Optimization of Enzymatic Hydrolysis Condition for Producing Black Gram Bean (*Vigna mungo)* Hydrolysate with High Antioxidant Activity

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Abstract

Black gram bean (*Vigna mungo*) hydrolysate was produced using commercial enzyme, Flavourzyme® to break down the peptide bonds. Hydrolysis conditions i.e. enzyme concentration of 1–7% (by dried weight of steamed bean) and hydrolysis time of 60–1200 min, were optimized for high antioxidant activity hydrolysate using response surface methodology based on central composite rotational design. The effect of hydrolysis conditions on degree of hydrolysis (DH), total phenolic content (TPC), browning and antioxidant activity as DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) was determined. The results of this study showed that increasing the enzyme concentration and hydrolysis time significantly affected DH as increasing DH influenced amount of free amino groups, released TPC and Maillard reaction products (MRPs); these components affected the antioxidant activity of black gram bean hydrolysate. The optimum hydrolysis condition to reach DH of 75% was 6.09% Flavourzyme® and 360 min of hydrolysis time, giving a predicted value of DPPH radical scavenging activity and FRAP values in the range of 80.47–80.48% and 1.42–1.43 µmol Trolox per gram of black gram beans (d.b.), respectively. The validation was confirmed using percentage error measurement. It was found that the observed values were different from the predict values within a range of 0.54–27.46% error. Thus, the obtained optimized model could be used for predicting desired responses for black gram bean hydrolysate production.

Keywords: Bean hydrolysate, Antioxidant activity, FRAP, Total phenolic content, *Vigna mungo*

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1. Introduction

Legumes are important food crops which provide the nutrition well-being as fundamental human diets. Grain legumes or pulses recognized as various types of common dry beans are produced and consumed in the largest quantity in the world. In Thailand, the *Vigna* bean e.g. mung bean, black gram bean, rice bean and adzuki bean, is one of the important industrial legumes. These beans are considered as food protein sources, containing 18–24% protein on dry basis (Nwokolo and Smartt, 1996). These beans are also rich in other nutrients such as complex carbohydrates, fiber, vitamins, certain minerals, and several bioactive compounds e.g., gallic acid, vanillic acid, chlorogenic acid, sinapic acid, etc. (Xu and Chang, 2009). These beans become an attractive source of proteins for producing hydrolysate. Nowadays, plant protein hydrolysate is widely used in food applications to improve functional properties of liquid foods (Clemente *et al.*, 1999) and meat products (Cumby *et al.*, 2008). In addition, this product has been considered as nutraceutical foods that possess biological and antioxidant activities. (Betancur-Ancona *et al.*, 2014)

Proteins show important roles in food product development and processing. Their physico-chemical, functional and biological properties, influence the consumer acceptability. The properties of protein can be improved by enzymatic modification under the controlled condition. It has been reported that the enzymatic modification improves the antioxidant activity based on degree of hydrolysis (DH). Commercial peptidases (Alcalase®, Pepsin®, Papain® and Flavourzyme®) were used for cleaving a peptide linkage in the primary structure of a protein into various sizes of small peptides and free amino acids in hydrolysate production from black bean (Do Evangelho *et al.*, 2016), kidney bean (Wani *et al.*, 2015) and rice bean (Sritongtae *et al.*, 2017). Several studies reported that small peptides and free amino acids obtained by enzymatic hydrolysis using Flavourzyme® (endo- and exopeptidase) also contributes to increasing antioxidant activity of hydrolysate from mung bean and adzuki bean sprouts (Sangsukiam and Duangmal, 2017), rice bean (Sritongtae et al., 2017) and okara (Sbroggio *et al.*, 2016). The antioxidant activity of hydrolysate depends on composition and sequence of amino acids in the peptides (Sarmadi and Ismail, 2010; Luna-Vital *et al.*, 2015). Moreover, the enzymatic hydrolysis affects the releasing of free phenolic compounds from the protein-bound polyphenols, resulting in the improvement of overall antioxidant activity (Sangsukiam and Duangmal, 2017). At the same time, the reaction of free amino groups with the reducing sugars leads to the formation of complex compounds known as Maillard reaction products (MRPs). It is well known that MRPs are able to modify important food properties such as aroma, color and stability. Researchers also found that MRPs in shrimp by-product protein

hydrolysate (Zha *et al.*, 2015) and soybean hydrolysate (Liu *et al.*, 2012) showed high antioxidant activity.

