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OPTIMISATION OF ENZYMATIC PROTEIN HYDROLYSIS OF MUD CRAB (*Scylla* sp.) TO OBTAIN MAXIMUM ANGIOTENSIN-CONVERTING ENZYME INHIBITORY (ACEI) ACTIVITY USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Optimisation is commonly employed to find the conditions that produce the best possible response, thus minimizing the cost and time of a particular process. Response Surface Methodology (RSM) is a widely used tool in optimisation. This study reported the optimisation of enzymatic hydrolysis of mud crab meat using RSM by employing a face-centered Central Composite Design (CCD) to obtain maximum angiotensin-converting enzyme inhibitory (ACEI) activity. Screening of commercial food grade proteinases was carried out using Alcalase®, Protamex®, Neutrase® and papain shows that Protamex® gave the highest ACEI activity. The enzymatic hydrolysis conditions used in the optimisation were temperature (45-65ºC), pH (pH 5.5-7.5), hydrolysis time (1-4 hours) and Protamex® enzyme to substrate (E/S) ratio (1-3%). It was found that quadratic model was able to explain the relationship between the hydrolysis conditions and ACEI activity. The optimum conditions were obtained at 65ºC, pH 5.6, 1% E/S ratio and 4 hours of hydrolysis time. The experimental ACEI activity (88.93±1.02%) was not significantly difference (p>0.05) with the predicted ACEI activity (91.10%). It was found that the IC_{50} of the mud crab hydrolysate prepared at optimum condition was 1.96±0.13 mg/mL. This study shows that RSM can be used to explain the relationship between enzymatic hydrolysis conditions of mud crab meat and its ACEI activity.

Key words: Mud crab, Enzymatic hydrolysis, protein hydrolysate, Response Surface Methodology (RSM), angiotensin-converting enzyme

INTRODUCTION

Hypertension is one of the risk factors contribute to cardiovascular disease, which can be prevented through the use of medicines as well as implementation of healthy diet and life style. Angiotensin-I-converting enzyme (ACE) occurs naturally in our cardiovascular system and it is the key enzyme in regulating our blood pressure and body's electrolyte. ACE converts inactive angiotensin I into potent vasoconstrictor, angiotensin II and deactivate bradykinin causing increase in blood pressure (Kawanding *et al.*, 2015). Thus, inhibition of ACE can become a potential strategy in the prevention and treatment of hypertension (Georgiadis *et al.*, 2003). Angiotensin-converting enzyme (ACE) occurs naturally in our body and it is the main cause of high blood pressure. Synthetic ACE inhibitors (ACEI) for example captopril and enalapril are widely used to treat hypertension. However, these synthetic ACEI incur adverse side effects such as mild azotemia at the start of therapy, hypotension, dizziness, renal dysfunction, non-productive cough, angioedema, hyperkalemia and renal insufficiency (Rui *et al.*, 2013).

ACEI peptides derived from natural food proteins are considered to be natural, milder and safer alternative as compared to synthetic drugs (Wang *et al.*, 2008). The resulting food-derived protein hydrolysate is generally recognized as safe (GRAS) and has been commercially used as food ingredient and supplement (Schaafsma, 2009). Thus, various sources of food proteins have been utilized

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to produce ACEI peptides for example from milk, egg, fish, soy, meat and whey protein (Miguel *et al.*, 2007). ACEI peptides also have been isolated from enzymatic hydrolysis of various marine products such as Alaska Pollack (Byun & Kim, 2001), cuttlefish (Balti *et al.*, 2015), tilapia (Raghavan and Kristinsson, 2009), sea cucumber (Zhao *et al.*, 2007), hard clam (Tsai *et al.*, 2008), microalgae (Ko *et al.*, 2012a) and big eye tuna (Lee *et al.*, 2010). Until now, no study has been reported on the optimisation of hydrolysis conditions of mud crab hydrolysis to obtain maximum ACEI activity.

An optimum conditions of enzymatic protein hydrolysis can be obtained using response surface methodology (RSM) (Silva *et al.*, 2010). Optimisation is necessary to minimize cost and time in a particular process. Optimisation is a useful tool to find the optimum conditions of variables including, temperature, pH and reagent concentration that might affect the response of the process (Bezerra *et al.*, 2008). Thus, the objective of this study was to optimize the enzymatic hydrolysis conditions of mud crab hydrolysis to obtain maximum ACEI activity.

