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Research Article

Preparation of Antihypertensive Peptide from Hydrolyzing Peanut Protein by Trypsin Covalently Immobilized on Chemically Modified Chitosan-coated Fe_3O_4 Particles

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Abstract: The aim of this study was mainly on apply immobilized trypsin which covalently linked with chemically modified chitosan-coated Fe_3O_4 particles to producing the antihypertensive peptide by hydrolyzing peanut protein. Response Surface Methodology (RSM) was employed to optimize the hydrolysis conditions, including Enzyme/substrate ratio, temperature, pH and time. Results showed that enzyme immobilization technology improved the thermal and pH stability of trypsin. The minimum IC_{50} value for Angiotensin Converting Enzyme (ACE) inhibitory activity (0.76 mg/mL) was obtained at the E/S ratio of 3.52, the temperature of 54.65°C, the time of 2.95 h and pH of 8.43, which was agreement with the predicted value (0.77 mg/mL) estimated by RSM within a 95% confidence interval. Moreover, a modest increase in the degree of hydrolysis promoted the ACE inhibitory activity of the hydrolysates, but excessive hydrolysis would lead to a decrease in ACE inhibitory activity.

Keywords: ACE inhibitory activity, degree of hydrolysis, enzyme immobilization, response surface methodology

INTRODUCTION

Peanut is the fourth most important oilseed in the world. China is the largest peanut producer, which had a share of about 43.86% of overall world peanut production in 2010 (USDA, 2012a). Sixty percent of the total produce in China is used for extraction of oil, leaving a large amount of residue as peanut meal, which contains 50-55% of protein. Peanut protein has good nutritional quality with high essential amino acid content (Wu *et al*., 2009). However, compared with other plant proteins, especially commercial soy protein isolate, its functional properties are weaker, which might greatly limit the application of peanut proteins in many food formulations (Liu *et al*., 2012). At present, most of peanut protein remains underutilized as animal feed in China.

To exploit the resources of peanut protein, enzymatic hydrolysis has been used to improve functional and nutritional properties of peanut protein, such as promoting solubility, removing antinutritional factors (Moure *et al*., 2006). Moreover, it has been recognized that some peptides that are released from peanut protein are antihypertensive effect (Jimsheena and Gowda, 2011; Jamdar *et al*., 2010). Although various proteases are employed to produce antihypertensive peanut peptides, trypsin is more suitable for this goal. Firstly, the peanut protein contains 11.42% of arginine residues and 3.63% of lysine residues (USDA, 2012b). Trypsin cleaves peptide chains mainly at lysine or arginine, so trypsin cleaves peanut protein easily. Moreover, because of the specificity of protease, protein could not be degraded to amino acids completely by single protease, so the trypic digestion in vivo has a minor influence on the structure of tryptic peptides which have been produced by hydrolysis in vitro, which is favorable in keeping the bioactivity of peptide.

However, the utilization of trypsin for producing antihypertensive peptide is hampered owing to its sensitiveness on temperature, pH and high cost compared with alkaline protease. Enzyme immobilization technology is an effective method to improve enzyme stability and reduce cost by recycling and reusing enzyme. With the development of technology, the magnetic particles for the immobilization of enzymes have been paid increasing attention due to their large specific surface area and easy separation from reaction mixture by magnetic field (Akgöl *et al*., 2001). Thanks to non-toxic, biodegradable and biocompatible properties and widely distribution, Chitosan is considered to be suitable for producing the magnetic particles as stabilizer to avoid the aggregation of magnetic Fe_3O_4. Therefore the combination of Fe_3O_4 and chitosan has been reported and applied in enzyme immobilization (Peniche *et al*., 2005; Wei *et al*., 2007). But practical application of immobilized trypsin has not been successful in hydrolyzing high molecular weight protein because of a significant decrease in catalytic efficiency. The reason

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why decrease in enzyme activity is that trypsin immobilized directly on a support suffers from steric hindrance of the substrate owing to its large molecular size. To promote the activity of immobilized trypsin, spacer introduction was an effective approach (Nouaimi *et al*., 2001). Yamamoto *et al*. (2005) reported as a spacer, 1, 6-Diaminohexane which was introduced between the trypsin and the support was the most effective in reducing the steric hindrance and allowing the β-lactoglobulin to approach the active site of immobilized trypsin.

