdown, thus, more hydrophobic peptide molecules such as alanine, valine, leucine, isoleucine, phenylalanine and methionine available against free radicals of the 1, 1- diphenyl-2-picrylhydrazyl (DPPH). Based on the graph obtained, the antioxidant activity of EPH had no significant different with the pH value of the hydrolysis mixture (pH 7 – pH 9). This was due to the high solubility of EPH which made the hydrophobic amino acids presence in EPH easily interact and inhibit DPPH activity. This result was in the same agreement with Gbogouri *et al*. (2004) which stated that the protein hydrolysates are soluble in wide pH range.

Effect of enzyme concentration and time on antioxidant activity of eel protein hydrolysate (EPH)

The effect of enzyme concentration and time on antioxidant activity of EPH at pH 8 and temperature of 50°C was shown in Figure 1(f). The antioxidant activity of EPH was increased with the increase of enzyme concentration. According to Centenaro *et al*. (2011), the DH had a significant influence on the antioxidant properties of the peptides where it increased with the increase of DH. The increase in DH means the number of broken down peptides from the protein peptide mass was getting higher and allows the increase of hydrophobic peptides released which would react against the DPPH free radicals.

As mentioned before, the DH of EPH increased with the increase of enzyme concentration (Figure 1(d)). Hence, the increase of antioxidant activity of EPH when enzyme concentration increased was in the same agreement with the findings by Centenaro *et al.* (2011). Meanwhile, as pH increase from pH 7 to pH 8.5, the antioxidant activity slightly increased but decreased slowly at pH higher than 8.5. The result obtained might be because of the presence of hydrophobic peptides at pH less than 8.5 were higher than these peptides at pH more than 8.5.

Optimization of EPH yield, degree of hydrolysis (DH) and antioxidant activity

Optimal response conditions

After limitations were set, optimization by using Design Expert 6.0.10 was conducted and solutions for optimization conditions were further evaluated. The optimum hydrolysis conditions suggested by RSM for EPH were time of 84.02 min, temperature of 50.18°C, pH of 7.89 and enzyme concentration of 2.26% was similar to the optimized conditions of grass carp skin hydrolysate which were 83.83min, 59.74°C, pH 8.25 and 1.70% of enzyme concentration (Wasswa *et al*., 2007).

Validation test

The yield, DH and antioxidant activity predicted from the optimum conditions of the hydrolysis of EPH were 10.03%, 83.23% and 89.25%, respectively. In order to validate the predicted responses, an additional experiment was conducted with three replicates for each response. The results obtained were 6.97% for yield, 74.45% for DH and 85.69% for antioxidant activity. The antioxidant activity of EPH was quite close to the predicted value. Although the

Table 4. Amino acid composition of eel flesh and eel protein hydrolysate

Amino acid	Eel flesh (%)	Eel protein hydrolysate (%)
Aspartic acid/ Asparagine	8.55±0.16[a]	8.82±0.11[a]
Threonine	3.60±0.04[b]	3.90±0.04[a]
Serine	3.21±0.05[b]	3.46±0.01[a]
Glutamic acid/ Glutamine	13.88±0.17[a]	13.28±0.16[a]
Proline	3.51±0.04[a]	3.25±0.33[a]
Glycine	5.09±0.04[a]	4.81±0.66[a]
Alanine	5.25±0.06[a]	5.01±0.13[a]
Valine	3.73±0.01[b]	4.40±0.15[a]
Methionine	2.50±0.03[b]	2.70±0.01[a]
Isoleucine	3.46±0.00[b]	4.35±0.19[a]
Leucine	6.06±0.05[a]	6.52±0.23[a]
Tyrosine	2.15±0.11[a]	2.61±0.12[a]
Phenylalanine	2.49±0.02[b]	3.47±0.10[a]
Histidine	1.95±0.02[b]	2.25±0.02[a]
Lysine	7.09±0.04[a]	7.76±0.18[a]
Arginine	5.77±0.05[a]	6.02±0.08[a]
Cystine	1.07±0.00[a]	1.70±0.44[a]
Tryptophan	0.21±0.01[b]	0.74±0.07[a]
Total	79.58±0.89[b]	85.06±0.40[a]

*Data with different letters within a row are significantly different ($p<0.05$, n=2)

yield and DH of EPH were lower than the predicted values but the values were still in the range of typical fish hydrolysate (Quaglia and Orban, 1990). Therefore, the suggested optimum condition by RSM is suitable for the preparation enzymatic hydrolysate of eel protein.

