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Optimization of angiotensin I-converting enzyme (ACE) inhibition by rice dregs hydrolysates using response surface methodology

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Abstract: Angiotensin I-converting enzyme (ACE) inhibitory peptides have been shown to have antihypertensive effects and have been utilized for physiologically functional foods and pharmaceuticals. The ACE inhibitory ability of a hydrolysate is determined by its peptide composition. However, the peptide composition of a hydrolysate depends on proteolytic enzyme and the hydrolysis conditions. In this study, the effect of process conditions on the ACE inhibitory activity of rice dregs hydrolyzed with a trypsin was investigated systematically using response surface methodology. It was shown that the ACE inhibitory activity of rice dregs hydrolysates could be controlled by regulation of five process conditions. Hydrolysis conditions for optimal ACE inhibition were defined using the response surface model of fractional factorial design (FFD), steepest ascent design, and central composite design (CCD).

Key words: Angiotensin I-converting enzyme inhibitor, Rice dregs, Response surface methodology
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INTRODUCTION

Angiotensin I-converting enzyme (ACE) can convert angiotensin I to angiotensin II which is known to be a strong vasopressor, besides inactivating bradykinin conducive to lowering blood pressure (Ondetti *et al*., 1977). This enzyme also plays physiological roles in the regulation of local levels of other endogenous peptides, such as enkephalins and substace P. Therefore, inhibition of ACE can reduce the activity of angiotensin II, but increase bradykinin and enkephalins levels, which results in lowering of blood pressure (Koike *et al*., 1980).

Many ACE inhibitory peptides have been characterized from food proteins, such as gelatin (Oshima *et al*., 1979), maize (Maruyama, *et al*., 1989), fish (Seki *et al*., 1995), eggs (Yoshii *et al*., 1999), pea and whey protein (Vermeissen *et al*., 2004).

This work studied ACE inhibitory activity peptides characterization from rice dregs mainly produced as by-products by many factories such as monosodium glutamate factory, glucose factory, fermentation industry, and so on. Although rice dregs are used as animal feed, it can cause a problem of environmental pollution. On the other hand, rice dregs contain 65% protein, which is higher than soybean protein content. Therefore, rice dreg is good material for producing the ACE inhibitory peptides.

As stated before, many peptides derived from food protein hydrolysis have ACE inhibitory activity, which is exhibited during the hydrolysis process. The hydrolysis process occurs in two parts, one peptide-forming process and another is peptide-degrading process. The two processes determine the maximum ACE inhibition. To optimize the peptides composition of hydrolysates, the hydrolysis processes have to be analyzed.

The peptides composition of hydrolysates depends on several process parameters such as proteolytic enzyme, enzyme to substrate ratio (E/S), hy-

drolysis time, hydrolysis pH, hydrolysis temperature and water to rice dregs ratio (W/R). Response surface modelling is a valuable tool for optimization of several processes such as, fermentation, hydrolysis and chemical reactions (Baek and Cadwallader, 1995; Diniz and Martin, 1996; Ibanoglu *et al*., 1998; Liu *et al*., 2000).

Response surface methodology (RSM) is a useful statistical technique for investigation of complex processes particularly in the fields of chemical and engineering processes, industrial research, biological investigations and agricultural processes, with emphasis on optimizing a process or a system (Khuri and Cormell, 1987). Response surface methodology can determine the optimal settings of the experimental factors that give the maximum (or minimum) value of the response.

MATERIALS AND METHODS

Enzymes and other material

Angiotensin converting enzyme (ACE) and N-hippuryl-His-Leu tetrahydrate were purchased from Sigma-Aldrich Co., trypsin from China Medicine (Group) Shanghai Chemical Reagent Co., rice dregs from Zhejiang Mi Feng Group Co., LTD.

Rice dregs hydrolysis

The rice dregs were hydrolyzed by trypsin. The hydrolysis process was terminated by heating in boiling water for 10 min, and the hydrolysate was centrifuged at 2500×g for 20 min to obtain the supernatant. After concentration, the hydrolysates were freeze-dried.

Protein determination

Protein concentration was measured by determination of total nitrogen on the nitrogen-analyzer. For calculation of protein concentration a Kjeldahl factor of 5.95 was used.

