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OPTIMISATION OF ENZYMATIC PROTEIN HYDROLYSIS OF MUD CRAB *(Scylla* sp.) TO OBTAIN MAXIMUM ANTIOXIDANT ACTIVITY USING RESPONSE SURFACE METHODOLOGY

ZALIHA HARUN[1], AMIZA MAT AMIN[1*], NORIZAH MHD SARBON[1], MOHAMAD KHAIRI MOHD ZAINOL[1] and KAZI NAZIRA SHARMIN[2]

[1]School of Food Science and Technology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
[2]Department of Applied Food Science and Nutrition, Faculty of Food Science and Technology, Chittagong Veterinary and Animal Science University, Khulshi, Chittagong 4225, Bangladesh
[*]E-mail: ama@umt.edu.my

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ABSTRACT

This study reported optimisation of enzymatic hydrolysis of mud crab meat using Protamex® to obtain maximum antioxidant activity using Response Surface Methodology (RSM). Prior to optimisation, screening of commercial food grade proteinases was carried out using Alcalase®, Protamex®, Neutrase® and papain. Protamex® was observed to give the highest DPPH scavenging activity. The enzymatic hydrolysis conditions used in the optimisation study were temperature (45-65ºC), pH (5.5-7.5), hydrolysis time (1-4 hours) and enzyme to substrate (E/S) ratio (1-3% Protamex®). A face-centered Central Composite Design (CCD) was employed. It was found that the relationship between hydrolysis conditions and DPPH scavenging activity could be explained by a quadratic model. Optimum condition was found to be at 54ºC, pH 5.5, 1% Protamex® and 1 hour of hydrolysis time. Validation experiment shows that the experimental DPPH scavenging activity (82.39 ± 0.16%) was close to the predicted value (82.64%). The hydrolysate prepared at optimum condition contained 5.52% moisture, 74.81% crude protein, 13.13% ash, 6.26% carbohydrate and 0.28% crude fat with IC_{50} for DPPH scavenging activity of 3.48 ± 0.05 mg/mL. This study shows that RSM can be used to explain the relationship between enzymatic hydrolysis conditions of mud crab meat and its antioxidant activity.

Key words: Mud crab, Enzymatic hydrolysis, Response Surface Methodology (RSM), antioxidant activity

INTRODUCTION

Lipid oxidation that leads to the development of undesirable off-flavours, odours and potentially toxic products has been a great concern in food industry and consumers (Lin & Liang, 2002). Besides, oxidation or free radical reactions in the human body can cause cancer, coronary heart disease and Alzheimer's disease (Butterfield *et al.*, 2002). Thus, it is very important to inhibit lipid peroxidation in foods and our body to prevent them from undergoing such deterioration and provide protection against serious diseases. Antioxidants are widely used to preserve food products from undergoing deterioration and retarding discoloration as a result of oxidation as well as to minimize free radical reactions in our body (Decker *et al.*, 2005). Synthetic antioxidant such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), t-butylhydroquinone (TBHQ) and propyl gallate have been used especially in food production and biological system to prevent the action of the free radicals and reported stronger antioxidative activity than natural antioxidants (Farvin *et al.*, 2014; Luo *et al.*, 2013). Nevertheless, due to potential health risk of the long term use of synthetic antioxidant and demand from consumers who prefer natural food without artificial food additives, there are growing interest in finding natural and safer antioxidants for human consumption (Wang *et al.*, 2013).

Besides the natural antioxidants such as Vitamin C and Vitamin E, antioxidant peptides may have great potential to control lipid oxidation in food products (Harnedy & FitzGerald, 2012). In recent studies, antioxidative peptides have been reported

* To whom correspondence should be addressed.

from seafoods such as sardinella (Bougatef *et al.*, 2010), loach (You *et al.*, 2010), tilapia (Zhang *et al.*, 2012), silver carp (Dong *et al.*, 2008), salmon (Girgih *et al.*, 2015), cod (Girgih *et al.*, 2013), *Sphyrna lewini* (Luo *et al.*, 2013) and oyster (Wang *et al.*, 2014). Until now, no study has been reported on optimisation of antioxidant peptide from mud crab. Mud crab is one of marine origin that has become one of the major constituent of the local crab fishery and popular for it delicacy and rich of nutrient as protein is the highest component in mud crab meat (Ikhwanuddin *et al.*, 2011; Sarower *et al.*, 2013).