Response surface methodology (RSM) is a mathematical and statistical tool for modeling and optimization of a multi-variable system. RSM has been successfully applied to optimize the hydrolysis conditions in many enzymatic hydrolysis systems i.e. hydrolysate from Bambara bean protein concentrate (Mune Mune, 2015), whey protein hydrolysate (Del Mar Contreras *et al.*, 2011) and eel protein hydrolysate (Jamil *et al.*, 2016). In this study, black gram bean hydrolysate was produced under various hydrolysis conditions to study the effect of hydrolysis conditions for black gram bean hydrolysate production using RSM and to optimize the hydrolysis condition for high antioxidant activity. Moreover, confirmation of generated model was conducted using percentage error measurement between predicted and experiment values in order to verify suitability and validity of model for predicting desired response.

2. Materials and Methods

2.1 Materials

Black gram bean seeds were purchased from Choomsin Food Industry Co., Ltd. (Nonthaburi, Thailand). The commercial enzymes, alpha-amylase BAN® 480L (EC 3.2.1.1) from *Bacillus amyloliquefaciens* was purchased from Novozyme (Mülheim, Germany). The specific activity of BAN® is 480 KNU g^{-1}, defined as amount of enzyme that hydrolyzes 5.26 g of starch per hour. Flavourzyme® 500 L (EC 3.4.11.1) from *Aspergillus oryzae* was purchased from Novozyme (Mülheim, Germany). The specific activity of Flavourzyme® is 500 LAPU g^{-1}, defined as amount of enzyme that hydrolyzes 1 µmol of L-leacine-*p*-nitroanilide per minute.

2.2 Production of black gram bean hydrolysate

The black gram bean hydrolysate was prepared according to the method of Sritongtae *et al*. (2017). Black gram beans were washed and subjected to a pre-treatment process (12 h soaking in tap water at the ratio of 1:10 (w/v) followed by 1 h steaming). Steamed beans were then ground thoroughly with a blender and were mixed with 0.1 M phosphate buffer solution containing 0.5 g L^{-1} $CaCl_2$, pH 6.0, at the ratio of 1:2.5 (w/v). Alpha-amylase was added at 1% steamed beans (d.b.) into the slurries in order to cleave glycosidic linkage in starch, followed by heating at 70 °C in an orbital shaking water bath for 2 h. Enzyme activity was then inactivated by heating at 85 °C for 15 min. After being cooled down to 50 °C, Flavourzyme® was added at 1–7% steamed beans (d.b.) into slurries. The reaction was continued at 50 °C with hydrolysis time ranging from 60 to 1200 min in an orbital shaking water bath. After enzyme was inactivated at 85 °C 10 min, the resulting slurry was centrifuged at 6,500 g for 15 min. The supernatant was collected as black gram bean hydrolysate.

2.3 Determination of chemical properties of black gram bean hydrolysate

The DH of hydrolysate was determined based on an increase in free amino groups using 2,4,6-trinitrobenzenesulfonic acid (TNBS) according to the method of Sritongtae *et al*. (2017). The amount of free amino groups in the supernatant was calculated as shown in Eq. (1) and DH was calculated based on the amount of free amino groups generated during hydrolysis as shown in Eq. (2)

$$\text{Free amino groups} = \frac{\text{Absorbance 420 nm} \times \text{mL of total reactant solution}}{\varepsilon \times 1\ \text{cm} \times \text{g of protein in sample}} \times 10^6 \quad (1)$$

$$\text{DH (\%)} = \frac{L_t - L_0}{L_{max} - L_0} \times 100 \quad (2)$$

where ε is molar extinction coefficient (20,300 $M^{-1}cm^{-1}$), L_0 and L_t are initial amount of free amino groups and amount of free amino groups in solution at time " t ", L_{max} is the amount of free amino groups obtained from acid hydrolysis.

TPC were determined using Folin-ciocalteu method as explained in Waterhouse (2005). Absorbance of sample was measured at wavelength of 765 nm. The released TPC was expressed as gallic acid equivalents per gram of black gram beans (d.b.).

Maillard reaction products (MRPs) produced during hydrolysis was monitored as browning via measuring absorbance at wavelength of 420 nm.

Antioxidant activity of beans hydrolysate was determined according to the method explained in Sritongtae *et al*. (2017). DPPH radical scavenging activity was measured at 517 nm and calculated as shown in Eq. (3). FRAP was measured at 593 nm, and expressed as μmol Trolox per gram of black gram beans (d.b.).

$$\text{DPPH radical scavenging activity } (\%) = \frac{A_0 - A}{A_0} \times 100 \quad (3)$$

where A_0 is absorbance of DPPH solution and A is absorbance of sample solution

2.4 Experimental design

RSM based on 2-factors, 5-levels central composite design (CCD) was employed to evaluate the degree of hydrolysis (DH), browning, total phenolic content (TPC), and antioxidant activity of the hydrolysate. The coded and actual levels of the two independent variables used in the RSM design are listed in Table 1. The results of three replicate experiments were analyzed using Minitab software version 17.1.0 (Minitab, Inc., State Collage, PA) for analysis of

variance (ANOVA) and regression analysis. The three-dimensional response surface plots were generated using Design-Expert® Software Version 11 (Stat-Ease, Inc., MN.).