MATERIALS AND METHODS

Materials

Mud crabs were purchased from a mud crab supplier in Sg. Petani, Kedah, Malaysia. Mud crabs were brought alive to laboratory. The crabs were cut into half and their bellies and gills were discarded. The crabs were then washed and steamed for four minutes and allowed to cool at room temperature before the meat was collected. The meat was homogenized using a food processor and kept in a freezer (-20ºC) until further use. Crude protein content of the homogenized meat was determined using Kjeldahl method (AOAC, 2000). Protein content is needed to calculate the weight of mud crab meat, water and proteinase needed in the hydrolysis mixture (Kristinsson & Rasco, 2000).

Chemicals and reagents

Commercial food grade enzymes, Alcalase®, Protamex® and Neutrase® were purchased from Novozymes Sdn Bhd., while papain, angiotensin converting enzyme from rabbit lung (ACE), Hippuryl-His-Leu acetate salt (HHL) were from Sigma-Aldrich. All the other chemicals used in this study were of analytical grades.

Screening of commercial food grade proteinases to yield maximum ACEI activity

The hydrolysis of mud crab meat for each commercial food grade proteinase was carried out at the suggested conditions by their manufacturer i.e. Alcalase® at pH 8.5 and 55ºC, Protamex® at pH 6.5 and 50ºC, Neutrase® at pH 7.0 and 55ºC, and papain at pH 6 and 50ºC. For each proteinase, 4 hydrolysis times were employed which were 1, 2, 3 and 4 hours. The proteinase giving the highest ACEI activity was used in the optimisation study.

Enzymatic hydrolysis of mud crab

Hydrolysis mixture of mud crab flesh, enzyme and water was prepared according to Kristinsson & Rasco (2000). First, the homogenized mud crab was boiled at 85ºC for 20 minutes to inactivate endogenous enzymes. The temperature and pH were adjusted accordingly following the screening and optimisation in water bath and using 1 M HCl and 1 M NaOH respectively. Then, the mixture was heated at 85ºC in order to stop the hydrolysis process and cooled at room temperature before centrifuged at 10,000 rpm for 5 minutes. Finally, the supernatant was lyophilized.

Experimental design for optimisation study

Based on screening study, Protamex® was chosen to be used in the optimisation study to yield maximum ACEI activity from mud crab meat. Response surface methodology was employed in this study. A three level face-centered central composite design (CCD) was used (Table 1). Design-Expert 8.0.7.1 software (Stat-Ease Inc.) was used to generate the experimental runs and data analysis. There were 30 experimental runs with 6 central points. Design-Expert 8.0.7.1 software (Stat-Ease Inc.) was used to evaluate the prediction of the model equation from the experimental data using polynomial function. Finally, the response surface and the counter plots of the response as a function of the independent variables were obtained and the optimum points were suggested by the software.

Table 1. Independent variables used in optimisation study

Independent variables	Symbol	Low actual	High actual	Low coded	High coded
Temperature	X_1	45	65	-1	+1
pH	X_2	5.5	7.5	-1	+1
Hydrolysis time	X_3	1	4	-1	+1
E/S ratio	X_4	1	3	-1	+1

For verification of model, six replications of mud crab meat hydrolysis prepared at the predicted optimum condition were carried out. The resulting supernatant from the hydrolysates were then freeze-dried prior to ACEI assay. This experimental value of ACEI activity was then compared with the predicted ACEI activity value obtained from RSM under optimum condition, using one sample t-test.

ACEI assay

ACEI assay measures the inhibition of the HHL conversion to hippuric acid (HA) by ACE. The ACE inhibitory assay was carried out according to Ahn *et al.* (2012). Firstly, the sample (50 µl of 10 mg/ml) and ACE solution (50 µl of 25 mU/mL) were pre-incubated at 37ºC for 10 minutes. Then, the substrate (150 µl of 8.3 mM HHL in 50 mM sodium borate buffer containing 0.3 M NaCl at pH 8.3) was added to the mixture and incubated for 30 minutes at 37ºC. At the end of the incubation, HCl (250 µl of 1 M) was added to the mixture to stop the reaction. HA acid was extracted using 500 µl of ethyl acetate, and the resulting 200 µl aliquot of the extract was evaporated in a dry oven at 80°C. Then HA was dissolved in 1 mL of distilled water and the absorbance was measured at 228 nm using UV-spectrophotometer. Half maximal inhibitory concentration (IC_{50}) is defined as an effective concentration of hydrolysate that is required to obtain 50% of ACEI activity (Liu *et al.*, 2013). The methodology to obtain IC_{50} was carried out by using a serial dilution of lyophilized mud crab meat hydrolysate prepared at optimum conditions (0, 0.3125, 0.625, 1.25, 2.5, 5 mg/mL). Finally, a plot of ACEI activity versus concentration of mud crab meat hydrolysate was plotted to obtain the IC_{50} value.

