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Original Article

Optimization of enzymatic protein hydrolysis conditions to obtain maximum angiotensin-I converting enzyme (ACE) inhibitory activity from flower crab (*Portunis pelagicus*) meat

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Abstract

In this study, optimization of enzymatic protein hydrolysis conditions of flower crab meat (FCM) to yield maximum angiotensin converting enzyme (ACE) inhibitory activity was carried out. First, screening of commercial food grade enzymes (Alcalase®, Neutrase®, Protamex® and papain) was carried out to select the most suitable proteinase to yield ACE inhibitory activity. A 3-level face-centered central composite design (CCD) was employed to optimize four hydrolysis conditions including temperature (45-55°C), hydrolysis time (1-3 hr), pH (6-8) and enzyme to substrate ratio (E/S) (1-3%). Half maximal inhibitory concentration (IC_{50}) of FCM hydrolysate prepared at optimum condition was also determined. It was found that the highest ACE inhibitory activity (85.52%) was given by Neutrase® after 2 hr hydrolysis. Hence, Neutrase® was used in the optimization study. It was found that the enzymatic hydrolysis condition of FCM towards ACE inhibitory activity could be predicted by a quadratic model. The optimum enzymatic hydrolysis condition to obtain maximum ACE inhibitory activity was at temperature of 54°C, E/S of 3%, pH of 7 and hydrolysis time of 1 hr. It was found that the predicted value of ACE inhibitory activity (97.21%) was close to that of experimental value (90.34%) with the IC_{50} of 0.425±0.05 mg/ml.

Keywords: Optimization, Angiotensin-I Converting Enzyme, Enzymatic hydrolysis, Flower crab

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Introduction

Hypertension is one of the chronic health problems contributing to arteriosclerosis, stroke, myocardial infarction and end stage renal disease (He et al., 2013). It was estimated that the number of people affected globally with hypertension will increase from year 2000 to 2025 (Poulter et al., 2015). In Malaysia, prevalence of hypertension among adults of age 30 and above was reported to increase from 32.9% as reported by National Health and Morbidity Survey II (NHMS II) to 42.6% in the NHMS III survey (Kiau et al., 2013; Institute for Public Health, 2015). Angiotensin-converting enzyme (ACE, peptidyldipeptide hydrolase E.C. 3.4.15.1) plays important role in regulating blood pressure by catalysing the conversion of the biologically inactive angiotensin I to the potent vasoconstrictor angiotensin

II and also inactive the potent vasodilator, bradykinin that cause blood pressure to increase (Guang et al., 2012). Hence, ACE inhibition has been an important pharmacological target for the treatment of hypertension (Izzo and Weir, 2011).

In hypertensive treatment, synthetic ACE inhibitor drugs such as captopril, enalapril and fosinopril may cause side effects, such as cough, skin rashes and angioedema (Wijesekara and Kim, 2010). Thus, efforts to search for safe, effective and natural ACE inhibitors are needed. To date, ACE inhibitory peptides derived from food proteins were proven to have excellent ACE inhibition activity and they were safer compared to synthetic ACE inhibitors (Li et al., 2004; Wijesekara and Kim, 2010). ACE inhibitory peptides have been identified in various types of food protein including from marine origin such as salmon by product protein (Ahn et al., 2012), squid gelatin (Alemán et al., 2011), grass carp (Chen et al., 2012), haruan (Ghassem et al., 2011), *Styela clava* (Ko et al., 2012), hard clam (Tsai et al., 2008) and oyster (Wang et al., 2008).

Bioactive peptides are inactive within the sequence of their precursor protein and need to be released to exert the bioactivities via hydrolysis (Bhat et al., 2015). Protein hydrolysis can be carried out using enzymatic process with the use of proteinase or by chemical reaction with acid or alkali (Sujith and Hymavathi, 2011). Enzymatic hydrolysis is strongly preferred for nutritional applications because it is carried out under milder conditions and produce specific hydrolysate of defined molecular weight distribution, peptide composition and degree of hydrolysis.