Although production of peanut antihypertensive peptide by various proteases have been extensively studied, preparation of antihypertensive peptide form hydrolyzing peanut protein by immobilized protease, especially introducing spacer between magnetic chitosan-coated Fe_3O_4 particles and protease is rarely reported. Thus, the aim of this study was to explore the potential application of immobilized trypsin which introduced 1, 6-Diaminohexane between chitosan-coated Fe_3O_4 particle and trypsin in producing ACE inhibitory peanut peptides by proteolysis. To obtain the most powerful ACE inhibitory peptide and analyze the relationship between ACE inhibitory activity and Degree of Hydrolysis (DH) during enzymatic hydrolysis, Response Surface Methodology (RSM) was employed to analyze and optimize hydrolysis parameters including E/S ratio, time, pH and temperature.

MATERIALS AND METHODS

Chemicals and enzyme: Defatted peanut flour was provided by Henan Liangjian Science and Technology Co. Ltd. hippuryl-L-histidyl-L-leucine (Hip-His-Leu), sodium Tripolyphosphate (TPP), bovine serum albumin, Nα-Benzoyl-DL-Arginine- 4-nitroanilide hydrochloride (BAPNA), Brilliant Blue G-250, 2, 4, 6-Trinitrobenzenesulfonic acid (TNBS) were purchased from Sigma-Aldrich (St. Louis, USA). Trypsin (215,000 U/g) was obtained from Amresco (Solon, USA). $FeSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, Glutaraldehyde (GA, 25% in v/v, aqueous solution), Chitosan, 1, 6-Diaminohexane, ammonium hydroxide were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All the other chemicals used were of analytical grade.

Methods:

Preparation of magnetic Fe_3O_4 particles and Fe_3O_4-chitosan particles: Fe_3O_4 particles were produced by the co-precipitating method (Pan *et al*., 2009). 4.0532 g $FeCl_3 \cdot 6H_2O$ and 4.1708 g $FeSO_4 \cdot 7H_2O$ were dissolved in 50 mL of deionized water by vigorous stirring at 25°C, pH was adjusted to 10 by adding ammonium hydroxide. Then this solution was incubated at 80°C for 30 min with N_2 as the protective gas. During this period, the pH was kept at 10 by adding NH_4OH solution. After incubation the mixture was cooled to room temperature by stirring. Fe_3O_4 particles formed were separated magnetically and washed several times with deionized water to remove the excess NH_3OH.

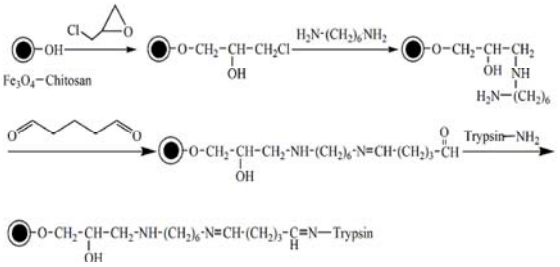

Fig. 1: Experimental procedures for introducing 1, 6-diamino-hexane on the surface of Fe_3O_4-chitosan particles and immobilization of trypsin

For preparation of Fe_3O_4-chitosan particles, Fe_3O_4 particles were dispersed in 100 mL 10 mg/mL chitosan solution (1% (v/v) acetate solution, pH 4.0) and 50 mL 2 mg/mL TPP was added simultaneously. The suspension was vigorously stirred for 50 min, then Fe_3O_4-chitosan particles were recovered from the mixture by placing on the magnetic field, washed with deionized water for several times, freeze-dried and stored at 4°C.

Preparation of chemically modified Fe_3O_4-chitosan particles by introducing spacer and immobilized trypsin: The procedure of preparation of chemically modified Fe_3O_4-chitosan particles by introducing spacer and immobilized trypsin was shown Fig. 1. 1, 6-Diaminohexane as the spacer was introduced to Fe_3O_4-chitosan particles according to the method of Habeeb (1967) and Watanabe *et al*. (1978). Three grams Fe_3O_4-chitosan particles were added to 36 mL 1 M NaOH, 12 mL epichlorohydrin dispersed in this suspension sequentially, this system reacted with stirring at 60°C for 60 min. Then 6.9726 g of 1, 6-Diaminohexane was entered into this reaction system and heated at 60°C for 120 min. After cooled to the room temperature with stirring, chemically modified Fe_3O_4-chitosan particles were separated from the suspension by an external magnetic field, washed with water for several times, lyophilized and store at 4°C.