Table 1. Experimental design for producing black gram bean hydrolysate

Run	Coded level		Actual level	
	X_1	X_2	Flavourzyme® (%w/w)	Hydrolysis time (min)
1	-1	-1	1.88	267
2	+1	-1	6.12	267
3	-1	+1	1.88	1033
4	+1	+1	6.12	1033
5	-1.412	0	1.00	630
6	+1.412	0	7.00	630
7	0	-1.412	4.00	60
8	0	+1.412	4.00	1200
9	0	0	4.00	630

The relationship of two independent variables (X_1 and X_2) each response (Y_i) was analyzed as second-order polynomial model using the following equation:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 X_1^2 + \beta_5 X_2^2$$

where X_1 and X_2 are enzyme concentration and hydrolysis time, respectively; β_0 is constant coefficient; β_1 and β_2 are linear coefficients; β_3 is interaction coefficient; β_4 and β_5 are quadratic coefficients.

2.5 Validation

An additional experiment with two conditions under the range of optimal hydrolysis condition was performed in order to validate the suitability and accuracy of generated response models. Confirmation of response models was conducted by considering percentage error measurement between the predicted and observed (experiment) values (Zhang *et al*., 2012).

2.6 Statistical analysis

Statistical differences were considered significant at $p < 0.05$. Comparison of means was performed with Tukey's test using SPSS Statistics software version 22.0 (SPSS Inc., Chicago, IL).

3. Results and Discussion

3.1 Effect of hydrolysis condition on DH, TPC, browning and antioxidant activity

Production of hydrolysate containing biological properties via enzymatic modification is known to be influenced by several factors e.g. enzyme concentration, hydrolysis time etc. In this study, the effect of two independent variables, (Flavourzyme® concentration of 1–7% (w/w) and hydrolysis time of 60–1200 min) on different response variables (DH, TPC, browning and antioxidant activity as DPPH radical scavenging activity and FRAP) using RSM was investigated. The observed values for all responses are shown in Table 2. The results showed that both independent variables had a significant effect on all responses ($p < 0.05$). The DH and released TPC were significantly higher as increasing both enzyme concentration and hydrolysis time ($p < 0.05$). Black gram bean hydrolysate showed the DH in the range of 27.57–92.60%. The greater value of DH indicated the presence of higher proteolytic activity resulted in more peptide bonds cleavage (Klompong *et al.*, 2007). The total number of peptide bonds cleaved is a reflection of the degree which a protein is hydrolyzed. A condition of 6.12% Flavourzyme® and hydrolysis time of 1042 min (run 4) showed the highest DH. The DH in this study was higher than DH (68.88%) of mung bean hydrolysate with 7% Flavourzyme® and hydrolysis time of 360 min (Sangsukiam and Duangmal, 2017). The difference in DH of these two studies could be due to the difference in bean variety and enzymatic hydrolysis conditions, resulting in the different extent of peptide bonds cleaved in the protein structure.

The protein-bound polyphenols in beans were released during enzyme hydrolysis, resulting in higher amount of free phenolic compounds. Increasing both enzyme concentration and hydrolysis time led to a significant increase in total phenolic compounds (TPC) of black gram bean hydrolysate ($p < 0.05$). The highest amount of released TPC was observed at hydrolysis condition of 7% Flavourzyme® for 630 min, (run 6). Enzymatic hydrolysis led to changes in tertiary structure of protein via hydrophobic interaction between protein and polyphenols, resulting in releasing of free polyphenols which may be buried in protein-bound polyphenols complex (Siebert et al., 1996). Similar report was shown in the hydrolysis of germinated adzuki bean with 7% Flavourzyme® and 360 min hydrolysis periods (Sangsukiam and Duangmal, 2017). Garcia-Mora *et al*. (2015) also reported the higher TPC in pinto bean hydrolysate with 2% alcalase as hydrolysis time increased.

During hydrolysis the formation of MRPs, the browning compounds, in black gram bean hydrolysate could be monitored spectrophotometric assay at 420 nm. Both independent variables significantly affected an increase in absorption values at 420 nm ($p < 0.05$). The results showed the highest absorption value at 4% Flavourzyme® and hydrolysis time of

60 min (run 7). Zha *et al*. (2015) reported that the MRPs showed a significant potent as antioxidant activity and radical scavenging activity in shrimp by-product protein hydrolysate.