Estimation of molecular weight estimation of protein hydrolysate

Molecular weight estimation of mud crab hydrolysate was carried out by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Tovar-Pérez *et al.* (2009) with slight modification. In this study, 20 µg of sample was loaded into 5% (w/v polyacrylamide) stacking gel and 12% (w/v polyacrylamide) separating gel. Electrophoresis was started with 100 V for 10 minutes and continued with 150 V for another 60 minutes. The gel was viewed with Coomassie brilliant blue R-250 under gel documentation (Bio-Rad, USA).

Statistical analysis

Analysis of variance of the RSM model was carried out using Design Expert (version 8.0.7.1 software) (StatEase Inc.). Mean values were accepted as significantly different at 95% level ($p < 0.05$). The mean of experimental ACEI activity and predicted ACEI activity under optimum conditions were compared using one-sample t-test (IBM SPSS v. 20).

RESULTS AND DISCUSSION

Screening of commercial food grade proteinases to yield ACEI activity

Alcalase®, Protamex® and Neutrase® are endopeptidases that break the peptide bond of non-terminal amino acids while papain is an exopeptidase that breaks the terminal amino acid (Fernandes, 2016). The results for screening study using 4 commercial food grade proteinases are summarized in Figure 1. This figure shows that Protamex® exhibited the highest ACEI activities for all hydrolysis times tested, while the lowest ACEI activity was given by papain hydrolysis. Hence, Protamex® was chosen to be used in the optimisation study. This study also shows that endopeptidases is more effective in producing ACEI peptide from mud crab meat.

Analysis of variance (ANOVA) of model from optimisation of enzymatic hydrolysis of mud crab meat

ACEI activities from 30 experimental runs ranged from 58.01–96.01%. Design Expert 8.0.7.1 software was used to analyze the data. Table 2 shows the ANOVA table of the quadratic model after model reduction ($p < 0.0001$). A non-significant lack-of-fit ($p=0.3419$) shows a good fit to the suggested model. The coefficient of determination ($p=0.8089$) was in good agreement with adjusted R-square ($p=0.7083$).

The final equation in terms of coded factors is as follows:

$$\text{ACEI activity} = +69.14 + 1.57\,A - 2.93\,B + 1.12\,C - 2.59\,D + 5.21\,A^2 - 3.42\,C^2 + 4.34\,AC - 5.87\,AD - 2.53\,CD$$

The final equation for the model in terms of actual factors is as follows:

$$\text{ACEI activity} = 210.23 - 5.12\,A - 4.84\,B - 9.15\,C + 33.92\,D + 0.05\,A^2 - 1.52\,C^2 + 0.30\,AC - 0.60\,AD - 0.77\,BC - 1.69\,CD$$

The equation in terms of actual factors shows that the ACEI activity was mostly affected by E/S ratio, followed by hydrolysis time, temperature and finally pH.

Quadratic model has been reported for optimisation of ACEI activity from many protein sources including Pacific hake fillet, *Porphyra*

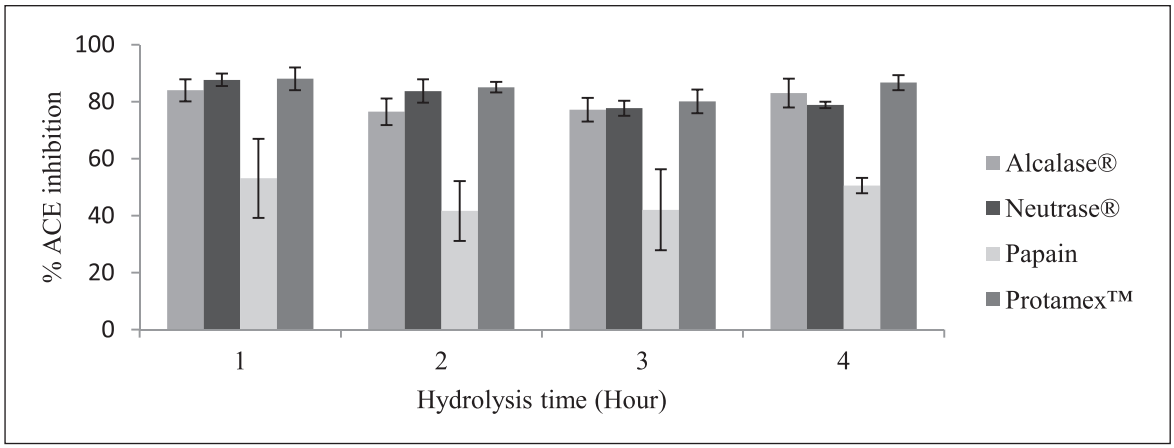

Fig. 1. Effect of commercial food grade proteinases on the ACEI activity of mud crab meat hydrolysate.