Enzymatic hydrolysis can be controlled and optimized to obtain the best response to save cost and time. Optimum enzymatic protein hydrolysis generally depend on the type of proteinase, pH, hydrolysis time, enzyme to substrate concentration and temperature (Sujith and Hymavathi, 2011). Response Surface Methodology (RSM) had been widely used in the optimization of enzymatic hydrolysis of marine protein to produce maximum ACE inhibitory activity. Previously, optimization of enzymatic of enzymatic hydrolysis produce maximum ACE inhibitory activity had been carried out on marine protein such as ribbonfish backbone (Wang et al., 2011), lizard fish (Wu et al., 2012), anglewing clam (Amiza et al., 2017), blood cockle (Aishah et al., 2017) and mud crab (Harun et al., 2017).

Flower crab or Blue swimming crab (*Portunus pelagicus*) is one of the important crab species in Malaysia. The total landing of flower crab in Malaysia was 1061 metric tons in 2016 (Department of Fisheries, 2016). According to Gökoðlu and Yerlikaya (2003), flower crab species contain high amount of protein compared to other species that might serve as the potential source of bioactive peptides. Thus, the aim of this study was to optimize the enzymatic protein hydrolysis condition of flower crab protein to obtain maximum ACE inhibitory activity.

Material and Methods

Material

Twenty five kilograms of whole fresh flower crabs were purchased from a wholesale fish market in Pulau Kambing, Kuala Terengganu. The flower crabs were cut into two and washed to remove all the contaminants. The flower crabs were then steamed for 3 minutes and the meat was separated from the carapace of the flower crab. Next, the flower crab meat was homogenized by using food processor at high speed for about 5 minutes until it turned into a paste form. Crude protein analysis was carried out based on AOAC methods (2000) using Kjeltech system (Foss Tecator Digestor DS 6 2006), in order to calculate for the mass of crab meat, water and proteinase in the enzymatic hydrolysis as described by Amiza and Masitah (2012). The flower crab meat was frozen at -18˚C until further use.

Screening of the protease for enzymatic hydrolysis

Screening study was carried out to determine the most suitable commercial food grade enzymes to produce hydrolysate with high ACE inhibitory activity. Four commercial enzymes which were Alcalase®, Protamex®, Neutrase® and papain were used to hydrolyse the flower crab meat at 2 and 4 hr of hydrolysis time. The enzyme giving the highest ACE inhibitory activity was optimized to produce hydrolysate with maximum ACE inhibitory activity. The pH and temperature used for each proteinase were in the range suggested by the manufacture i.e. Alcalase® at pH 8 and 55ºC, Protamex® at pH 6.5 and 55ºC, Neutrase® at pH 6.5 and 50ºC and papain at pH 6 and 60ºC. The enzyme to substrate (E/S) ratio was set to 1% (w/w) for all proteinase.

Preparation of flower crab meat hydrolysate

Flower crab meat hydrolysates were prepared using enzymatic protein hydrolysis. Firstly 59.18 g of crab meat was mixed with 28.82 g of water by using a homogenizer and was heated at 85°C for 20 min in water bath (Shaker bath-903, Protech®, UK) to inactive the endogenous enzyme in the sample. pH was adjusted according to the designated pH using 2 M NaOH using a pH meter (InoLab pH 720, WTW GmbH, Germany) and the mixture was placed in the water bath that was set at the designated temperature. The hydrolysis was started by adding 20 g proteinase solution (proteinase and distilled water) to the mixture. After the enzymatic hydrolysis was completed, the mixture was heated at 85˚C for 20 min to inactivate the enzyme. Next, the hydrolysate was centrifuged at 10000 rpm for 10 min and the supernatant was collected and lyophilized. The flower crab meat hydrolysate powder was then stored in airtight container for further analysis.

ACE inhibitory activity assay

The assay of ACE inhibitory activity was measured according to Ahn et al. (2012) and according to Cushman and Cheung (1971). It is based on inhibition of ACE that hydrolyses hippuryl-L-histidyl-L-leucine (HHL) to hippuric acid (HA) and histidyl-leucine (HL). For each assay, 50 µL of the sample hydrolysate (10 mg/ml) was preincubated with 50 µL of ACE solution (25 mU/mL) at 37˚C for 10 min in water bath. Then, 150 µL of the substrate (8.3 mM HHL in 0.5M sodium borate buffer which containing 0.3 M NaCl at pH 8.3) was added to the mixture and incubated for 30 min at 37˚C. The reaction was terminated by adding 250 µL of 1 M HCl and 500 µL of ethyl acetate was added to extract HA from the mixture. The mixture was centrifuged at 100 rpm for 2 min before 200 µL of the upper layer containing the extracted HA was took out and it was evaporated in a dry oven at 80˚C for 30 min until it forms residue. The residue was dissolved in 1 ml of distilled water and the absorbance was measured at 228 nm.