Table 2. Effect of hydrolysis condition on DH, TPC, browning and antioxidant activity

Run*	%DH	TPC (mg GAE g^{-1}, db.)	Absorbance at 420 nm	Antioxidant activity	
				% DPPH radical scavenging activity	FRAP (μmol Trolox g^{-1}, db.)
1	$33.92^{de} \pm 0.75$	$1.01^{abc} \pm 0.21$	$0.487^{c} \pm 0.01$	$79.47^{a} \pm 0.99$	$1.06^{d} \pm 0.18$
2	$70.65^{c} \pm 7.78$	$1.60^{abc} \pm 0.41$	$0.591^{b} \pm 0.00$	$79.95^{a} \pm 0.08$	$1.28^{bc} \pm 0.30$
3	$71.40^{bc} \pm 1.58$	$1.38^{abc} \pm 0.24$	$0.448^{cd} \pm 0.05$	$79.90^{a} \pm 0.11$	$1.30^{ab} \pm 0.17$
4	$92.60^{a} \pm 2.05$	$1.76^{ab} \pm 0.43$	$0.412^{cd} \pm 0.05$	$79.93^{a} \pm 0.01$	$1.36^{ab} \pm 0.38$
5	$47.94^{d} \pm 1.64$	$0.85^{c} \pm 0.30$	$0.364^{d} \pm 0.00$	$76.66^{b} \pm 0.15$	$1.06^{d} \pm 0.18$
6	$91.96^{a} \pm 6.15$	$1.90^{a} \pm 0.39$	$0.383^{d} \pm 0.00$	$80.43^{a} \pm 0.16$	$1.42^{a} \pm 0.37$
7	$27.57^{e} \pm 0.33$	$0.96^{bc} \pm 0.20$	$0.674^{a} \pm 0.02$	$77.09^{b} \pm 1.97$	$1.19^{cd} \pm 0.21$
8	$82.56^{ab} \pm 0.49$	$1.73^{abc} \pm 0.28$	$0.484^{c} \pm 0.01$	$80.44^{a} \pm 0.21$	$1.46^{a} \pm 0.44$
9	$75.83^{abc} \pm 2.53$	$1.51^{abc} \pm 0.29$	$0.436^{cd} \pm 0.00$	$80.01^{a} \pm 0.82$	$1.29^{bc} \pm 0.32$

Note: * Condition of run 1 - 9 was explained in Table 1

Increasing the antioxidant activity of black gram bean hydrolysate may be due to the increasing peptides containing smaller molecular weight, phenolic compounds and the generated browning compounds. The antioxidant activities of the bean hydrolysate were determined using DPPH radical scavenging and FRAP techniques. The results are shown in Table 2. The bean hydrolysate showed high DPPH radical scavenging activity and FRAP in the range of 76.66–80.44% and 1.06–1.46 μmol Trolox per gram of black gram beans (d.b.), respectively. Their antioxidant activities were highest with %DH in the range of 82.56–91.96 (run 6 and run 8). An increase in both enzyme concentration and hydrolysis time significantly influenced increasing DPPH radical scavenging activity and FRAP ($p < 0.05$). The greater antioxidant activity of hydrolysate may be due to higher the amount of released TPC, smaller peptides, free amino acids, and generated MRPs. Particularly, the presence of amino acids containing aromatic side chains (tryptophan, tyrosine, phenylalanine) or nucleophilic-sulfur containing side chains (cysteine, methionine) in the sequence of peptides after hydrolysis is proven to increase the antioxidant activities as their hydrogen atoms are easily to be removed. Phenolic compounds also exhibited the ability to scavenge free radicals and inhibited lipid oxidation in food system by donating hydrogen and electron to stabilize free radicals (Rice-Evans *et al*., 1997). Sbroggio *et al*. (2016) reported that DPPH radical scavenging activity and FRAP of okara protein hydrolysate prepared using Flavourzyme® increased from 9.5 to 18.5%

and 168.0 to 360.3 μmol Trolox per litre, respectively as DH increased up to 5.8%. Porcine skin collagen hydrolysate obtaining from the action of protease exhibited the antioxidative properties due to the small peptides (Li *et al*., 2007).

