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# Optimization of enzymatic protein hydrolysis conditions on Angiotensin-converting enzyme inhibitory (ACEI) activity from blood cockle *(Anadara granosa)* meat

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**Abstract**

The aim of this study is to determine the optimization of enzymatic protein hydrolysis conditions on the angiotensin-converting enzyme inhibitory (ACEI) activity of blood cockle meat. Preliminary study was carried out to evaluate the effect of different commercial proteinases (Alcalase®, Protamex™, Neutrase®, pepsin, papain, trypsin and α-chymotrypsin) and hydrolysis time on the ACEI activity of cockle's meat. The proteinase with the highest ACEI activity will be used in the optimization study using Response Surface Methodology. A face-centered central composite design was employed to investigate the effect of hydrolysis conditions parameters (i.e hydrolysis time, pH, hydrolysis temperature and enzyme to substrate (E/S) ratio) towards ACEI activity of cockle's meat. Preliminary study found that the highest ACEI activity was given by Protamex™ at 6 hrs. Therefore, optimization study was carried out using Protamex™ at 4-8 hrs hydrolysis times with temperature of 35-60oC, pH of 5.5-7.5 and E/S of 0.5-1.5%. All variables were successfully fitted with the quadratic model ($p < 0.0001$) with a non-significant lack-of-fit ($p = 0.3665$), and also possess good coefficient of determination ($p = 0.8666$) and adjusted R-square ($p = 0.8158$). The optimum hydrolysis conditions were found to be at temperature of 59.8°C, time of 4.69 hrs, pH of 5.59 and E/S ratio of 0.9%. ACEI activity of the experimental value (97.8%) agreed with the predicted value (99.2%) within 95% confidence interval.



## Introduction

Angiotensin-converting enzyme (ACE) inhibitory peptide isolated from natural food protein sources has captured interest among researchers due to potent antihypertensive activity and minimal adverse effect to human health (Lee *et al*., 2010; Chen *et al*., 2012; Zhou *et al*., 2013). This peptide could efficiently be released by enzymatic hydrolysis that involves mild hydrolysis condition and easily controlled process with high reproducibility for consistence production of ACEI peptide (Liaset *et al*., 2000; Hammershoj *et al*., 2008; Contreras *et al*., 2011). The resulting food-derived protein hydrolysate is generally recognized as safe (GRAS) and has been commercially used as food ingredient (Olav *et al*., 2014).

Among the available food protein sources, peptide derived from marine sources is one of the identified sources of ACEI peptide (He *et al*., 2007; Ko *et al*., 2012; Wu *et al*., 2015). Blood cockle or commonly known as *Anadara granosa* is a source of cheap marine protein that is commercially cultured in the tidal mudflats along the western coast of Peninsular Malaysia and it is a good revenue source for the local population (Ibrahim, 1995). These cockles contained 15.99% of protein, 1.51% of carbohydrate, 1.93% of fat, 78.94% of moisture and 1.63% of ash (Nurnadia *et al*., 2011). Although these cockles are known as one of sources of cheap protein, only two study has been reported on optimization of blood cockle hydrolysate whereby Amiza and Masitah (2012) reported on the optimization of blood cockle hydrolysate on degree of hydrolysis of cockle while Haslaniza *et al*. (2013) reported on the optimization of cockle meat wash water precipitate for development of seafood flavour. To date, no study has been reported on its potential as a source of ACEI peptide.

Screening of proteinases have been reported in previous studies as preliminary step to determine suitable proteinase prior to preparation, isolation and characterization of the potential ACE inhibitory peptide (He *et al*., 2007; Bougatef *et al*., 2008; Lee *et al*., 2010; Qu *et al*., 2010; Jimsheena and Gowda, 2011; Ko *et al*., 2012). Many of studies demonstrated

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that the selection of proteinase highly affect the bioactivity of peptide (Qu *et al*., 2010; Ko *et al*., 2012). In fact, the adequate match of proteinase and raw material used is one of the most critical factors for bioactive peptide production (Lourenço da Costa *et al*., 2007). This is due to the protease cleavage specificity that subsequently affects resulting amino acid composition, sequence and the bioactivity of peptide (Chen *et al.*, 1998; Jimsheena and Gowda, 2011).