Many factors contribute to antioxidant activity of peptides such as its composition, structure, and hydrophobicity whereby Tyr, Trp, Met, Lys, Cys and His are example of amino acids with antioxidant activities (Chalamaiah *et al.*, 2012). The antioxidant mechanism of amino acids including, proton donation from aromatic residues of amino acids that improve radical scavenging properties. Besides, type of proteinases to hydrolyze the protein and molecular weight also affect the antioxidant activity of the peptides (Sarmadi & Ismail, 2010; Ren *et al*., 2008).

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). Response surface methodology (RSM) has been used in optimisation of hydrolysis condition to produce high anti-oxidative activity of various protein sources of marine origin including flying squid muscle, grass carp sarcoplasmic, shrimp waste, fish gelatin, jellyfish and saithe (*Pollachius virens*) (Fang *et al*., 2012; Ren *et al*., 2008; Sowmya *et al.*, 2012; You *et al.*, 2010; Zhuang *et al.*, 2009; Chabeaud *et al.*, 2009). Thus, the objective of this study was to optimize the enzymatic hydrolysis conditions of mud crab hydrolysis to obtain maximum anti-oxidant 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 and were killed immediately by cutting them into halves. The bellies and gills were discarded prior to washing to remove any contaminants. Next, the mud crabs were steamed for four minutes and leaved to cool at room temperature before the meat was separated from their carapaces. The meat were homogenized using food processor and kept at freezer (-20ºC) until further use.

Chemicals and reagents

Protamex® and Neutrase® were purchased from Novozyme Sdn Bhd., while Alcalase®, papain and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich. All other chemicals used in this study were of analytical grades.

Proximate analysis

Proximate analysis of mud crab meat and its hydrolysate prepared at optimum condition were determined using method as described in AOAC (2000). Moisture content was determined using oven drying method while ash content was determined using dry ashing method by means of a muffle furnace. Crude protein content was determined by Kjeldahl method while crude fat content was carried out using Soxhlet method. Crude protein content was used in the calculation of hydrolysis mixture (Kristinsson & Rasco, 2000).

Screening of commercial food grade proteinases to yield maximum antioxidant 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 commercial proteinase giving the highest antioxidant activity (DPPH scavenging activity) was used in the optimisation study.

Enzymatic hydrolysis of mud crab

Hydrolysis mixture of mud crab meat, proteinase and distilled water was calculated and prepared according to Kristinsson & Rasco (2000). First, the homogenized mud crab was boiled at 85ºC for 20 minutes to inactivate endogenous enzymes. Then the temperature and pH were adjusted accordingly. Enzymatic hydrolysis was carried out in a water bath. Once the enzymatic hydrolysis has completed, 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.

DPPH scavenging activity

The DPPH scavenging activity was carried-out according to the method described by Polatoğ lu *et al*. (2013) using a 96-well plate. The sample was dissolved in distilled water and 100 µL (10 mg/mL) was mixed with 100 µL DPPH solution (100 µM) prepared in ethanol. The mixture was left 30 minutes

in dark. The absorbance of the mixture was determined using spectrophotometer at 517 nm using an ELISA microplate reader (Bio-Rad, USA). Half maximal inhibitory concentration (IC_{50}) is defined as an effective concentration of hydrolysate that is required to scavenge 50% of radical activity (Zhang *et al.*, 2012). 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, 10 mg/mL). Finally, a plot of DPPH scavenging activity versus concentration of mud crab meat hydrolysate was plotted to obtain the IC_{50} value which refers to the minimum concentration of substrate that is required to inhibit 50% of DPPH free radicals (Muniandy *et al*., 2016)

Experimental design for optimisation

Response surface methodology (RSM) was used to optimize the hydrolysis condition of mud crab meat hydrolysis using Protamex®. A face-centered central composite design (CCD) comprised of 30 experimental runs with 6 center points was employed using four experimental variables as stated in Table 1. Design-Expert 8.0.7.1 software (Stat-Ease Inc.) was used to generate the experimental runs and data analysis. The software generates model equation, response surface interaction and optimum conditions. 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 DPPH scavenging assay. This experimental value of antioxidant activity was then compared with the predicted antioxidant activity value obtained from RSM under optimum condition, using one sample t-test (IBM SPSS v. 20).