Amino acid composition of eel flesh and eel protein hydrolysate (EPH)

Amino acid profile can be used to determine the functional and bioactive quality of the hydrolysate produced. Table 4 shows the amino acid composition of eel flesh and eel protein hydrolysate. It can be seen that eel flesh and EPH contain both hydrophilic and hydrophobic amino acids with the higher amount compared to other amino acids found were aspartic acid and glutamic acid. The finding was in the same agreement with most of the amino acid composition from different sources of fish protein hydrolysates (Klompong *et al*., 2009; Yin *et al*., 2010; Hou *et al*., 2011). The hydrolysis of eel protein not only increase its protein content from 16.88% to 98.53%, the total amino acid content of EPH (85.06%) was also significantly ($p< 0.05$) increased as compared to amino acid in eel flesh (79.58%).

Based on the result in Table 4, most of the amino acids were increased after hydrolysis with alcalase with significant increase ($p<0.05$) was found on threonine, serine, valine, methionine, isoleucine, phenylalanine and tryptophan. It can also be seen that several amino acids such as glutamine, proline, glycine and alanine were decrease hydrolysis of eel protein, however, the decrease these amino acids was not significant ($p>0.05$).

It had been discussed that the hydrophobic amino acids are important to improve the bioactivity such as antioxidant activity and antiproliferative acitvitiy of hydrolysates (Samarayanaka and Li-Chan, 2008; Picot *et al.*, 2010; Song *et al*., 2011). Several amino acids namely tyrosine, methionine, cystine, leucine, valine, glycine, glutamine and aspartic acid are important in radical scavenging activity as proton donors which quench to the unpaired electrons and inhibit the activity (Fan *et al.*, 2012). Besides that, Kumar *et al.* (2011) reported that aromatic amino acid phenylalanine can act as radical scavenger while cystine acts directly with free radicals by donating sulphur hydrogen. Based on the result, most of the hydrophobic amino acids (valine, isoleucine, methionine and phenylalanine) were significantly ($p<0.05$) increased. Previous studies reported the peptides sequences which exhibit antioxidant activities found in fish protein hydrolysates, for examples, tuna dark muscle by-product (Pro-Val-Ser-His-Asp-His-Ala-Pro-Glu-Tyr) (Hsu, 2010) and grass carp muscle (Pro-Ser-Lys-Ty-Glu-Pro-Phe-Val) (Ren *et al.*, 2008). The result of optimization had shown that EPH has the antioxidative ability and hence, hydrolysis process is important in producing amino acids which are responsible to inhibit oxidation.

The amino acids found in fish protein hydrolysates are also involved in other biological activities such as antiproliferative activity and antihypertensive activity. Hsu *et al.* (2011) had isolated amino acid sequence in peptides of tuna dark muscle by-product with antiproliferative potential which was Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr. Meanwhile, there are many studies reported

the isolated peptides with the ability to inhibit angiotensin I-converting enzyme (ACE) activity. For example, the peptides found in lizard fish hydrolysate (Ser-Pro-Arg-Cys-Arg) (Wu *et al*., 2012) and Channa striata hydrolysate (Val-Pro-Ala-Ala-Pro-Pro-Lys) (Ghassem et al., 2011). According to Ghassem *et al.* (2011), the presence of aromatic (Trp, Tyr, Phe) and aliphatic (Ile, Ala, Leu, Met) residues is suggested in the ultimate position to increase ACE activity of peptides.

In terms of functional properties, the presence of amino acids in fish protein hydrolysates is important especially in solubility, emulsifying properties, water holding capacity and fat binding capacity. According to Souissi *et al.* (2007), the difference in solubility can be due to peptide length and the ratio of hydrophilic and hydrophobic peptides. Hydrolysis process exposes some hydrophobic group to the surface and it also converts some hydrophobic groups to hydrophilic by generating two-end carbonyl and amino groups (Betty *et al.*, 2014). Meanwhile, hydrophobic peptides contribute the emulsion stability and fat binding capacity (Tanuja *et al*., 2012; Cho *et al*., 2008). The water holding capacity is affected by the presence of polar side chains such as COOH and NH_2 as found in glutamic acid and aspartic acid (Taheri *et al*., 2013). In short, hydrolysis is needed for the native protein to demonstrate its biological function (Suarez-Jimenez *et al.*, 2012). Hence, the result obtained provides potential of the peptides to undergo their bioactivity and improve their functional properties.

Conclusion

In conclusion, the eel protein hydrolysate (EPH) produced from the study has significantly higher protein content and ash content and lower fat and moisture content as compared to the eel flesh. The RSM applied suggested that the optimum condition of EPH was time of 84.02 min, temperature of 50.18°C, pH of 7.89 and enzyme concentration of 2.26%. After validation test was conducted, it was found that the antioxidant activity of EPH was quite similar with the predicted value generated by RSM but lower in terms of its yield and DH. However, the values of these responses were still in the range of typical fish hydrolysates, thus, the optimum conditions suggested by RSM could be used in the production of EPH. In terms of amino acid composition, aspartic acid and glutamic acid showed higher amount as compared to other amino acids in eel flesh and EPH. The enzymatic hydrolysis of eel protein had improved the protein content of EPH with potential as new natural antioxidant.

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