The production of protein hydrolysate involve specific hydrolysis conditions that need to be carefully control to obtained desired final ACEI activity (Guo *et al*., 2009). Response Surface Methodology (RSM) is an efficient and widely utilized tool for optimization of enzymatic hydrolysis condition (i.e. enzyme to substrate ratio, hydrolysis time, hydrolysis temperature and pH) and other process conditions to achieve desired response (Qu *et al.*, 2010; Shafisolani *et al*., 2014). Thus, the current study aims to investigate the effect of seven commercial proteinases (Alcalase®, Protamex™, Neutrase®, pepsin, papain, trypsin and α-chymotrypsin) and hydrolysis time on ACEI activity of resulting cockle's peptide; and to use the proteinase giving the highest ACEI activity for optimization of cockle's hydrolysis condition using RSM to achieve maximum ACEI activity.

## Materials and Methods

*Raw materials*

Blood cockles were obtained from cultivated farm in Sungai Kerang, Perak. Alcalase®, Protamex™ and Neutrase® were purchased from Novo Nordisk A/S Co. (Bagsvaerd, Denmark). Hippuric acid, hippuryl-L-histidyl-L-leucine (HHL), Angiotensin-converting enzyme (ACE), captopril, trypsin, pepsin, papain and α-chymotrypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents were of analytical grades.

*Screening of commercial proteinases*

Blood cockle's meat was hydrolyzed using different commercial proteinases at the recommended pH and temperature as suggested by the manufacturer (i.e. Alcalase® (pH 8.5, 55°C), Neutrase® (pH 7, 55°C), Protamex™ (pH 6.5, 50°C), pepsin (pH 2, 37°C), papain (pH 6, 60°C), trypsin (pH 8, 37°C) and α-chymotrypsin (pH 7.5, 37°C), respectively). This enzymatic hydrolysis was carried out at constant enzyme to substrate ratio (E/S = 1%) according to the method of Amiza and Masitah (2012). Protein hydrolysis was carried out in shaking water bath under constant shaking (100 rpm) for 0, 2, 4, 6 and 8 hrs. The resulting supernatants were then lyophilized prior to determination of ACEI activity. For screening study, the concentration of lyophilized hydrolysate and captopril was 1 mg/ml and 5 x $10^{-6}$ mg/ml, respectively.

*Determination of ACEI activity*

The ACEI activity was assayed by measuring the concentration of hippuric acid liberated from HHL using the method by Cushman and Cheung (1971). For each assay, 10 µl of ACE solution (2 mU/ml) was pre-incubated at 37°C for 10 min, and then incubated with a 10 µl of the sample solution (1 mg/ml) and 50 µl of substrate (25 mM HHL in 50 mM sodium borate buffer containing 500 mM NaCl at pH 8.3) at 37°C for 30 min. The reaction stopped by adding 85 µl of 1 N HCl. Hippuric acid was extracted with 500 µl of ethyl acetate. Then a 200 µl aliquot of the extract was removed by evaporation in a dry-oven at 80°C. The residue was dissolved in 1 ml distilled water and its UV spectra absorbance was measured at 228 nm.

The ACEI activity was calculated as follows:

$$\text{Inhibition (\%)} = (Ac - As)/ (Ac - Ab)^{*}100$$

where Ac is the absorbance of the control, As is the absorbance of the sample, and Ab is the absorbance of the blank.