Before the immobilization of trypsin onto chemically modified magetic chitosan particles, 2 g of dry particles were activated using 100 mL glutaraldehyde agent (3% (v/v) glutaraldehyde in a 50 mM phosphate buffer at pH 8.0) with stirring at 30°C for 2 h. The particles were separated by magnetic decantation and washed with water for several times. The above glutaraldehyde-activated carriers were transferred to 100 mL of 0.1 mg/mL trypsin solution (50 mM phosphate buffer at pH 8.0). This resulting mixture was shaken at 20°C for 4 h. At the end of binding, the magnetic particles bound with trypsin were collected by application of an external magnetic field and washed several times with phosphate buffer (pH 8, 50 Mm) to remove free trypsin. The immobilized trypsin was thus obtained and used for hydrolysis after

drying by lyophilization. The activity of immobilized trypsin was 700.04 U/g.

Preparation of peanut protein: Peanut protein was prepared using isoelectric precipitation, as described by Yu *et al*. (2007) with slight modification. Defatted peanut flour was mixed with water in the ratio of 1/12 (w/v), pH of the mixture was adjusted to 10 with 1 M NaOH. The mixture was stirred at room temperature for 2 h and then centrifuged at 5, 000 *g* for 20 min.

The supernatant was adjusted to pH 4.4 with 1 M HCl. The suspension was centrifuged at 5,000 g for 20 min. The precipitate was washed with water to remove the residual acid. After lyophilization, the peanut protein served as the sample for subsequent enzymatic hydrolysis. The protein content of peanut protein was 92.75 %.

Hydrolysis of peanut protein using immobilized trypsin: The hydrolysis experiments were carried out in a 250 mL glass reactor under controlled conditions (pH, temperature and stirring speed). Three grams peanut protein was mixed with 97 mL 0.05 M phosphate buffer whose pH was equal to the required pH. Then a certain amount of immobilized trypsin was added to this system, the final pH of the mixture was adjusted to the required pH with 0.1 M NaOH or 0.1 M HCl. The system incubated at the specified temperature and time. Reaction was terminated by isolating immobilized trypsin form the system by an external magnet. The residual suspension was heated at 100°C for 20 min. the hydrolysate was then centrifuged at 10,000 g for 30 min. The supernatant was filtrated using a microfilter of 0.45 μm. The analysis of hydrolysate was then carried out.

Determination of DH of peanut protein: DH was measured by the method of Adler-Nissen (1979). A spectrophotometric assay of a chromophore formed by the reaction of TNBS with primary amines under alkaline conditions with maximum absorption at 340 nm was calculated. L-Leucine (0-50 mM) was used to generate a standard curve of absorbance at 340 nm against amino nitrogen (mg/L) to calculate the amount of free amino groups. DH was calculated using Eq. (1):

$$DH(\%) = \frac{h_t - h_o}{h_{max} - h_o} \times 100 \quad (1)$$

h_t = The amount of a specific free amino acid at time t
h_o = The amount of the specific amino acid in the original substrate (blank)
h_{max} = The total amount of the specific amino acid in the substrate obtained after complete hydrolyzing by using 6 M HCl at 120°C for 24 h

Assay for ACE inhibitory activity: The ACE inhibitory activity was assayed by modification of the method of Cushman and Cheung (1971). Fifty μL of the hydrolysate solution with 50 μL of the ACE solution (25 mU/mL) was pre-incubated at 37°C for 10 min, then incubated with 150 μL of the substrate (8.3 mM Hip-His-Leu in a 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) at 37°C for 60 min. The reaction was stopped by the addition of 250 μL of 1 M HCl. The resulting hippuric acid was extracted with 1.5 mL of ethyl acetate. After centrifugation (8,000 g, 15 min), 1 mL of the upper layer was transferred into a glass tube and evaporated at room temperature for 2 h under vacuum. The hippuric acid was dissolved in 3.0 mL of distilled water. The absorbance was measured at 228 nm using a UV/Vis spectrophotometer. The ACE-inhibition activity was determined using Eq. (2):

$$AA(\%) = (1 - \frac{B - C}{D}) \times 100 \quad (2)$$

AA = ACE inhibitory activity
B = The absorbance of reaction mixture
C = The absorbance of blank solution
D = The absorbance of ACE solution only

The IC_{50} for ACE inhibitory activity was defined as the concentration of the hydrolysate that is required to inhibit 50% of the ACE inhibitory activity. It could be obtained by analyzing the plot of inhibiting activity against concentration of hydrolysate.