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 DPPH scavenging activity and that of predicted activity under optimum conditions was compared using one-sample t-test (IBM SPSS v. 20).

RESULTS AND DISCUSSION

Proximate analysis

Proximate analysis of mud crab meat shows that it contained 79.72±0.44% moisture, 14.63±1.06% crude protein, 2.32±0.21% ash, 0.04±0.03% crude fat and 3.30±1.53% carbohydrate. This results were in agreement with previous study by Sarower *et al*. (2013) whom reported that wild mud crab contained 16.60–19.38% protein, 75.31–78.02% moisture, 3.20–3.63% ash and 1.32–1.17% fat.

Type of proteinases used in protein hydrolysis influences the antioxidant activity of the hydrolysate (Sarmadi & Ismail, 2010). Screening study shows that Protamex® gave the highest DPPH scavenging activity, followed by Alcalase® and Neutrase® and finally papain (Figure 1). Hence, Protamex® was chosen to be used in optimisation study. Alcalase®, Neutrase® and Protamex® are endopeptidases, while papain is an exopeptidase. Endopeptidase breaks the peptide bond of non-terminal amino acids, while exopeptidase breaks the terminal amino acid (Fernandes, 2016). This study shows that endopeptidase is more effective in hydrolyzing mud crab meat to yield DPPH scavenging activity.

Optimisation of hydrolysis conditions to obtain maximum antioxidant activity

DPPH scavenging activities obtained from 30 experimental runs ranged from 66.81%–81.18%. Design Expert software was used to analyze the data further. Table 2 summarizes the ANOVA of the quadratic model after model reduction (elimination of insignificant model terms). The table shows that the model was significant ($p<0.001$) with 99.99% confident interval. A non-significant lack-of-fit ($p=0.3504$) shows a good data fitting to the suggested model. The coefficient of determination ($p=0.8592$) was in good agreement with adjusted R-square ($p=0.8144$).

Final equation of DPPH scavenging activity in terms of coded factors is as follows:

$$\text{DPPH scavenging activity (\%)} = 74.73 + 0.05\ A - 4.61\ B + 0.82\ C - 0.73\ D - 3.3\ A^2 + 1.83\ C^2 + 1.69\ BD$$

Table 1. Optimisation conditions of mud crab meat hydrolysis using Protamex®

Independent variables	Symbol	Low actual	High actual	Low coded	High coded
Temperature	A	45	65	-1	+1
pH	B	5.5	7.5	-1	+1
Hydrolysis time	C	1	4	-1	+1
E/S	D	1	3	-1	+1

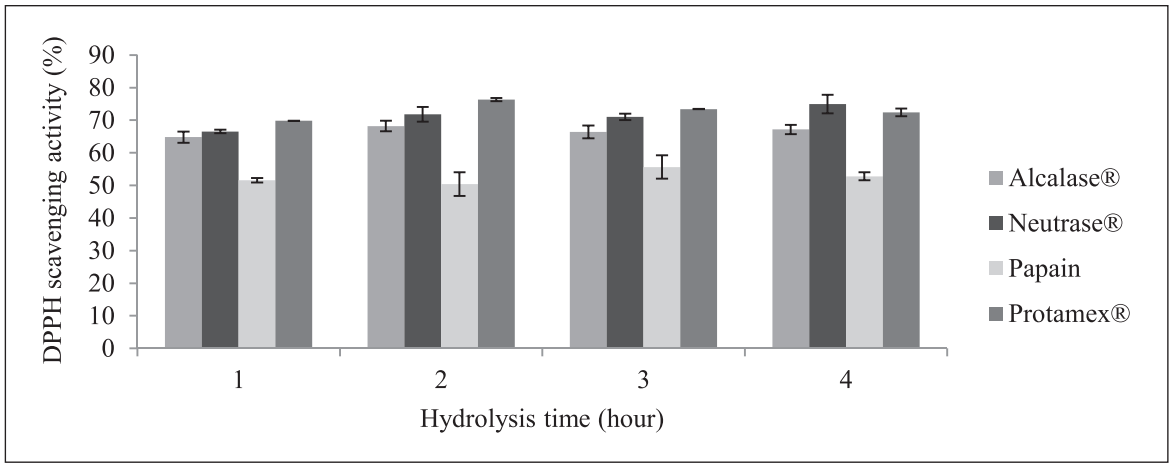

Fig. 1. Screening of commercial food grade proteinases to obtain maximum DPPH scavenging activity from mud crab meat hydrolysis.