*Optimization using response surface methodology*

The optimization of hydrolysis conditions was employed by using Protamex™, as suggested by the preliminary study. A face-centered central composite design was used. Hydrolysis conditions mainly consisted of four independent variables i.e. hydrolysis time (4-8 hrs), pH (5.5-7.5), hydrolysis temperature (35-60°C) and E/S ratio (0.5-1.5%) with respect to ACEI activity (%) as the response. Thirty runs of experiment consisted of three levels of each process parameter (-1, 0, +1) were coded from 1 to 30. Experimental runs were randomised to minimise the effects of unexpected variability in the observed response.

*Verification of Model*

Five replications of blood cockle 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 ACEI activity assay. Experimental values of ACEI activities were then compared with the predicted value obtained from RSM, using one

Table 1.The data obtained for ACEI activity (%) from meat cockle using face-centered Central Composite Design

| Std Order | Run Order | A: Temp | B: Time | C: E/S | D:pH | ACEI (%) |
|---|---|---|---|---|---|---|
| 5 | 1 | 35.0 | 4 | 1.5 | 5.5 | 98.02 |
| 6 | 2 | 60.0 | 4 | 1.5 | 5.5 | 82.31 |
| 11 | 3 | 35.0 | 8 | 0.5 | 7.5 | 44.90 |
| 4 | 4 | 60.0 | 8 | 0.5 | 5.5 | 92.43 |
| 24 | 5 | 47.5 | 6 | 1.0 | 7.5 | 88.58 |
| 12 | 6 | 60.0 | 8 | 0.5 | 7.5 | 78.94 |
| 16 | 7 | 60.0 | 8 | 1.5 | 7.5 | 64.95 |
| 7 | 8 | 35.0 | 8 | 1.5 | 5.5 | 99.23 |
| 15 | 9 | 35.0 | 8 | 1.5 | 7.5 | 73.04 |
| 1 | 10 | 35.0 | 4 | 0.5 | 5.5 | 69.25 |
| 18 | 11 | 60.0 | 6 | 1.0 | 6.5 | 97.46 |
| 29 | 12 | 47.5 | 6 | 1.0 | 6.5 | 79.42 |
| 9 | 13 | 35.0 | 4 | 0.5 | 7.5 | 60.68 |
| 25 | 14 | 47.5 | 6 | 1.0 | 6.5 | 87.17 |
| 10 | 15 | 60.0 | 4 | 0.5 | 7.5 | 68.62 |
| 27 | 16 | 47.5 | 6 | 1.0 | 6.5 | 91.72 |
| 22 | 17 | 47.5 | 6 | 1.5 | 6.5 | 77.28 |
| 13 | 18 | 35.0 | 4 | 1.5 | 7.5 | 87.00 |
| 2 | 19 | 60.0 | 4 | 0.5 | 5.5 | 74.68 |
| 23 | 20 | 47.5 | 6 | 1.0 | 5.5 | 85.82 |
| 19 | 21 | 47.5 | 4 | 1.0 | 6.5 | 76.45 |
| 21 | 22 | 47.5 | 6 | 0.5 | 6.5 | 46.57 |
| 26 | 23 | 47.5 | 6 | 1.0 | 6.5 | 84.79 |
| 17 | 24 | 35.0 | 6 | 1.0 | 6.5 | 93.68 |
| 28 | 25 | 47.5 | 6 | 1.0 | 6.5 | 92.85 |
| 3 | 26 | 35.0 | 8 | 0.5 | 5.5 | 58.21 |
| 30 | 27 | 47.5 | 6 | 1.0 | 6.5 | 81.17 |
| 8 | 28 | 60.0 | 8 | 1.5 | 5.5 | 86.59 |
| 20 | 29 | 47.5 | 8 | 1.0 | 6.5 | 81.54 |
| 14 | 30 | 60.0 | 4 | 1.5 | 7.5 | 55.45 |

sample t-test.

*Data analysis*

Statistical Package for Social Science (SPSS) and Design Expert 8.0.7.1 software were used for data processing and statistical analysis. For screening of enzymes, all analyses were carried out in triplicate and data were stated in mean ± standard deviation. For optimization study, data were subjected to analysis of variance (ANOVA) using Design Expert 8.0.7.1 software. Mean values were accepted as significantly different at 95% level ($p < 0.05$).