ACE inhibitory activity measurements

The ACE inhibitory activity assay was performed using a modified version of the method of Cushman and Cheung. The reaction mixture (50 μl) contained 5 mmol/L Hip-His-Leu as a substrate, 0.3 mol/L NaCl and 5 mU enzyme in 50 mmol/L sodium borate buffer (pH 8.3). A sample (50 μl) was added to the above reaction mixture. After incubation at 37 °C for 180 min, further reaction was stopped by addition of 1.0 mol/L HCl (250 μl). The resulting hippuric acid was extracted by addition of 1.5 ml ethyl acetate. After centrifugation (2500×g, 15 min), 1 ml of the upper layer was transferred into a glass tube and evaporated at room temperature for 3 h in vacuum. The hippuric acid was redissolved in 3.0 ml distilled water, and absorbance was measured at 228 nm using spectrophotometer.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Response surface methodology (RSM) was used to optimize the hydrolysis reaction parameters. To optimize the hydrolysis reaction, a three-step design was used that included fractional factorial design (FFD), steepest ascent design, and central composite design (CCD). FFD was used for screening the most important factor of the hydrolysis reaction, steepest ascent design was used to determine the direction toward predicted higher responses, and CCD was used to optimize the important factor and maximize the ACE inhibitory activity (Chen *et al*., 2002). In the FFD, a set of 12 experiments was carried out. The following five hydrolysis parameters were chosen for screening design: pH, temperature, enzyme to substrate ratio (E/S), water to rice dregs ratio (W/R), and time of hydrolysis. The factors were varied at three levels coded as −1, 0, +1 (Table 1).

Table 1 Factors and levels of FFD applied in rice dregs hydrolysis

Factor	Level		
	−1	0	+1
pH	5.5	7.0	8.5
Temperature (°C)	32.0	37.0	42.0
E/*S* (%w/w)	1.0	1.5	2.0
W/*R* (w/w)	4.0	5.0	6.0
Hydrolysis time (h)	2.0	4.0	6.0

Statistical analysis was performed with SAS 8.0 software.

The variables were coded according to the following equation (Montagomery, 1991).

$$x_i=(X_i - X_0)/\Delta x_i \quad (1)$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and Δx_i is the step change value.

The first-order model was obtained from FFD, and second-order model was obtained from CCD.

The model proposed for the response (Y) was:

$$Y=b_0+\Sigma b_i x_i+\Sigma b_{ii} x_i^2+\Sigma b_{ij} x_i x_j \quad (2)$$

where b_0 is the value of the fixed response at the central point of the experiment, which is the point (0,0); b_i, b_{ii} and b_{ij} are the linear, quadratic and cross product coefficients, respectively.

RESULTS AND DISCUSSION

The screening design

In this experiment, FFD was used to screen the factors of hydrolysis process. According to several studies, the most important parameters for optimizing the hydrolysis reaction were pH, temperature, enzyme to substrate ratio (E/S), water to rice dregs ratio (W/R), and time of hydrolysis (Haaland, 1989). In this study, these parameters were selected and set to three levels coded as −1, 0, +1. Table 2 gives the results of the scheme involving 12 experiments, including 4 replicates of the center point experiment. The ACE inhibitory activity varied from 20.0% to 86.0% (Table 2).

The effects of the parameters were analyzed by multiple regression techniques. The application of response surface methodology (RSM) yielded the following regression equation of empirical relationship between the ACE inhibitory activity values and the variables in coded units.

$$Y=54.17+13.76x_1-5.26x_2-4.26x_3-15.26x_4+5.76x_5 \quad (3)$$

where Y is the response (ACE inhibitory activity) and x_1, x_2, x_3, x_4 and x_5 the coded values of test variables pH, temperature, enzyme to substrate ratio (E/S), water to rice dregs ratio (W/R), and time of hydrolysis, respectively.

The values of the regression coefficient were calculated and a first order equation with coefficient of determination, R^2=0.8770 was obtained.

The significance of each coefficient was determined by $|T|$-value and P-values which are listed in Table 3. These results show that pH and water to rice dregs ratio (W/R) were more significant than other factors at 95% confidence level, (the P-values were 0.0069 and 0.0043, respectively) as shown in Table 3. These results suggest that the more important independent variables are pH and W/R in hydrolysis reaction.