Optimization of enzymatic hydrolysis using Neutrase®

Optimization of four parameters of enzymatic hydrolysis of flower mud crab using Neutrase® was carried out using Response Surface Methodology (RSM). A three-level face-centered Central Composite Design (CCD) was employed. The independent experimental variables were temperature (45-55°C), hydrolysis time (1-3 hr), pH (6-8) and Neutrase® to substrate ratio (E/S) (1-3%), which were employed at three equidistant levels (-1, 0 and +1), with ACE inhibitory activity as the response variable. In this study, a total of 30 runs of FCM enzymatic hydrolysis were employed. Experimental runs were randomised to minimise the effects of unexpected variability in the observed response.

Verification of the model

Four replications of flower crab meat hydrolysis prepared at the predicted optimum condition were carried out to validate the model. The resulting supernatant from the hydrolysates were then freeze dried prior to determination of ACE inhibitory activity. The experimental values of ACE inhibitory activity were then compared with the predicted value obtained from RSM, using one-sample t-test.

Determination of half maximal inhibitory concentration (IC_{50})

Half maximal inhibitory concentration (IC_{50}) is the concentration of inhibitor or sample to inhibit 50% of ACE activity. Several ranges of hydrolysate concentration from 0.2 to 1.0 mg/mL were used to determine the IC_{50} value. A plot of ACE inhibitory activity versus concentration of flower crab meat hydrolysate was plotted to obtain the IC_{50} value.

Statistical analysis

Data from screening study was analysed using one-way ANOVA. For the optimization data, Design Expert 6.0.10 software (StatEase Inc. USA) was used to carry out statistical analysis. The software gave important analysis including the sequential model sums of squares, ANOVA table, final equation, suggested solutions for optimization, response surface plots and point prediction table. Significance level was set at 95% probability level. Data for verification of the model was analysed using one-sample t-test.

Results and Discussion

Effect of different types of commercial food grade proteinase and hydrolysis time on the ACE inhibitory activity of flower crab meat

Figure 1 shows the ACE inhibitory activity of flower crab hydrolysate prepared using different types of commercial food grade proteinases and hydrolysis time. Hydrolysis with papain for 2 hr gave the highest ACE inhibitory activity (90.5%±0.81) followed by Neutrase® for 2 hr (85.52%±5.13), Neutrase® for 4 hr (80.76%±3.06), papain for 4 hr (75.52%±3.73), Protamex™ for 4 hr (71.03%±1.61), Protamex™ for 2 hr (64.48%±1.18), Alcalase® for 2 hr (55.72%±4.42) and lastly Alcalase® for 4 hr (50.76%±6.71). There were no significant ($p>0.05$) difference in ACE inhibitory activities between Protamex® and Alcalase® at 2 and 4 hr of hydrolysis. However, Neutrase® and papain gave significantly higher ACE inhibitory activities ($p<0.05$) compared to Protamex® and Alcalase®. For optimization study, Neutrase® was chosen due to economical reason whereby Neutrase® is cheaper than other food grade enzymes. Besides, the hydrolysis of protein using Neutrase® produced from a strain of *Bacillus amyloliquefaciens* require a neutral pH or slightly acidic pH which produces ACE inhibitory peptide that is more stable in acidic and slightly alkaline pH (Qu et al., 2010). The ACE inhibitory activity also stable over time with Neutrase® hydrolysis because the result shows the highest ACE inhibitory activity at four hr of hydrolysis time compared to other proteases. Moreover, previous studies also reported that food protein hydrolysate by Neutrase® showed potent ACE inhibitory activities such as from modified egg protein by-products (Pokora *et al.*, 2013), chicken collagen (Soladoye *et al.*, 2015) and cottonseed (Goa et al., 2010). [Figure 1]