## Results and Discussion

*Screening of proteinases*

Apparently, all samples display ACEI activity with an increasing trend until a maximum ACEI was reached and then decreased afterwards (Figure 1). Maximum ACEI activity was obtained at 4 hrs for trypsin, pepsin, Alcalase® and Neutrase®. For papain, Protamex™ and α-chymotrypsin, the maximum ACEI activity was obtained at 6 hrs. Blood cockle hydrolysed using Protamex™ for 6 hrs gave the highest ACEI activity (93.68%); followed by papain at 6 hrs (77.92%), pepsin at 4 hrs (73.54%), chymotrypsin at 6 hrs (55.17%), trypsin at 4 hrs (50.41%), Alcalase® at 4 hrs (44.62%) and Neutrase® at 4 hrs (36.25%). ACEI activity given by hydrolysate prepared using Protamex™ at 6 hrs hydrolysis (1 mg/ml) was higher compared to that of captopril (synthetic drug commonly used as ACE inhibitors) at 5 x 10-6 mg/ml (83.58%).

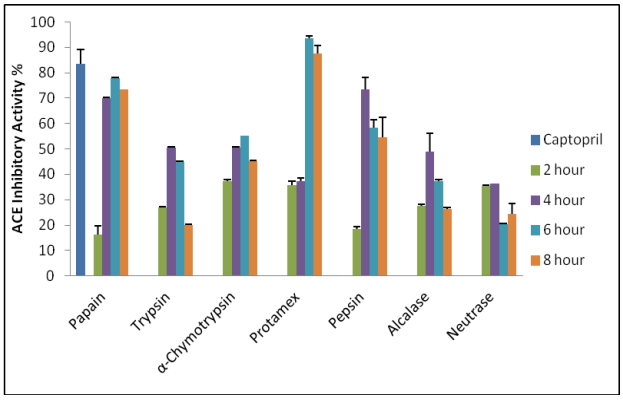


Figure 1. ACEI activity of blood cockle hydrolysates produced using several commercial proteinases at 2 - 8 hours of hydrolysis time as compared to that of captopril

Longer hydrolysis time generally will result in increased degree of hydrolysis (DH). However, ACEI activity is not proportionate to DH (Chiang *et al*., 2006). This is in line with study by Guo *et al.* (2009) whom found that proper hydrolysis conditions (i.e. hydrolysis temperature, pH, time and E/S) to produce ACEI peptides were different from the proper hydrolysis conditions to reach high DH.

In the present study, Protamex™ was chosen as the best proteinase for hydrolysis of blood cockle based on high ACEI activity. He *et al*. (2007) reported that for different kind of marine food materials including fish, shrimp, seashell, algae and seafood wastes, Protamex™ and SM98011 much higher ACEI activity were obtained than Alcalase® and Flavourzyme™, indicating that these proteinases were more suitable for marine protein digestion and production of ACEI peptides. Similar finding have been observed in Styela clava flesh tissue hydrolysate

Table 2. Analysis of variance table for reduced quadratic model

| Source of Variation Model | Sum of Squares | df | Mean Square | F Value | p-value Prob>F | |
|---|---|---|---|---|---|---|
| Model | 5407.82 | 8 | 675.98 | 17.05 | <0.0001 | Significant |
| A-Temp | 16.86 | 1 | 16.86 | 0.43 | 0.5214 | |
| B-Time | 3.02 | 1 | 3.02 | 0.076 | 0.7853 | |
| C-E/S | 932.98 | 1 | 932.98 | 23.53 | < 0.0001 | |
| D-pH | 859.47 | 1 | 859.47 | 21.68 | 0.0001 | |
| AB | 414.33 | 1 | 414.33 | 10.45 | 0.0040 | |
| AC | 1399.13 | 1 | 1399.13 | 35.29 | < 0.0001 | |
| $A^2$ | 471.73 | 1 | 471.73 | 11.90 | 0.0024 | |
| $C^2$ | 1658.37 | 1 | 1658.37 | 41.83 | < 0.0001 | |
| Residual | 832.56 | 21 | 39.65 | | | |
| Lack of Fit | 683.67 | 16 | 42.73 | 1.43 | 0.3665 | not significant |
| Pure Error | 148.89 | 5 | 29.78 | | | |
| Cor Total | 6240.38 | 29 | | | | |