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

Source	Sum of squares	DF	Mean square	F	Prob > F
Model	490.75	7	70.11	19.18	< 0.0001
A – Temperature	0.045	1	0.045	0.012	0.9128
B – pH	383.28	1	383.28	104.87	< 0.0001
C – Hydrolysis time	12.16	1	12.16	3.33	0.0818
D – E/S ratio	9.61	1	9.61	2.63	0.1192
A^2	37.55	1	37.55	10.27	0.0041
C^2	11.58	1	11.58	3.17	0.0889
BD	45.93	1	45.93	12.57	0.0018
Residual	80.41	22	3.65		
Lack of Fit	67.12	17	3.95	1.49	0.3504
Pure Error	13.28	5	2.66		
Correlation Total	571.16	29			
R-Squared	0.8592				
Adjusted R-Square	0.8144				
Predicted R-Square	0.7184				
Adequate Precision	14.57				

Final equation of DPPH scavenging activity in terms of actual factors is as follows:

$$\text{DPPH scavenging activity (\%)} = +31.78247 + 3.63694\,A - 8.00294\,B - 3.52591\,C - 11.7432\,D - 0.03302\,A^2 + 0.081477\,C^2 + 1.69425\,BD$$

The equation in terms of actual factors shows that the most influencing experimental factors in producing DPPH scavenging activity from mud crab meat hydrolysis using Protamex® are E/S ratio, followed by pH, temperature and hydrolysis time. Hydrolysis time had little effect on DPPH scavenging activity. This was in agreement with study by Sowmya *et al.* (2012) whom reported shorter time was sufficient to obtain higher DPPH scavenging activity of shrimp waste hydrolysis.

Quadratic model has been reported for optimisation of enzymatic hydrolysis conditions towards antioxidant activity from jellyfish collagen (Zhuang *et al*., 2009), while both linear and quadratic model was reported for those of saithe and grass carp sarcoplasmic protein (Chabeaud *et al.*, 2009; Ren *et al*., 2008).

Analysis of the response surface methodology

Figure 2 shows the effect of pH and E/S at constant hydrolysis time and temperature. The figure shows that there is a synergistic effect of DPPH scavenging activity at low pH and low E/S ratio. Although Protamex® was reported to be active at wide range of pH (pH 5- pH 11), lower pH range is preferable to produce antioxidant peptide from mud crab meat.

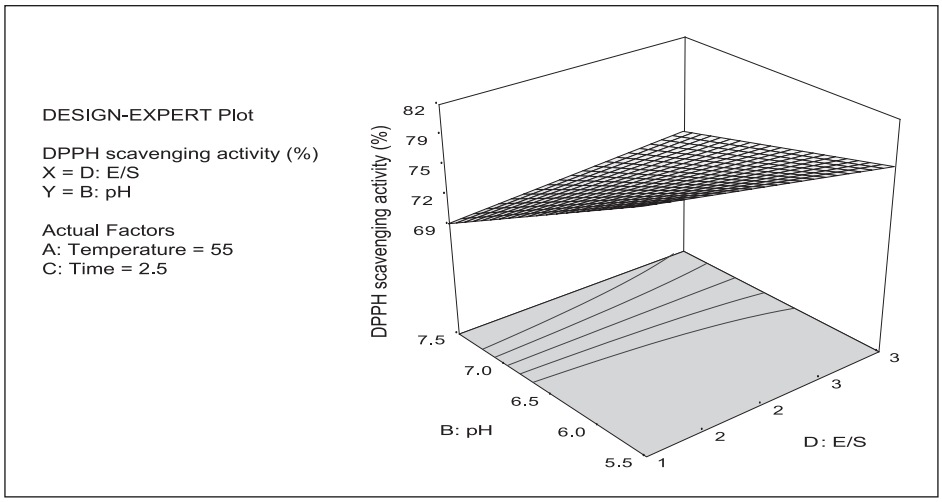

Fig. 2. Effect of E/S ratio and pH on DPPH scavenging activity.