Experimental design: By virtue of providing the accurate and visualizing information under relative few experiment numbers, RSM has widely applied to the field of food science and technology (Adeleye *et al*., 2012). RSM was applied to evaluate effects of the hydrolysis factors on the DH (Y_1) and IC_{50} for ACE inhibitory activity (Y_2). The parameters and their ranges were chosen on the basis of the preliminary experiment results (Table 1). The experiments were designed according to the central composite design using a 2^n factorial and star design with 7 central points (Table 2).

Data were analyzed by multiple regressions, to fit the following second-order polynomial Eq. (3):

$$Y = b_o + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} b_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} b_{ij} X_i X_j + e_i \quad (3)$$

Y = The response variable
b_o, b_i, b_{ii}, b_{ij} = Constant (intercept), the linear (main effect), the quadratic and cross-product regression coefficients
e_i = The random error
X_i, X_j = The Independent variables

Table 1: Coded and uncoded levels of variables used in the RSM design

	Uncoded level			
Coded level	Enzyme/substrate ratio (E/S) (w/w)	Temperature (°C)	Time (h)	pH
-2	1	35.0	1.0	6
-1	2	42.5	1.5	7
0	3	50.0	2.0	8
1	4	57.5	2.5	9
2	5	65.0	3.0	10

Table 2: Experimental design and responses of the dependent variables to the hydrolysis conditions

	Independent variables				Dependent variables	
No.	X_1 E/S (w/w)	X_2 temperature (°C)	X_3 time (h)	X_4 pH	Y_1 DH%	Y_2 IC_{50} for ACE inhibition (mg/mL)
1	2	42.5	1.5	7	11.42	1.01
2	2	42.5	1.5	9	13.13	0.94
3	2	42.5	2.5	7	14.25	0.89
4	2	42.5	2.5	9	15.08	0.86
5	2	57.5	1.5	7	12.26	0.97
6	2	57.5	1.5	9	14.74	0.92
7	2	57.5	2.5	7	14.36	0.89
8	2	57.5	2.5	9	15.37	0.85
9	4	42.5	1.5	7	12.96	0.97
10	4	42.5	1.5	9	15.12	0.90
11	4	42.5	2.5	7	14.94	0.91
12	4	42.5	2.5	9	15.47	0.89
13	4	57.5	1.5	7	13.98	0.91
14	4	57.5	1.5	9	15.53	0.87
15	4	57.5	2.5	7	15.27	0.86
16	4	57.5	2.5	9	16.12	0.79
17	1	50	2	8	13.75	0.94
18	5	50	2	8	16.01	0.80
19	3	35	2	8	10.45	1.06
20	3	65	2	8	14.21	0.90
21	3	50	1	8	12.62	0.96
22	3	50	3	8	16.23	0.78
23	3	50	2	6	11.53	1.00
24	3	50	2	10	15.06	0.86
25	3	50	2	8	15.63	0.85
26	3	50	2	8	16.18	0.83
27	3	50	2	8	15.42	0.84
28	3	50	2	8	16.29	0.81
29	3	50	2	8	15.63	0.83
30	3	50	2	8	15.45	0.84
31	3	50	2	8	16.07	0.81

Chemical analysis: The protein content of peanut protein was determined by the method of Misra (2001). Soluble protein content in the hydrolysates was determined according to the procedure based on Bradford method, using bovine serum albumin as the standard (Bradford, 1976). Activities of Trypsin and immobilized trypsin were measured according to the method of Erlanger *et al*. (1961) as modified by Benjakul *et al*. (2000) using BAPNA as a substrate.

Statistical analysis: Without explanation, all experiments were repeated three times. Data were processed by the SAS system (Version 9.1.3, SAS Inst., Cary, NC, USA). Significant difference level was 0.05. Highly significant difference level was 0.01.