ANOVA analysis show that the P-value of the model is 0.0106, indicating that the model is statistically significant at 95% confidence level (Table 4).

T-test for variance between the average observation of two-level experiment and center point showed that the difference was not significant. This result indicated that optimum point is not in the domain

Table 2 Experimental designs for the optimization

Experiment	pH	T (°C)	E/S (%w/w)	W/R (w/w)	Hydrolysis time (h)	$ACEI$ (%)
	x_1	x_2	x_3	x_4	x_5	Y
1	1	−1	−1	1	1	66.0
2	−1	−1	1	1	−1	20.0
3	−1	1	1	−1	1	50.0
4	−1	−1	−1	−1	1	80.0
5	−1	1	−1	1	−1	26.0
6	1	1	1	1	1	58.0
7	1	−1	1	−1	−1	86.0
8	1	1	−1	−1	−1	76.0
9	0	0	0	0	0	42.0
10	0	0	0	0	0	52.0
11	0	0	0	0	0	44.0
12	0	0	0	0	0	50.0

Table 3 Parameters estimates from the regression analysis

Variable	DF	Parameter estimate	Standard error	T for H0: parameter=0	*P*-values
Intercept	1	54.17	2.79	19.40	0.0001
pH	1	13.76	3.42	4.02	0.0069
Temperature (°C)	1	−5.26	3.42	−1.54	0.1756
E/*S* (%w/w)	1	−4.26	3.42	−1.24	0.2602
W/*R* (w/w)	1	−15.26	3.42	−4.46	0.0043
Hydrolysis time (h)	1	5.76	3.42	1.68	0.1436

Table 4 Analysis of variance (ANOVA) for the model

Source	DF	Sum of squares	Mean square	*F*-value	*P*-values
Model	5	4002.50	800.50	8.559	0.0106
Error	6	561.17	93.53		
Cor. total	11	4563.67			

of our experiment. Experimentation on steepest ascent path is necessary to reach optimum domain (Table 5).

Table 5 Analysis of *T*-test for the model

Factor	*N*	Mean	Std error	*T*	d.f.	Prob>|*T*|
pH	8	57.75	8.65	1.19	8	0.2650
W/*R* (w/w)	4	47.00	2.38	0.85	10	0.4147

The steepest ascent experiment and analysis

The experimental data showed that the optimal region is outside the current design space (Table 2 and Table 3). In this situation, a directional search method was used to determine the next set of experiments. Steepest ascent experiment uses the sign of the linear effects to determine the direction of the experiment. This experiment started at the center point of the current design and stretches beyond of the current design. Since statistical analysis showed that pH and W/R were more significant than other factors at 95% confidence level, so these two factors were selected for the steepest ascent experiment and the other factors were fixed at zero level. The experimental results indicated that ACE inhibitory activity was increased when pH increased and that W/R decreased during the first experiment, but after the second step in the experiment, the ACE inhibitory activity decreased (Table 6). This suggests that the highest ACE inhibitory activity was achieved during the second step of the experiment.

Table 6 Experimental designs of steepest ascent and experimental data

Experiment	pH (x_1)	*W*/*R* (w/w) (x_4)	*ACEI* (%)
1	7.5	4.5	78.0
2	8.0	4.0	87.0
3	8.5	3.5	60.0
4	9.0	3.0	42.0
5	9.5	2.5	36.0

Central composite design (CCD) and response surface analysis

Further optimization of ACE inhibitory activity was devised by using central composite design (CCD). Based on results of the screening and steepest ascent experiment, the effect of variables x_1 (pH) and x_4 (water to rice dregs ratio) at five variation levels (Table 7) in the process of hydrolysis were investigated in CCD by response surface methodology (RSM). A set of 13 experiments was carried out, as shown in Table 8. The following regression equation was obtained after the analysis of variance (ANOVA).

$$Y=84.96+1.65x_1+2.85x_4-6.36x_1^2-4.85x_4x_1-6.89x_4^2 \quad (4)$$

where Y is the response (ACE inhibitory activity) and the x_1 and x_4 are coded values of the test variables pH and water to rice dregs ratio (W/R), respectively.