3.2 Polynomial models of responses

Experiment data were analyzed using RSM. Relationship of both independent variables with responses was evaluated using multiple regression analysis. The results of ANOVA (Table 3) showed that regression coefficient of both enzyme concentration and hydrolysis time had a significant relation with responses ($p < 0.05$). The equation models for the response variables of black gram bean hydrolysate were derived using the regression coefficient of linear, quadratic and the interaction terms of both independent variables to fit the second-order polynomial model. The regression models are shown as follows:

Primary response models

$Y_1 = -28.48 + 16.45X_1 + 0.1458X_2 - 0.875X_1^2 - 0.000071X_2^2 - 0.00372X_1X_2$

$r^2 = 0.9557$

$Y_2 = -0.009 + 0.299X_1 + 0.001881X_2 - 0.0113X_1^2 - 0.000001X_2^2 - 0.000069X_1X_2$

$r^2 = 0.8717$

$Y_3 = 0.5166 + 0.0776X_1 - 0.000631X_2 - 0.00622X_1^2 - 0.000032X_1X_2 r^2 = 0.9358$

Secondary response models

$Y_4 = 77.62 + 0.515X_1 + 0.00896X_2 - 0.0283X_1^2 - 0.000007X_2^2 - 0.000724X_1X_2$

$r^2 = 0.7760$

$Y_5 = 0.7967 + 0.1682X_1 + 0.000759X_2 - 0.01581X_1^2 - 0.000013X_1X_2$

$r^2 = 0.7793$

where X_1 and X_2 are enzyme concentration and hydrolysis time, Y_{1-5} are dependent variables which are DH, TPC, browning, DPPH radical scavenging activity and FRAP, respectively.

The polynomial model for responses could be evaluated the suitability, fitness and significance using Fisher's test (F-test) as shown in Table 3. Evaluation the fitness of response models with experiment data was conducted using “lack of fit” parameter (Jamil *et al*., 2016). The corresponding *p* values for the models were in a range of 0.102–0.359, which were not significant ($p > 0.05$), indicating that the models fitted the data well. Furthermore, the coefficients of determination (r^2) values of response models were found in the range of 0.7760–0.9557, indicating a good fit model. Therefore, these models can be used for predicting the optimal hydrolysis condition to obtain the desirable response values. The three-dimensional

(3D) response surface plots showed that DH, TPC, DPPH radical scavenging activity and FRAP increased as increasing enzyme concentration and hydrolysis time. The 3D response surface plots for DH, DPPH radical scavenging activity and FRAP represented surfaces where the maximum plot was located within the experimental region. However, the maximum point of TPC was outside the experimental region as the amount of TPC depended on the increasing DH. MRPs increased as enzyme concentration increased, this plot presented a saddle point as critical point, an inflexion point between a relative maximum and a relative minimum.

Table 3. Results of ANOVA for second-order polynomial models

Variable	Source	Model	X_1	X_2	X_1X_1	X_2X_2	X_1X_2	Residual	Lack of fit	Pure error	Total
	DF†	5	1	1	1	1	1	21	3	18	26
DH	SS	12980.10	5452.50	6588.40	133.20	1126.40	114.70	601.00	171.70	429.30	13581.10
	MS	2596.03	5452.50	6588.37	133.16	1126.41	114.69	28.62	57.23	23.85	
	F	90.71	190.52	230.21	4.65	39.36	4.01		2.40		
	p	0.000*	0.000*	0.000*	0.043*	0.000*	0.058		0.102		
TPC	SS	4.71	2.94	1.68	0.02	0.12	0.04	0.69	0.17	0.52	5.40
	MS	0.94	2.94	1.68	0.02	0.12	0.04	0.03	0.06	0.03	
	F	28.54	89.17	50.92	0.67	3.77	1.21		2.02		
	p	0.000*	0.000*	0.000*	0.422	0.066	0.284		0.148		
Browning	SS	0.241	0.006	0.100	0.007	0.050	0.008	0.017	0.003	0.013	0.258
	MS	0.048	0.006	0.100	0.007	0.050	0.008	0.001	0.001	0.001	
	F	61.25	8.13	127.12	8.55	63.89	10.67		1.51		
	p	0.000*	0.010*	0.000*	0.008*	0.000*	0.004*		0.245		
DPPH	SS	46.32	3.05	20.99	0.14	10.10	4.34	13.37	2.14	11.23	59.69
	MS	9.26	3.05	20.99	0.14	10.10	4.34	0.64	0.71	0.62	
	F	14.55	4.79	32.96	0.22	15.85	6.81		1.14		
	p	0.000*	0.040*	0.000*	0.644	0.001*	0.016*		0.359		
FRAP	SS	0.210	0.121	0.037	0.044	0.053	0.001	0.060	0.018	0.042	0.270
	MS	0.042	0.121	0.037	0.044	0.053	0.001	0.003	0.006	0.002	
	F	14.83	42.82	12.89	15.36	18.58	0.50		2.55		
	p	0.000*	0.000*	0.002*	0.001*	0.000*	0.489		0.088		

Note: where X_1 and X_2 are enzyme concentration and hydrolysis time, respectively.