RESULTS AND DISCUSSION

Statistical analysis for the mathematical models of DH and IC_{50}: To analyze the influence of E/S ratio, temperature, time and pH on DH of peanut protein and IC_{50} for ACE inhibition by hydrolysate, the statistic analysis of RSM for the linear, the quadratic and the interaction of these variables (X_1, X_2, X_3 and X_4) on the DH (Y_1) and IC_{50} (Y_2) presented in Table 3 and 4.

The p value of model of DH was less than 0.01, which indicated the model was highly significant. Moreover and lack of fit was not significant relative to pure error ($p = 0.058>0.05$) and the R^2 was 0.9224, which demonstrated that this model has a good fit with the experimental data and the experiment error was small. Therefore, this model was suitable for analyzing the actual relationship between hydrolysis parameters and DH, monitoring and optimizing the hydrolysis of peanut protein using immobilized trypsin.

From the p value, it concluded that E/S ratio, temperature, time and pH were highly significant factors ($p<0.01$). According to the F and p value, the most significant factor was time, then followed by pH, E/S ratio, temperature. The regression equation for the DH of peanut protein as a function of the independent variables (X_1, X_2, X_3, X_4) and their interactions was as follow Eq. (4):

Table 3: Statistic analysis for the mathematical models of DH from RSM design

	Y_1				
Source	D.F.	S.S.[a]	M.S.[b]	F value	p
X_1	1	7.37	7.37	20.59	0.0003
X_2	1	6.80	6.80	19.01	0.0005
X_3	1	14.95	14.95	41.76	0.0001
X_4	1	13.77	13.77	38.48	0.0001
X_1*X_1	1	0.45	0.45	1.26	0.2788
X_1*X_2	1	0.01	0.01	0.03	0.8564
X_1*X_3	1	0.68	0.68	1.90	0.1869
X_1*X_4	1	0.06	0.06	0.15	0.6996
X_2*X_2	1	16.64	16.64	46.50	0.0001
X_2*X_3	1	0.39	0.39	1.09	0.3117
X_2*X_4	1	0.03	0.03	0.08	0.7862
X_3*X_3	1	1.64	1.64	4.57	0.0483
X_3*X_4	1	1.37	1.37	3.82	0.0682
X_4*X_4	1	7.78	7.78	21.74	0.0003
Model	14	68.08	4.86	13.57	0.0001
Linear	4	42.89	10.72	29.96	0.0001
Quadratic	4	22.65	5.66	15.82	0.0001
Cross product	6	2.53	0.42	1.18	0.3648
Error	16	5.73	0.36		
Lack of fit	10	4.94	0.49	3.80	0.0580
Pure error	6	0.78	0.13		
Total	30	73.80			

[a]: S.S. Means sum of square; [b]: M.S. Means mean square

Table 4: Statistic analysis for the mathematical models of IC_{50} obtained from RSM design

	Y_2				
Source	D.F.	S.S.	M.S.	F value	p
X_1	1	0.01	0.01	18.20	0.0006
X_2	1	0.02	0.02	27.78	0.0001
X_3	1	0.03	0.03	57.96	0.0001
X_4	1	0.02	0.02	31.42	0.0001
X_1*X_1	1	0.19×10^{-2}	0.19×10^{-2}	3.25	0.0901
X_1*X_2	1	0.18×10^{-2}	0.18×10^{-2}	3.03	0.1007
X_1*X_3	1	0.14×10^{-2}	0.14×10^{-2}	2.36	0.1438
X_1*X_4	1	6.25×10^{-6}	6.25×10^{-6}	0.01	0.9197
X_2*X_2	1	0.04	0.04	61.32	0.0001
X_2*X_3	1	6.25×10^{-6}	6.25×10^{-6}	0.01	0.9197
X_2*X_4	1	6.25×10^{-6}	6.25×10^{-6}	0.01	0.9197
X_3*X_3	1	0.19×10^{-2}	0.19×10^{-2}	3.25	0.0902
X_3*X_4	1	0.31×10^{-3}	0.31×10^{-3}	0.51	0.4836
X_4*X_4	1	0.02	0.02	25.92	0.0001
Model	14	0.13	0.94×10^{-2}	15.82	0.0001
Linear	4	0.08	0.02	33.84	0.0001
Quadratic	4	0.05	0.01	20.06	0.0001
Cross product	6	0.35×10^{-2}	0.59×10^{-3}	0.99	0.4641
Error	16	0.95×10^{-2}	0.6×10^{-3}		
Lack of fit	10	0.81×10^{-2}	0.81×10^{-3}	3.48	0.0701
Pure error	6	0.14×10^{-2}	0.23×10^{-3}		
Total	30	0.14			