Table 2. Analysis of variance table of the quadratic model after model reduction

Source	Sum of squares	DF	Mean square	F	Prob > F
Model	1412.33	10	141.23	8.04	< 0.0001**
A – temperature	44.15	1	44.15	2.51	0.1294
B – pH	154.47	1	154.47	8.79	0.0079**
C – hydrolysis time	22.58	1	22.58	1.29	0.271
D – E/S ratio	120.69	1	120.69	6.87	0.0168**
A^2	93.61	1	93.61	5.33	0.0324*
C^2	40.33	1	40.33	2.3	0.1462
AC	300.76	1	300.76	17.12	0.0006**
AD	551.66	1	551.66	31.41	< 0.0001**
BC	21.09	1	21.09	1.2	0.2869
CD	102.47	1	102.47	5.83	0.026*
Residual	333.74	19	17.57		
Lack of Fit	269.91	14	19.28	1.51	0.3419
Pure Error	63.83	5	12.77		
Cor Total	1746.07	29			
R-Squared	0.8089				
Adj R-Square	0.7083				
Pred R-Square	0.5240				
Adeq Precision	13.276				

*Significant within a 95% confidence interval.
**Significant within a 99% confidence interval.

yezoenis, goldstripe sardine and salmon skin (Cinq-Mars & Li-Chan, 2007; Taheri *et al.*, 2011; Qu *et al.*, 2010; See *et al.*, 2011).

Analysis of the response surface methodology

To better understand the influence of the independent variables on the ACEI activity, the data were presented as the response surface graph in which two variables were set to constant. Figure 2 shows the antagonistic effects between temperature and E/S ratio whereby higher ACEI activity was obtained at higher temperature (above 55ºC) and lower E/S as well as at lower temperature and higher E/S. Figure 2 also shows that increase in temperature between 55ºC to 65ºC caused the ACEI activity to increase particularly at 1–2% of E/S ratio while increased in E/S ratio from 2–3% at temperature below 50ºC could also increase ACEI activity. On the contrary, Figure 3 shows that maximum ACEI activity was obtained at high temperature and longer hydrolysis time. Figure 4 shows that ACEI activity is maximum at moderate hydrolysis time and lower pH. It is also noted that lower ACEI activity was obtained at extreme hydrolysis time and higher pH.

Design Expert software suggested 10 optimum conditions of mud crab hydrolysis using Protamex® to yield maximum ACEI activity (Table 3). Solution number 1 which is 65ºC, pH 5.6, 1% E/S and 4 hours of hydrolysis time was chosen as the optimum condition because it gave the desirability closest to

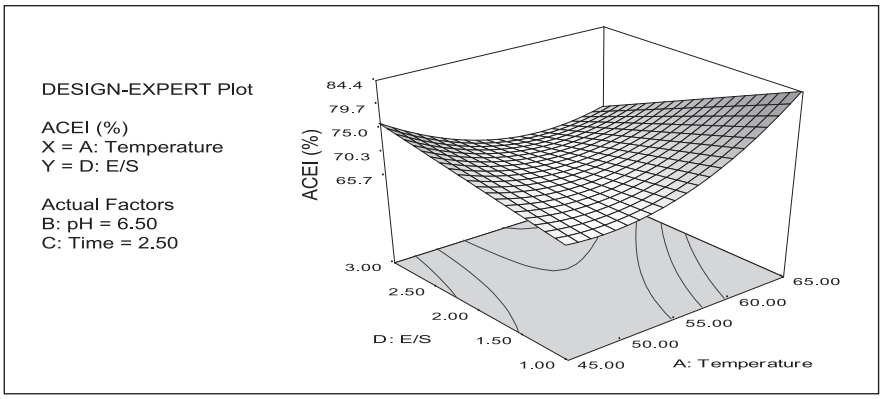

Fig. 2. Effect of temperature and E/S on ACEI activity.