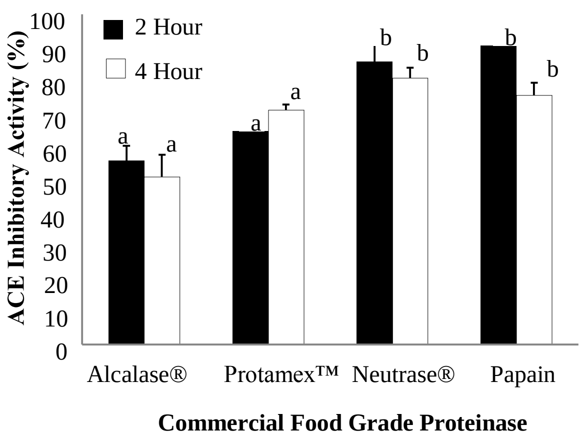

Fig. 1. ACE inhibitory activity (%) of flower crab hydrolysate prepared using different types of commercial food grade proteinase

Optimization of enzymatic hydrolysis of flower crab using Neutrase® to obtain maximum ACE inhibitory activity

Response surface methodology (RSM) was used to optimize the enzymatic protein hydrolysis of flower crab meat using Neutrase®. The optimization data obtained were analyzed using Design Expert 6.0.10 software (StatEase Inc. USA). There were four independent variables which were temperature, hydrolysis time, pH and Neutrase® to substrate (E/S) ratio that were tested simultaneously to obtain maximum ACE inhibitory activity. It was found that ACE inhibitory activity obtained from 30 experimental runs ranged from 43.99% to 97.26%.

Table 1 summarizes the analysis of variance (ANOVA) of the reduced quadratic model of the optimization study. The table shows that the model was significant ($p<0.01$) and the data could be fitted to a quadratic model as represented by the insignificant lack of fit ($p>0.05$). In addition the coefficient determination ($R^2 = 0.8237$) was high and in agreement with the adjusted R^2 ($R^2 = 0.7566$). [Table 1]

The final equation for the model in coded factors is given as:

ACE inhibitory activity = $+87.81 - 2.73A - 0.73B - 2.74C + 0.86D + 21.11A^2 - 5.53AB - 6.04BC + 10.06CD$

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

ACE inhibitory activity = $-900.0137 + 303.87{*}pH + 98.38{*}time - 2.10{*}temperature - 99.76903{*}E/S - 21.11{*}pH^2 - 5.53312{*}pH{*}time - 1.20762{*}time{*}temperature + 2.01263{*}temperature{*}enzyme\ to\ substrate\ ratio$.

Table 1 shows that the model term that were significant in this model included A^2, AB, BC and CD. As stated in the final equation for the model in coded factors, temperature is the most influential variable affecting the ACE inhibitory activity, followed pH, enzyme to substrate ratio (E/S) and hydrolysis time.

Response surface plot and effect of independent variables on ACE inhibitory activity

The response surface plot was used to examine the interaction between independent variables on ACE inhibitory activity. Figure 2 shows the response surface plot of hydrolysis time and pH on ACE inhibitory activity at 50°C and 2% of enzyme to substrate (E/S) ratio. The plot shows that ACE inhibitory activity was highest at intermediate pH, while temperature shows only slight effect on the ACE inhibitory activity. Nevertheless, linear contour shows that there is an antagonistic interaction between pH and hydrolysis time. ACE inhibitory activity increased with increasing hydrolysis time at lower pH, but decreased with decreasing hydrolysis time at high pH. This is in agreement with optimization of enzymatic hydrolysis of mud crab and angle wing clam that reported high ACE inhibitory activity at lower pH and longer hydrolysis time (Harun et al., 2017; Amiza et al., 2017). According to Guo et al. (2009), initially, the hydrolysis may release the ACE inhibitory peptides resulting in an increase in ACE inhibitory activity. However, prolonged treatment may result in the hydrolysis of ACE inhibitory peptides subsequently in which the activity increased with increased of temperature and hydrolysis time. However, there was a significant antagonistic interaction between temperature and hydrolysis time at high temperature and high hydrolysis time. High temperature may cause denature of the protein and disruption of the active site structure. According to Qu et al. (2010), the ACE inhibitory activity will start to decrease and cease in prolonged hydrolysis. In addition, increase temperature of hydrolysis time will increase the ACE inhibitory activity until it reached an optimum point and beyond the optimum point, the ACE inhibitory activity reduced (Wu et al., 2012). Nevertheless Guo et al. (2009) also reported high ACE inhibitory activity at low and high temperatures. [Figure 3]