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

Number	Temperature	pH	Hydrolysis time	E/S	DPPH scavenging activity (%)	Desirability
1	54	5.5	1.0	1	82.64	1.000
2	54	5.5	1.1	1	82.54	0.995
3	55	5.5	1.0	1	82.51	0.994
4	55	5.5	1.5	1	82.07	0.969
5	55	5.5	1.5	1	82.07	0.969
6	54	5.5	1.6	1	81.9	0.960
7	55	5.5	1.7	1	81.82	0.956
8	56	5.5	1.8	1	81.81	0.955
9	55	5.5	2.0	1	81.64	0.946
10	51	5.5	2.0	2	81.24	0.924

Predicted optimum condition

Design Expert software suggested ten solutions for optimum conditions for Protamex® hydrolysis of mud crab (Table 3). Solution number 1 was selected due to its desirability is 1 and it gave highest DPPH scavenging activity.

Verification of the model

This model was further verified by comparing the experimental DPPH scavenging activities of hydrolysates prepared using the suggested optimum condition with that of the predicted DPPH scavenging activity at optimum condition. One-sample t-test shows that, under the optimum condition, the experimental DPPH scavenging activity (82.39 ± 0.16 %) is close to the predicted DPPH scavenging activity (82.64%) ($p>0.05$). 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 DPPH scavenging activity.

Proximate analysis and IC_{50} of mud crab hydrolysate prepared at optimum condition

Proximate analysis of mud crab meat hydrolysate prepared at optimum condition (wet basis) shows that it contained 5.52% moisture, 74.81% crude protein, 13.13% ash, 6.26% carbohydrate and 0.28% crude fat. This result shows that mud crab hydrolysate is very high in protein content.

The IC_{50} of the DPPH scavenging activity of the mud crab hydrolysate was 3.48 ± 0.05 mg/mL (R^2=0.978). This value was comparable with other marine origin such as *Sphyrna lewini* muscle protein hydrolysate (3.06 mg/mL) (Luo *et al.*, 2013) and better compare to *Arca subcrenata* hydrolysate (6.23 mg/mL) (Song *et al.*, 2008). However more potent DPPH scavenging activity was reported in hydrolysate from salmon byproducts (0.721 mg/mL) (Wu *et al.*, 2017) and grass carp sarcoplasmic protein hydrolyzed using papain (0.597 mg/mL) (Ren *et al.*, 2008). Further purification/fractionation can be used

to reduce the IC_{50} value of protein hydrolysate (Wu *et al*., 2017).

CONCLUSION

Quadratic model can be used to explain the relationship between hydrolysis conditions of mud crab meat and antioxidant activity. The optimum condition was obtained at 54ºC, pH 5.5, 1% Protamex® and 1 hr of hydrolysis time. Mud crab meat hydrolysate prepared at optimum condition contained 5.52% moisture, 74.81% crude protein, 13.13% ash, 6.26% carbohydrate and 0.28% crude fat (wet basis). The IC_{50} of the DPPH scavenging activity of the mud crab hydrolysate was 3.48 ± 0.05 mg/mL. This study shows that optimisation using Response Surface Methodology can be used to predict the effect of hydrolysis conditions to produce the best yield of natural and safer antioxidant peptide from mud crab meat. Further study should be carried out on purification of DPPH scavenging peptide from mud crab, determination of its amino acid sequence, efficacy of DPPH scavenging peptide from mud crab on cell culture model or spontaneously hypertensive rats, intervention study on human subject as well as the stability of mud crab DPPH scavenging peptide against temperature, pH and digestive enzymes. Furthermore, comparison of antioxidant activity between natural antioxidant peptide from mud crab and that of synthesized peptide with amino acid sequence similar to antioxidant peptide from mud crab can be carried out as well.

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