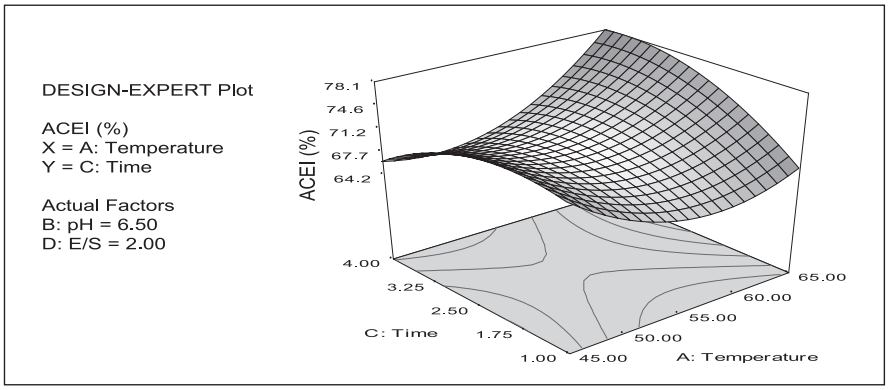

Fig. 3. Effect of hydrolysis time and temperature on ACEI activity.

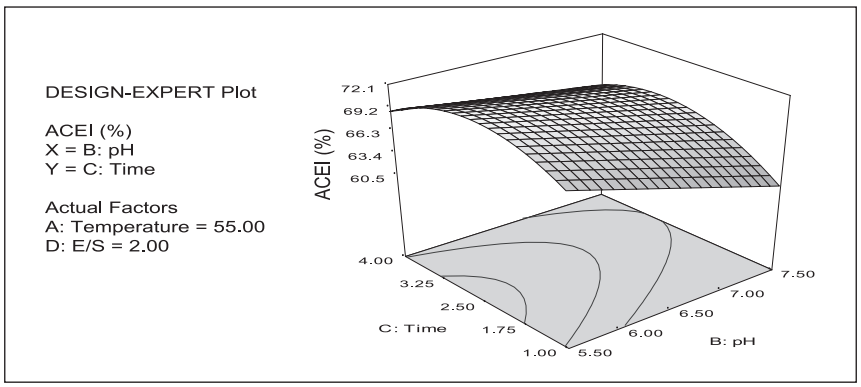

Fig. 4. Effect of hydrolysis time and pH on ACEI activity.

Table 3. Suggested optimum conditions to produce maximum ACEI activity from mud crab meat hydrolysis using Protamex®

Number	Temperature	pH	Hydrolysis time	E/S	ACEI (%)	Desirability
1	65	5.6	4	1	91.10	0.867
2	65	5.5	4	1	90.9	0.866
3	65	5.8	4	1	90.3	0.851
4	65	5.8	4	1	90.2	0.848
5	65	7.4	4	1	85.7	0.727
6	65	5.5	3	1	84.7	0.702
7	45	5.5	1	3	80.5	0.592
8	45	5.6	1	3	80.3	0.585
9	45	5.5	1	3	78.7	0.543
10	45	5.5	1	2	77.5	0.511

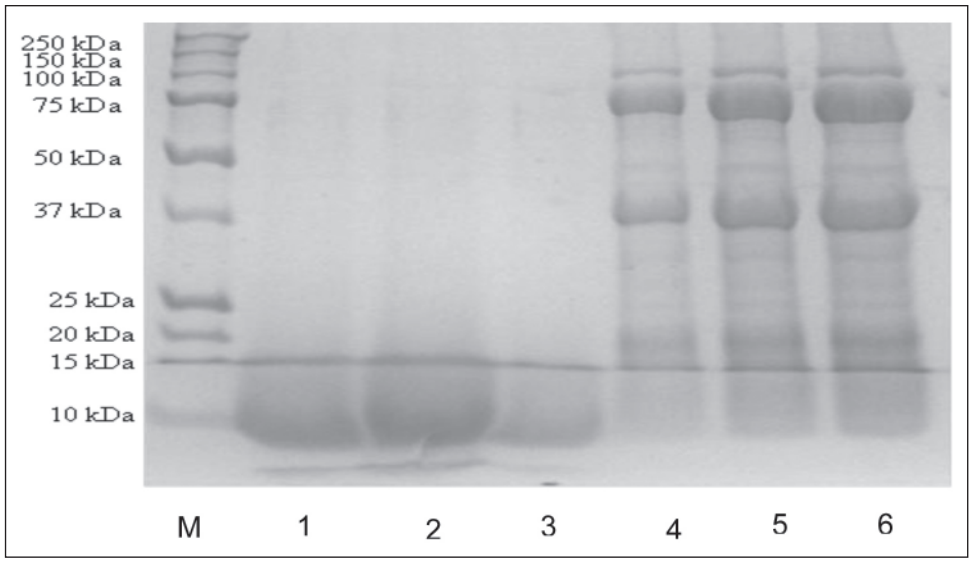

Fig. 5. Molecular weight of protein marker (well M), mud crab hydrolysate (well 1–3) and mud crab meat (well 4–6).