Figure 4 shows the response surface plot of enzyme to substrate (E/S) ratio and temperature ACE inhibitory activity that influence. It shows that both E/S and temperature gave linear relationship on ACE inhibitory activity. The graph shows that at high temperature (55°C), increase of E/S ratio will increase the ACE inhibitory activity. Similarly, at high E/S ratio (3%), increase of temperature caused the ACE inhibitory activity to increase. A synergistic effect could be observed in the plot that shows low E/S ratio interaction with temperature to produce high ACE inhibitory activity. Increasing E/S ratio will increase the rate of reaction, as more enzymes will be colliding with substrate molecules. However, further increased in E/S ratio will not improve ACE inhibitory activity (Guo et al., 2009; Aishah et al., 2017). Besides, Cinq-Mars et al. (2007) proved that protease at high concentration cleaved protein at sites that do not facilitate ACE-inhibitory activity, thus reduce the ACE inhibitory activity. In addition, too high temperature may cause denature of the protease and disruption of its active site structure that eventually cause reduced the production of ACE inhibitory peptides. [Figure 4]

In order to get the optimum condition of enzymatic hydrolysis of flower crab using Neutrase®, a defined goal of the variables were set to produce maximum ACE inhibitory activity. The independent variables degraded the peptides. In addition, optimum pH at neutral pH will produce ACE inhibitory peptides that are stable in acidic and slightly alkaline pH (Qu et al., 2010). [Figure 2]

Figure 3 shows response surface plot of temperature and hydrolysis time on ACE inhibitory activity at pH 7 and 2% of enzyme to substrate (E/S) ratio. Both variables show linear effect on ACE inhibitory activity were set as "in range" while for the ACE inhibitory activity, it was set as "maximum". Based on the optimization study, the optimum conditions that were extracted by Design Expert 6.0.10, software were at pH 7, 1 hour hydrolysis time, temperature of 54°C and 3% of E/S ratio with the expected ACE inhibitory activity of 97.31%.

Verification of the optimum conditions for ACE inhibitory activity

In order to verify the optimum conditions of enzymatic hydrolysis of flower crab using Neutrase® that were suggested by RSM, actual hydrolysis of flower crab using the optimum conditions were carried out. The hydrolysis was conducted in quadruplicate producing four hydrolysates that subjected to be verified with the suggested optimum conditions. The mean of ACE inhibitory activity for quadruplicate hydrolysates was 90.52%±3.13. This value was close to the predicted value (97.31%) and there was no significant difference ($p>0.05$) between the values. This indicates that the suggested quadratic model can be used to predict the ACE inhibitory activity of enzymatic hydrolysis of flower crab meat using Neutrase®.

Half maximal inhibitory concentration (IC50) of flower crab meat hydrolysate prepared under optimal conditions

To evaluate the potential of ACE inhibitory activity of flower crab hydrolysed by Neutrase®, half maximal inhibition of ACE was determined. The IC_{50} value was defined as the concentration of peptide required to reduce 50% of ACE activity, which was determined by plotting a linear graph of ACE inhibitory activity (%) versus peptide concentration (Goa et al., 2010). A higher ACE inhibitory activity or lower IC_{50} value indicates a stronger antihypertensive activity and could become the potential source of ACE inhibitory peptides (Qu et al., 2010; Asoodeh et al., 2012).

It was found that the IC_{50} of flower crab hydrolysed by Neutrase® was 0.425 mg/ml±0.05 which shows better inhibition activity compared to other protein sources for example *Styela clava* flesh (0.455 mg/mL) (Ko et al., 2012), grass carp (0.872 mg/mL) (Chen et al., 2012), mud crab (1.96 mg/mL) (Harun et al., 2017) and angle wing clam (3.63 mg/mL) (Amiza et al., 2017). However, the ACE inhibitory activity of flower crab was lower compared to squid gelatin (0.34 mg/mL) (Alemán et al., 2011), skate skin hydrolysate (95-148 µM) (Lee et al., 2011), lysozyme hydolysates (0.03 mg/mL) (Asoodeh et al., 2012) and cottonseed (0.16 mg/mL) (Goa et al., 2010).