for hydrolysis using nine proteinases (Protamex™, Kojizyme, Neutrase®, Flavourzyme®, Alcalase®, pepsin, trypsin, α-chymotrypsin and papain), whereby Protamex™ produced peptide with the highest ACEI activity with an $IC_{50}$ of 1.023 mg/ml as compared to other proteinases (1.781-2.481 mg/ml) (Ko *et al*., 2012).

*Optimization of hydrolysis conditions*

Table 1 shows the data obtained for ACEI activity (%) from meat cockle using face-centered Central Composite Design with ACEI activity ranging from 44-98%. Thirty runs of experiment were carried out according to four factors (temperature, time, Enzyme to substrate concentration (E/S) and pH) and the response variable was ACEI activity (%).

*Effect of hydrolysis conditions on the ACEI activity of blood cockle's meat*

Table 2 shows the analysis of variance table for reduced quadratic model ($p < 0.0001$). A non-significant lack-of-fit ($p = 0.3665$) showed the good fitness of the model. The coefficient of determination ($p = 0.8666$) was in good agreement with adjusted R-square ($p = 0.8158$). In this model, the significant terms are C, D, AB, AC, $A^2$, $C^2$ with "p-value" less than 0.05 (Table 2). Insignificant term A and B (temperature and time) with "p-value" more than 0.05 was not excluded as it was required to support hierarchy. This is because term AB and AC were significant, thus term A and B must be included in the model. Thus, the significant model terms in this study for ACEI activity response were model terms A, B, C, D, AB and AC, $A^2$ and $C^2$. The mathematical model representing each parameter was expressed by the following equations;

Final Equation in Terms of Coded Factors:

$$ACEI = + 84.77 + 0.97^{*} A + 0.41^{*} C - 6.91 B + 7.20^{*} D + 5.09^{*} AB - 9.35^{*} AC + 11.70^{*} A^2 - 21.94^{*} C^2$$

Final Equation in Terms of Actual Factors:

$$ACEI = + 178.54141 - 6.76294^{*} \text{Temperature} - 9.46390^{*} \text{Time} + 261.00645^{*} \text{E/S} - 6.91000^{*} \text{pH} + 0.20355^{*} \text{Temperature} * \text{Time} - 1.49620^{*} \text{Temperature} * \text{E/S} + 0.074898^{*} \text{Temperature2} - 87.76903^{*} \text{E/S2}$$

The equation showed that pH and enzyme to substrate ratio (E/S) significantly affect the ACEI activity. This finding is in agreement with optimization study on ACEI activity of whey protein (Guo *et al*., 2009). According to Adler-Nissen (1982), pH exerts an independent influence on properties of hydrolysate and a change in pH have affect to both the enzyme and substrate by changing the charge distribution and conformation of molecules.