† DF is degree of freedom of each response.

* Statistical significance ($p < 0.05$)

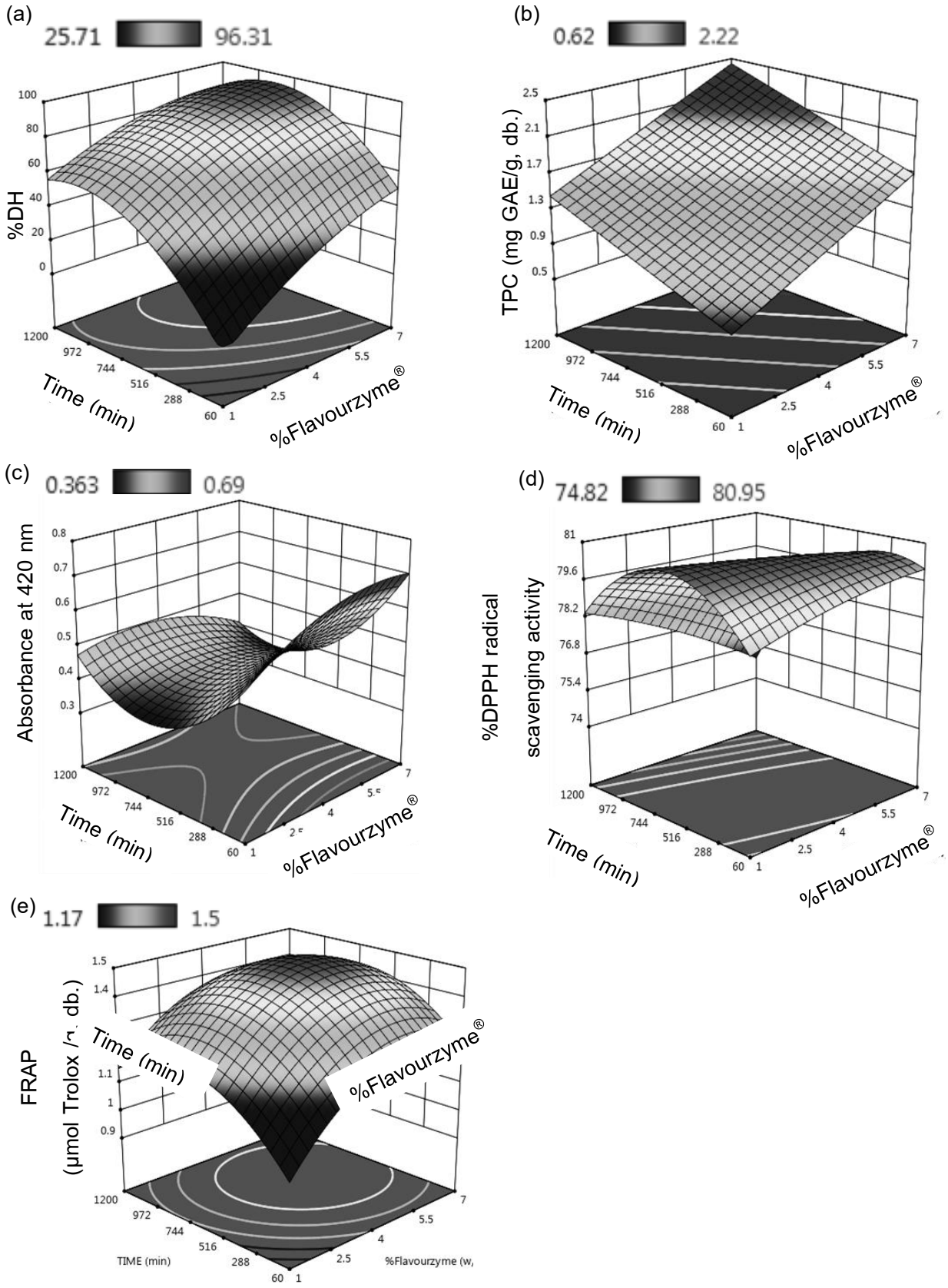

Figure 1 Response surface plots of black gram bean hydrolysate on DH (a), TPC (b), browning (c) antioxidant activity as DPPH radical scavenging activity (d) and FRAP (e).