The coefficient of determination R^2 value provides a measure of variability in the observed response values and can be explained by the experimental factors and their interactions. The closer the R^2 value is to 1.00, the better the model. It can predict the response (Khuri and Cormell, 1987). R^2 was calculated to be 0.9145 for the ACE inhibitory activity. This implies that the sample variation of 91.45% could be attributed to the independent variables and

the model did not explain only 8.5% of the total variations (Table 9). The model's *F*-value of 14.97 and the *P*-value of 0.0013 were less than 0.05 implying that the model is significant.

Table 7 Factor and levels of central composite design applied to rice dregs hydrolysis

Factor	Level				
	−1.41421 (−α)	−1	0	+1	+1.41421 (α)
pH	7.29	7.50	8.00	8.50	8.71
W/*R* (w/w)	2.6	3.0	4.0	5.0	5.4

Table 8 Experimental design for the optimization experiment and experimental data

Experiment	pH	*W*/*R* (w/w)	*ACEI* (%)
	x_1	x_4	*Y*
1	−1.00000	1.00000	76.2
2	0.00000	0.00000	85.7
3	1.00000	−1.00000	80.1
4	−1.00000	−1.00000	64.3
5	0.00000	0.00000	86.9
6	−1.41421	0.00000	70.3
7	0.00000	0.00000	84.3
8	0.00000	1.41421	76.1
9	0.00000	0.00000	83.5
10	1.41421	0.00000	71.0
11	0.00000	0.00000	84.4
12	0.00000	−1.41421	63.1
13	1.00000	1.00000	72.6

Table 9 Analysis of variance (ANOVA) for the model

Regression	*DF*	R^2	*F*-value	*P*-values
Linear	2	0.1098	4.49	0.0556
Quadratic	2	0.6855	28.05	0.0005
Cross product	1	0.1192	9.76	0.0168
Total model	5	0.9145	14.97	0.0013

To elucidate the interaction of pH and water and rice dregs ratio (W/R) and determine the level of these two factors, which are required for optimum ACE inhibitory activity, we obtained the three-dimensional response surface curve by the statistically significant model (Fig.1). The plot shows that the ACE inhibitory activity reached its maximum value (ACE inhibitory activity 85.27%) at a combination of coded level 0.041 (x_1) and 0.131 (x_4). To examine the possibility hydrolysis conditions of 8.02 (pH) and 4.13 (W/R) could optimize ACE inhibitory activity, ACE inhibitory activity under such conditions was examined. The data showed that the ACE inhibitory activity (mean) was 84.95%. The above results indicate that the model is adequate under these hydrolysis conditions and is useful for produce ACE inhibitory activity peptides.

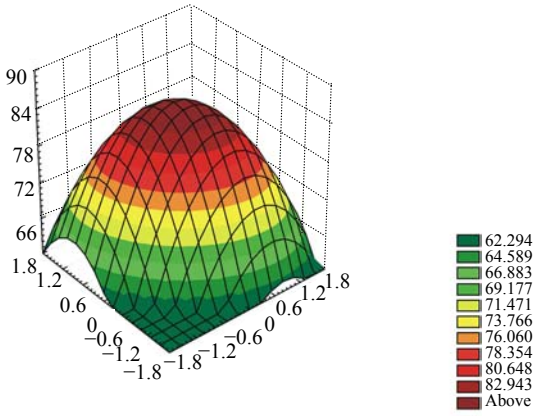

Fig. 1 Three-dimension plot for ACE inhibitory activity as a function of pH and W/R by keeping the other factor constant

CONCLUSION

Optimum conditions for rice dregs hydrolysis are pH of 8.02, temperature of 37.0 °C, enzyme to substrate ratio (E/S) of 1.5%, water to rice dregs ratio (W/R) of 4.13, and hydrolysis time span of 4.0 h. Using these conditions, the ACE inhibitory activity reached to 85.27%. Predicted values using the model were shown to correspond well with the experimental results. Hence by using a statistical experimental design it is possible to determine the optimum conditions for obtaining improved ACE inhibitory activity.

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