$$DH = - 86.63 + 2.78X_1 + 1.48X_2 + 13.40X_3 + 10.18X_4 - 0.12X_1X_1 - 0.37\times10^{-2}X_1X_2 - 0.41X_1X_3 - 0.06X_1X_4 - 0.01X_2X_2 - 0.04X_2X_3 + 0.55\times10^{-2}X_2X_4 - 0.96X_3X_3 - 0.58X_3X_4 - 0.52X_4X_4 \quad (4)$$

Due to high R^2 value (0.9326), insignificant lack of fit ($p = 0.07>0.05$) and low p value of model ($p<0.01$), the mathematical model of IC_{50} obtained from RSM design was able to explain the data variation and significantly represent the actual relationships between the hydrolysis factors and IC_{50} (Table 4). Time was the first significant factor, pH was the second significant factors, temperature was the third significant factor, E/S ratio was the fourth significant factors. The regression equation for the IC_{50} as a function of the independent variables and their interactions was as follow Eq. (5):

$$IC_{50} = 4.70 - 0.03X_1 - 0.06X_2 - 0.32X_3 - 0.41X_4 + 0.82\times10^{-2}X_1X_1 - 0.14\times10^{-2}X_1X_2 + 0.02X_1X_3 - 0.62\times10^{-3}X_1X_4 + 0.64\times10^{-3}X_2X_2 - 0.17\times10^{-3}X_2X_3 - 0.83\times10^{-4}X_2X_4 + 0.03X_3X_3 + 0.88\times10^{-2}X_3X_4 + 0.02X_4X_4 \quad (5)$$

Influence of hydrolytic parameters on the DH of peanut protein: To visualize the effect of the hydrolytic factors on the DH, surface response plots of the quadric polynomial models were generated by varying two of hydrolysis factors within the

experimental range while keeping the other independents at the centre level.

As shown in Fig. 2a, increasing E/S ratio was beneficial to enhance the DH due to greater hydrolysis of the protein when more protease was added during the same period. However, along with the growth of E/S ratio, the slope of curve for DH as a function of E/S ratio dropped gradually, especially when the E/S ratio was more than 4.0, there was no remarkable rise in the DH curve. That was because peanut protein concentration was kept constant and the trypsin concentration was gradually increased in this case, peanut protein concentration became the rate limiting of hydrolysis. When most of peanut protein took part in the hydrolysis, active sites of excess trypsin are no longer saturated. So adding excess trypsin was in vain to enhance the rate of hydrolysis, which corresponded to the fall in growth rate of DH.

Prolonging time was effective in promoting the DH (Fig. 2a). It could be explained the quantity of protein bonds cleaved by protease accumulated as the proceeding of hydrolytic reaction, so the percentage of cleaved peptide bonds increased with time, which suggested DH increase.

The effect of pH on DH was quite complicate. The DH increased with the growth of pH below 8.4 and then decreased thereafter (Fig. 2b). It could be explained that the pH affect the activity of action site of immobilized trypsin. At the extremely high or low pH, the conformational changes of trypsin took place and trypsin could not bind to the substrate properly. The effect of temperature on DH is shown in Fig. 2b. Initially The DH increased with temperature to a maximum level and then decreased with further growth of temperature. Proper increasing temperature could promote the rate of hydrolysis, which led to the rise in DH. The loss in DH was presumably caused by thermal denaturation of immobilized trypsin.