According to Ambigaipalan et al. (2015), differences in the ACE inhibitor activity of these peptides could be attributed to their different amino acid compositions and hydrophobicity. In several studies, the hydrophobic amino acid residues (leucine, valine, alanine, tryptophan, tyrosine, proline or phenylalanine) are more preferably bind with catalytic sites of ACE, which act as strong competitive ACE inhibitors. In addition, according to Jamdar et al. (2010), peptide isolation from hydrolysate produced higher ACE inhibitory activity compared to hydrolysate itself. McCarthy et al. (2013) found that ACE inhibitory activity of soy protein was higher in smaller molecular weight peptides. Thus, to obtain a better ACE inhibitory activity, the flower crab meat hydrolysate can be fractionated to get smaller molecular size of ACE inhibitory peptides.

Table.1. Analysis of variance (ANOVA) of the reduced quadratic model

Source	**Sum of Square**	**DF**	**Mean Square**	**F Value**	**Prob > F**	
Model	6168.80	8	771.11	12.27	<0.0001	significant
A: pH	134.37	1	134.37	2.14	0.1585	
B: Hydrolysis time	9.62	1	9.62	0.15	0.6996	
C: Temperature	109.52	1	109.52	1.74	0.2010	
D: E/S ratio	13.38	1	13.38	0.21	0.6493	
A^2	1197.39	1	3208.55	51.05	<0.0001	
AB	489.85	1	489.95	7.79	0.0109	
BC	583.34	1	583.34	9.28	0.0061	
CD	1620.26	1	1620.26	25.78	<0.0001	
Residual	1319.99	15	71.27			
Lack of Fit	1277.96	10	97.71	5.31	0.0611	Not significant
Pure Error	92.03	5	18.41			
Cor total	7488.89	29				
R-Squared	0.8237					
Adjusted R-Squared	0.7566					
Predicted R-Squared	0.4955					
Adequate Precision	12.359					

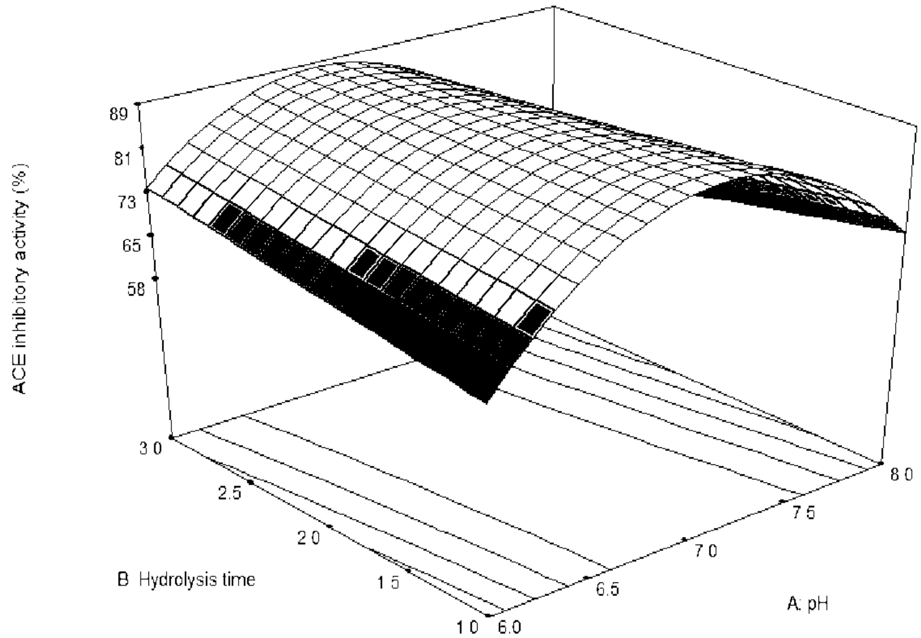

Fig. 2. Response surface plot of hydrolysis time and pH on ACE inhibitory activity