3.3 Validation

To obtain the optimization of hydrolysis condition on responses, DH, TPC, browning and antioxidant activity via DPPH radical scavenging activity and FRAP were considered. In this study, DH, specified as main primary response, was set at 75–80% whereas other responses were specified target value of responses with the highest value. An additional experiment with two conditions was performed with three replicates using optimal hydrolysis conditions with 6.03–6.09% enzyme concentration and hydrolysis time of 360–395 min. Results are shown in Table 4. The validation test was conducted using percentage error measurement between the predicted and observed values, In order to verify the suitability and validity of obtained response models.

Table 4. The predicted and observed values of hydrolysis responses in verification test

Flavourzyme® %(w/w)	Time (min)	Responses	Predicted value	Observed value	%Error
6.09	360	%DH	74.71	76.91 ± 1.56	2.94
		TPC	1.82	1.76 ± 0.03	3.30
		Browning	0.523	0.515 ± 0.06	1.53
		%DPPH	80.48	71.44 ± 0.31	11.23
		FRAP	1.42	1.03 ± 0.03	27.46
6.03	395	%DH	76.55	79.22 ± 0.94	3.48
		TPC	1.85	1.84 ± 0.05	0.54
		Browning	0.507	0.494 ± 0.02	2.56
		%DPPH	80.47	70.62 ± 1.25	12.24
		FRAP	1.43	1.13 ± 0.05	20.98

As shown in Table 4, the percentage error for primary (DH, TPC and browning) and secondary (DPPH radical scavenging activity and FRAP) responses were different in range of 0.54–3.48% and 11.23–27.46%, respectively. The higher percentage error of secondary responses could be because (1) FRAP value might be partly affected by primary responses, (2) other components in hydrolysate also contributed antioxidant activity as reducing power in the form of FRAP. The percentage error of this study was quite low (0.54–3.48%) for the prediction of primary responses, indicating a good fit of model. The percentage error of DPPH radical scavenging activity was also low (12%). Thus, this model was acceptable to use for prediction of condition to produce black gram bean hydrolysate with high DPPH radical scavenging

activity. The results for succinic acid production with optimal condition using RSM showed that the percentage error of primary responses was lower than percentage error of 3.02–6.38 (Zhang *et al*., 2012).

4. Conclusions

RSM was conducted to optimize enzymatic hydrolysis condition for high antioxidant black gram bean hydrolysate production and the polynomial model for all responses was considered to be adequate and valid for predicting the targeted responses. The results showed that the hydrolysis responses of black gram bean hydrolysate were influenced by enzyme concentration and hydrolysis time. The optimal hydrolysis condition was at enzyme concentration of 6.09% with hydrolysis time of 360 min. Under this hydrolysis condition the DH was reached 75–80% with the predicted released TPC of 1.82 mg GAE per gram of black gram beans (d.b.), DPPH radical scavenging activity of 80.48% and FRAP of 1.42 μmol Trolox per gram of black gram beans (d.b.).

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References

Betancur-Ancona, D., Sosa-Espinoza, T., Ruiz-Ruiz, J., Segura-Campos, M. and Chel-Guerrero, L. 2014. Enzymatic hydrolysis of hard-to-cook bean (*Phaseolus vulgaris* L.) protein concentrates and its effects on biological and functional properties. International Journal of Food Science and Technology. 49: 2–8.

Clemente, A., Vioquea, J., Sánchez-Vioquea, R., Pedrochea, J., Bautistab, J. and Millána, F. 1999. Protein quality of chickpea (*Cicer arietinum* L.) protein hydrolysates. Food Chemistry. 67: 269–274.

Cumby, N., Zhong, Y., Naczk, M. and Shahidi, F. 2008. Antioxidant activity and water-holding capacity of canola protein hydrolysates. Food Chemistry. 109: 144–148.

Del Mar Contreras, M., Hernández-Ledesma, B., Amigo, L., MartínÁlvarez, P.J. and Recio, I. 2011. Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: Optimization by response surface methodology. LWT-Food Science and Technology. 44(1): 9–15.

Do Evangelho, J. A., Berrios, J. D., Pinto, V. Z., Dias-Atunes, M., Levien-Vanier, N. and Zavareze, E. 2016. Antioxidant activity of black bean (*Phaseolus vulgaris* L.) protein hydrolysates. Journal of Food Science and Technology. 36(1): 23–27.

Garcia-Mora, P., Frias, J., Peñas, E., Zieliński, H., Giménez-Bastida, J.A., Wiczkowski, W., Zielińska, D. and Martínez-Villaluenga, C. 2015. Simultaneous release of peptides and phenolics with antioxidant, ACE-inhibitory and anti-inflammatory activities from pinto bean (*Phaseolus vulgaris* L. var. pinto) proteins by subtilisins. Journal of Functional Foods. 18: 319–332.