1. Protamex® is active in a wide pH range from pH 5 to pH 11 (Olajuyigbe & Ajele, 2008) and it is 95% stable at 60ºC and may retain 50% of its activity at temperature of 80ºC (Olajuyigbe & Ajele, 2008). Similar range of optimum hydrolysis time (4 hrs) and pH (pH 6.2) has been reported for Pacific hake fillet hydrolysis using Protamex® to yield maximum ACEI activity. However, lower optimum temperature (40-50ºC) and higher optimum E/S ratio (2–4.5%) have been reported for hydrolysis of marine microalgae, salmon by product protein, Pacific hake fillet and hard clam using Protamex® to yield maximum ACEI activity (Ahn *et al.*, 2012; Ko *et al.*, 2012a; Cinq-Mars & Li-Chan, 2007; Tsai *et al.*, 2008).

Verification of the model

Verification of the model was conducted with six replicates of the hydrolysis carried out at the predicted optimum condition. The average ACEI activity was 88.93±1.02%. One-sample t-test shows that, under the optimum condition, there is no significant difference (p>0.05) between the experimental ACEI activity (88.93±1.02%) and predicted ACEI activity (91.10%). This shows that this model is valid and it can be used to explain the relationship between the experimental conditions of mud crab meat hydrolysis by Protamex towards ACEI activity.

The IC_{50} of the mud crab hydrolysate prepared at optimum condition was found to be 1.96±0.13 mg/mL, which was higher than IC_{50} values that has been reported for protein hydrolysate from seaweed (1.6 mg/ml) and *Chlorella ellipsoidea* (1.47 mg/mL) (Qu *et al.*, 2010; Ko *et al.*, 2012a). In addition, other hydrolysis that was reported more potent ACEI activity than mud crab hydrolysate including hydrolysate of *Styela clava* flesh (0.455 mg/mL), grass carp (0.872 mg/mL), skate skin (0.68 mg/mL) and hard clam (0.036 mg/mL) (Chen *et al.*, 2012; Ko *et al.*, 2012b; Lee *et al.*, 2011; Tsai *et al.*, 2008). Further purification/fractionation can be used to reduce the IC_{50} value of protein hydrolysate (Cinq-Mars & Li-Chan, 2007).

Molecular weight of protein in mud crab meat and its hydrolysate

The molecular weight (MW) of protein from of mud crab hydrolysate was distributed mostly at 15 kDa and below (Figure 5). The molecular weights of the hydrolysate were more intense around 10 kDa and below compared to mud crab meat, which gave higher MW including 100 kDa, 75 kDa, and 37 kDa. It was noted that there was no protein resolved around 10 kDa and below in the mud crab meat. These results suggest that mud crab meat protein had been hydrolyzed into peptides during the enzymatic hydrolysis using Protamex. This finding is consistent with the fact that ACEI peptide is usually comprised of peptide with 3–20 amino acids, which correlates with lower molecular weight (normally < 10 kDa) (Ko *et al.*, 2012b; Jang & Lee, 2005; Esteve *et al.*, 2015).

CONCLUSION

Quadratic model can be used to explain the relationship between hydrolysis conditions of mud crab meat using Protamex® towards its ACEI

activity. The optimum condition was obtained at 65ºC, pH 5.6, 1% E/S and 4 hours of hydrolysis time. This study shows that Response Surface Methodology can be used to predict the effect of hydrolysis conditions to produce the best yield of natural and safer ACEI peptide from mud crab meat. Further study should be carried out on purification of ACEI peptide from mud crab, determination of its amino acid sequence, efficacy of ACEI peptide on spontaneously hypertensive rats, tissue culture model and human subject as well as the stability of mud crab ACEI peptide against temperature, pH and digestive enzymes. Comparison of ACEI activity between natural ACEI peptide from mud crab and that of synthesized peptide with amino acid sequence similar to ACEI peptide from mud crab can be carried out as well.

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