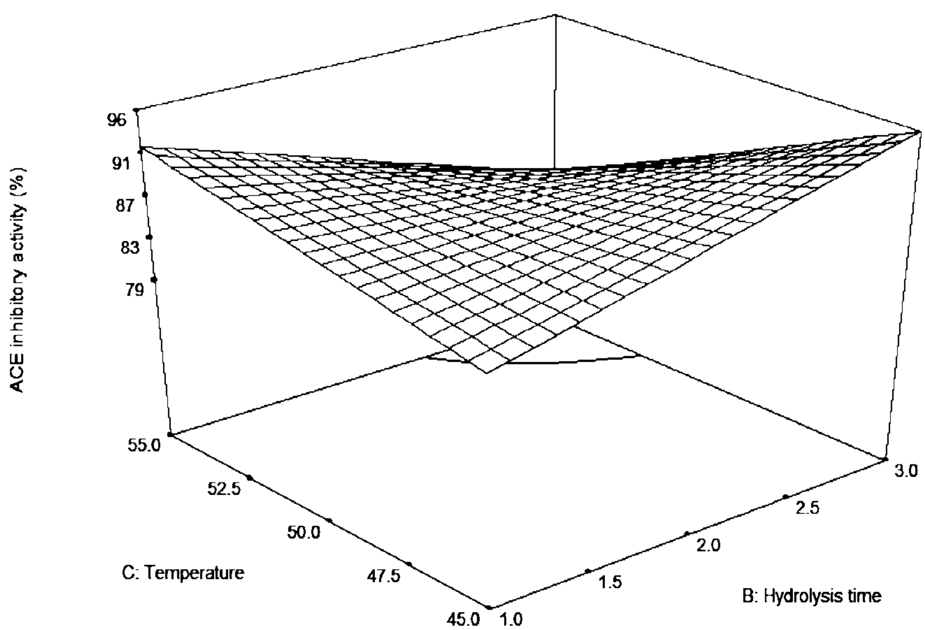

Fig. 3. Response surface plot of temperature and hydrolysis time on ACE inhibitory activity

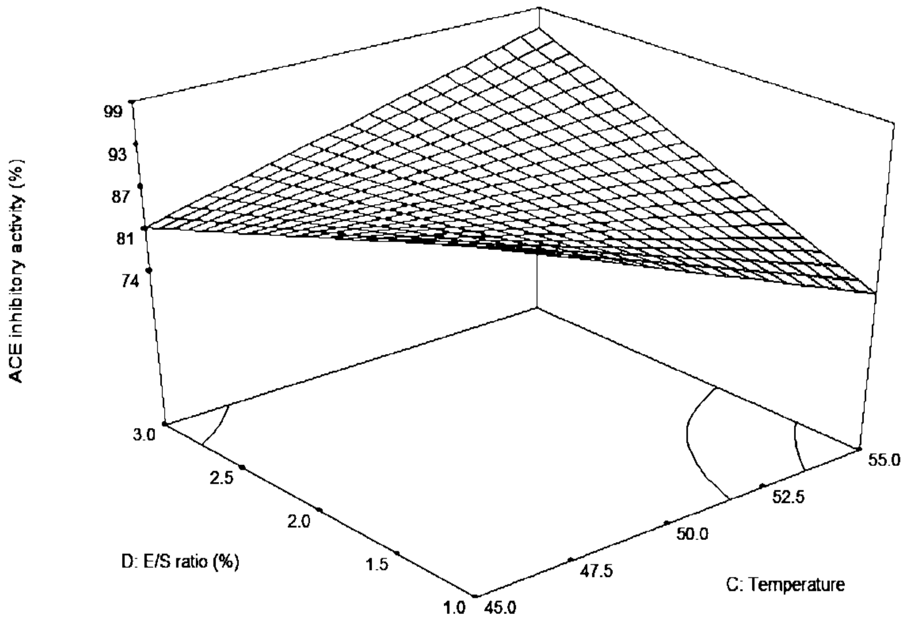

Fig. 4. Response surface plot of enzyme concentration and temperature on ACE inhibitory activity

Conclusion

This study shows that the relationship between four variables of enzymatic hydrolysis of flower crab meat using Neutrase® can be predicted using a quadratic model. Optimum condition to obtain maximum ACE inhibitory activity was found at temperature of 50°C, pH of 7, Neutrase® concentration of 3% and hydrolysis time of 1 hr. The experimental ACE inhibitory activity obtained under this optimum condition was 90.34% which was close to the predicted value of 97.21%. The low IC_{50} value of ACE inhibitory activity (0.425 ±0.05 mg/mL) of flower crab meat hydrolysate indicated that it could become a potential source of ACE inhibitory peptide.

Contribution of Authors

Amiza MA: Author of the manuscript and advised on experimental design and technical aspect.
Harun Z: Author of the manuscript and advised on data analysis.
Intan Liyana MR: Author of the manuscript and conducted laboratory work.

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