Jamil, N. H., Halim, N. R. A. and Sarbon, N. M. 2016. Optimization of enzymatic hydrolysis condition and functional properties of eel (*Monopterus* sp.) protein using response surface methodology (RSM). International Food Research Journal. 23(1): 1–9.

Klompong, V., Benjakul, S., Kantachote, D. and Shahidi, F. 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chemistry. 102: 1317–1327.

Li, B., Chen, F., Wang, X., Ji, B. and Wu, Y. 2007. Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. Food Chemistry. 102(4): 1135–1143.

Liu, P., Huang, M., Song, S., Hayat, K., Zhang, X., Xia, S. and Jia, C. 2012. Sensory characteristics and antioxidant activities of Maillard reaction products from soy protein hydrolysates with different molecular weight distribution. Food Bioprocess Technology. 5: 1775–1789.

Luna-Vital, D.A., Mojica, L., González de Mejía, E., Mendoza, S. and Loarca-Piña, G. 2015. Biological potential of protein hydrolysates and peptides from common bean (*Phaseolus vulgaris* L.): A review. Food Research International. 76: 39–50.

Mune Mune, M.A. 2015. Optimizing functional properties of bambara bean protein concentrate by enzymatic hydrolysis using pancreatin. Journal of Food Processing and Preservation. 39(6): 2572–2580.

Nwokolo, E. and Smartt, J. 1996. Food and feed from legumes and oilseeds (pp. 12–32). Chapman and Hall, London.

Rice-Evans, C., Miller, N. and Paganga, G. 1997. Antioxidant properties of phenolic compounds. Trends in Plant Science. 2(4): 152–159.

Sangsukiam, T. and Duangmal, K. 2017. A comparative study of physico-chemical properties and antioxidant activity of freeze-dried mung bean (*Vigna radiata*) and adzuki bean

(*Vigna angularis*) sprout hydrolysate powders. International Journal of Food Science and Technology. 52: 1971–1982.

Sarmadi, B.H. and Ismail, A. 2010. Antioxidative peptides from food proteins: A review. Peptides. 31(10): 1949–1956.

Sbroggio, M.F., Montilha, M.S., Figueiredo, V.R.G., Georgetti, S.R. and Kurozawa, L.E. 2016. Influence of the degree of hydrolysis and type of enzyme on antioxidant activity of okara protein hydrolysates. Food Science and Technology. 36(2): 375–381.

Siebert, K.J., Troukhanova, N.V. and Lynn, P.Y. 1996. Nature of polyphenol-protein interactions. Journal of Agricultural and Food Chemistry. 44: 80-85.

Sritongtae, B., Sangsukiam, T., Morgan, M.R. and Duangmal, K. 2017. Effect of acid pretreatment and the germination period on the composition and antioxidant activity of rice bean (*Vigna umbellate*). Food Chemistry. 227: 280–288.

Wani, I.A., Sogi, D.S., Shivhare, U.S. and Gill, B.S. 2015. Physico-chemical and functional properties of native and hydrolyzed kidney bean (*Phaseolus vulgaris* L.) protein isolates. Food Research International. 76: 11–18.

Waterhouse, A.L. 2005. Polyphenolics. In Wrolstad, R.E., Terry, E.A., Decker, E.A., Penner, M.H., Reid, D.S., Schwartz, S.J., Shoemaker, C.F., Smith, D. and Sporns, P. (Eds.). Handbook of Food Analytical Chemistry (pp. 461–470). John Wiley and Sons, London.

Xu, B., and Chang, S.K.C. 2009. Total phenolic, phenolic acid, anthocyanin, flavan-3-ol, and flavonol profiles and antioxidant properties of pinto and black beans (*Phaseolus vulgaris* L.) as affected by thermal processing. Journal of Agricultural and Food Chemistry. 57: 4754–4764.

Zha, F., Wei, B., Chen, S., Dong, S., Zenga., M. and Liua, Z. 2015. The Maillard reaction of a shrimp by-product protein hydrolysate: chemical changes and inhibiting effects of reactive oxygen specie s in human HepG2 cells. Food and Function. 6: 1919–1927.

Zhang, Y.J., Li Q., Zhang, Y.X., Wang, D. and Xing, J.M. 2012. Optimization of succinic acid fermentation with *Actinobacillus succinogenes* by response surface methodology (RSM). Journal of Zheijang University SCIENCE B - Biomedicine and Biotechnology. 13(2): 103–110.