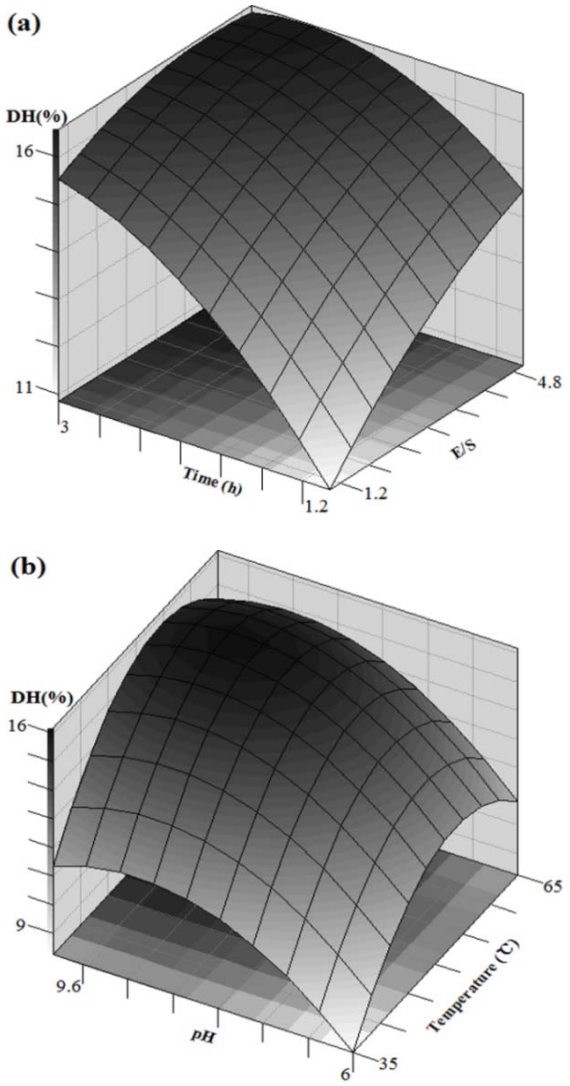

Fig. 2: Surface response plots for effects of hydrolysis factors on the DH of peanut protein, (a) effects of time and E/S ratio on the DH. The temperature and pH were set at 50°C and 8, (b) effects of pH and temperature on the DH
The E/S ratio and time are fixed at 3 and 2 h

Optimization of hydrolysis factors for the highest DH: According to the mathematic model of RSM, the highest DH of 16.73±0.38% was obtained at the following parameters, E/S ratio of 4.59, temperature of 52.74°C, time of 2.29 h and pH of 8.48. To confirm the validity of the model, three assays were performed under the optimal conditions given above. The experimental DH was 16.68±0.29%.This value agreed with the value predicted by the model within a 95% confidence interval ($p = 0.865>0.05$). The above results confirmed that the model was powerful and suitable for the estimation of experimental value.

Moreover, the optimal operating pH and temperature of typically trypsin were about 7.5-8.0, 37-40°C, respectively (Chiang *et al*., 2006; Otte *et al*., 2007; Quist *et al*., 2009), while the optimal operating pH and temperature of immobilized trypsin were 8.48 and 52.74°C, which indicated that the thermal and pH stability of trypsin was improved by covalently immobilizing on chemically modified chitosan-coated Fe_3O_4 particles. Therefore, immobilized trypsin has great potential for preparing bioactive peptide.

Effects of hydrolytic factors on the IC_{50} for ACE inhibition: For the effect of E/S ratio on the IC_{50} for ACE inhibition, it was clear that increasing the E/S ratio could decrease the IC_{50} value, which corresponded to an enhancement in ACE inhibition of the hydrolysate (Fig. 3a). That was because more hydrolysate generated in the high E/S ratio during the same period. This result