Response surface plot shows that at 35°C and 60°C, ACEI activity increases with increase in E/S until optimum point is reached (Figure 2), then further increase in E/S does not result in an increased in ACEI activity, due to specificity of proteinases on protein substrate (Adler-Nissen, 1986). The proteinase is able to hydrolyse fewer peptide bonds when E/S is low and the increment of E/S may result in an increase of the rate of splitting certain peptide bonds that contribute to ACEI activity more than

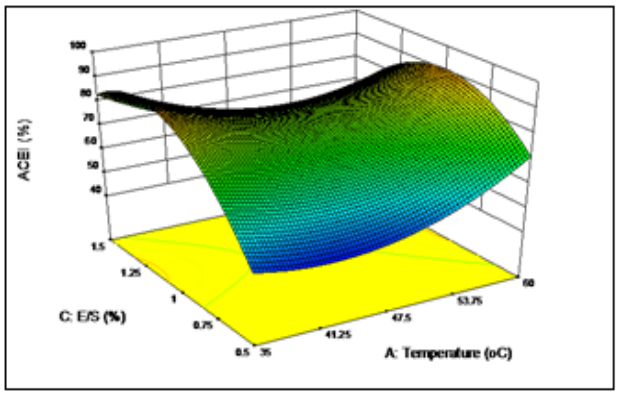


Figure 2. Response Surface Plot for temperature and E/S on ACEI activity

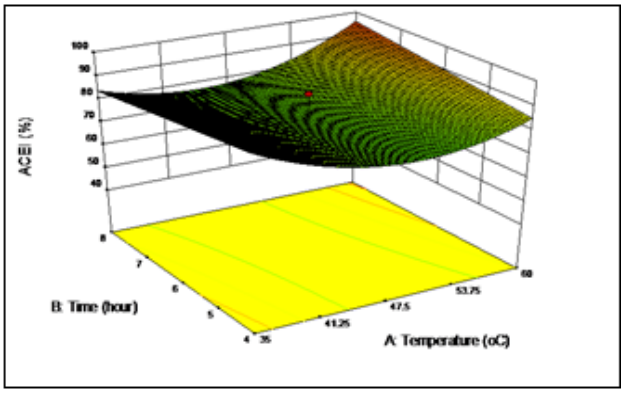


Figure 3. Response Surface Plot for temperature and time on ACEI activity

proportionally to E/S until optimum point (Guo *et al*., 2009). This is due to unspecific interaction between enzyme and substrate that does not involve the formation of Michaelis complex (i.e. through weak van der Waals attraction between other regions on the enzyme molecule and the substrate) (Adler-Nissen, 1986).

Although the main effect of temperature and time is not significant, the interaction effect of temperature-time was found to be positively and significantly ($p = 0.014$) affect the ACEI activity (Figure 3). A change in temperature will affect the hydrolysis reaction rate resulting in different ACEI activity. For example, at 60oC, higher hydrolysis time resulted in increment of ACEI activity. In contrary, there was a negative but significant ($p = 0.001$) interaction effect of hydrolysis temperature-E/S ratio on ACEI activity. This shows that in a specified reaction condition, enzyme is utilised most efficiently at optimum temperature and the optimum temperature decreases with reaction time.

*Optimum point and verification of model*

Optimization of hydrolysis conditions of blood cockle's meat was set to obtain maximum ACEI activity. The goals for independent variables (i.e. hydrolysis time, pH, hydrolysis temperature and E/S ratio) were set to be in range. All independent variables were rate for importance value at scale 3. Meanwhile, ACEI activity was rate for obtaining maximum value at scale 5. Enzymatic hydrolysis of cockle meat using optimum hydrolysis condition (hydrolysis time of 4.69 hrs, pH of 5.59, hydrolysis temperature of 59.8°C and E/S ratio of 0.9%) was run in five replicates for verification. The ACEI activity result was 97.9% ± 0.81. Validation using one sample t-test shows that the experimental value agreed with the predicted value within a 95% confidence interval.

## Conclusions

Preliminary study shows that cockle's hydrolysate prepared using Protamex™ at 6 hrs hydrolysis time yielded the highest ACEI activity. Optimum hydrolysis condition to obtain maximum ACEI activity from blood cockle's meat was at temperature of 59.8°C, time of 4.69 hrs, pH of 5.59 and E/S ratio of 0.9%. The predicted ACEI activity (99.2%) was close to the experimental value (97.8%), suggesting a good fit between predicted and experimental data.

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