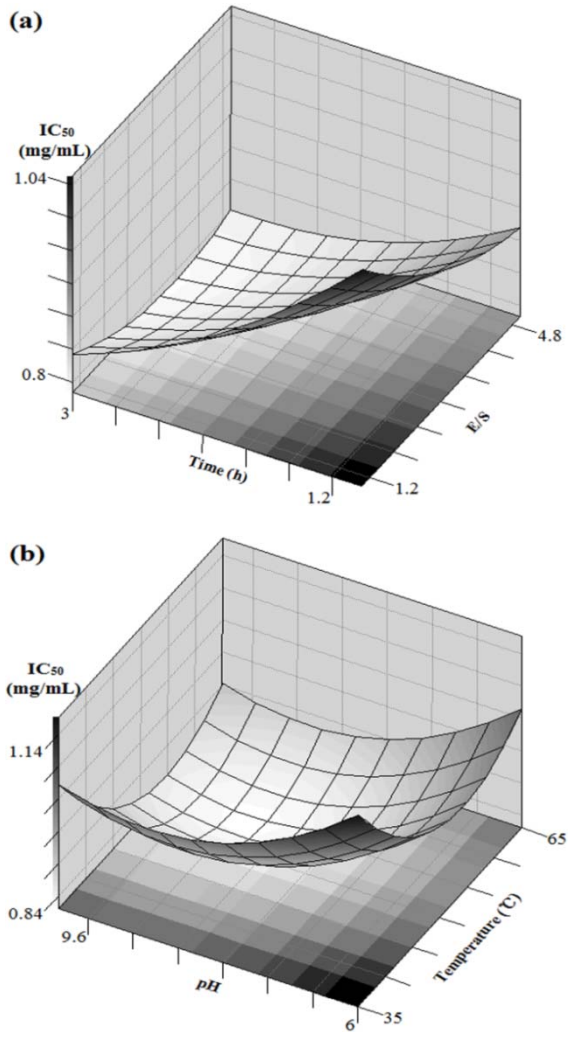

Fig. 3: Surface response plots for effects of hydrolysis factors on the IC_{50} for ACE inhibitory activity by hydrolysate, (a) Effects of time and E/S ratio on the IC_{50}. The temperature and pH were set at 50°C and 8, (b) effects of pH and temperature on the IC_{50} The E/S ratio and time are fixed at 3 and 2 h

was consistent with the report of Cornelly *et al*. (2002) who found that high protease concentration increase ACE inhibition by the hydrolysates derived from whey protein.

In terms of time, prolonging time increased ACE inhibitory activity due to more hydrolysate accumulation in the system as the proceeding of enzymatic reaction. However, the slope of plot for IC_{50} as a function of time went down as time prolonged, indicating that the extent of growth for ACE inhibition was decrease (Fig. 3a). It could be explained that the preparation ACE inhibitory peptide by hydrolysis was a complex reaction system, including main reaction and side reaction. The main reaction was releasing the ACE inhibitory peptide, which resulted in an increase in ACE inhibitory activity. The side reaction was degradation of ACE inhibitory peptide by further enzymatic hydrolysis, which resulted in a decrease in ACE inhibitory activity. Initially, the main reaction was in the dominance in the system, while the rate of side reaction increased as the reaction product accumulated, which reduced the extent of growth for ACE inhibitory activity.

Temperature had impact on the ACE inhibition which illustrated the growth of the ACE inhibition with an increase of temperature from 30 to 50°C, while the ACE inhibition gradually decreased at the temperature over 50°C (Fig. 3b). That was because proper temperature increased the rate of enzymatic reaction, hence more hydrolysate generated. Moreover, peanut protein would unfold and exposed the hydrophobic or proton-donating residues buried inside the protein molecular at high temperature, which facilitated cleavage of the peanut protein and contributed to the ACE inhibitory activity of the hydrolysates. But the operation at over 50°C would cause denaturaion of protease, which adversely affected the generation of hydrolysate. Ren *et al*. (2008) had the similar result. They found that high or low temperature wasn't suitable for improving the antioxidant activity of hydrolysate producing from grass carp sarcoplasmic protein.

It was clear that proper pH could increase the ACE inhibition of hydrolysate. When pH was close to 9.0, the ACE inhibition of hydrolysate reached the maximum level. Reducing pH or increasing pH led to weak the ACE inhibitory activity (Fig. 3b). By the reason of enzyme activity affected by pH, extremely high or low pH brought adverse effect to the proceeding of enzymatic reaction and consequently less ACE inhibitory hydrolysate generated.

Optimization of hydrolysis factors for the lowest IC_{50}: On the basis of data analysis of SAS, the optimal IC_{50} of 0.77±0.02 mg/mL was obtained at the following factors, E/S ratio of 3.52, temperature of 53.49°C, time of 2.95 h and pH of 8.43. To confirm the validity of the model, the parallel tests were operated under the optimal conditions given above. The experimental IC_{50} was 0.76±0.02 mg/mL. This data was consistent with the predicted data within a 95% confidence interval ($p = 0.573 > 0.05$), which confirmed that the model was powerful and suitable for the estimation of experimental value.

Compared with IC_{50} for ACE inhibition by hydrolysate derived from other protein (Korhonen and Pihlanto, 2006; Matsui and Matsumoto, 2006), the hydrolysate of peanut protein has a significant ACE inhibitory activity. In addition, the resource of peanut protein is abundant and inexpensive, so there are great potential for exploiting the ACE inhibitory peanut peptide.

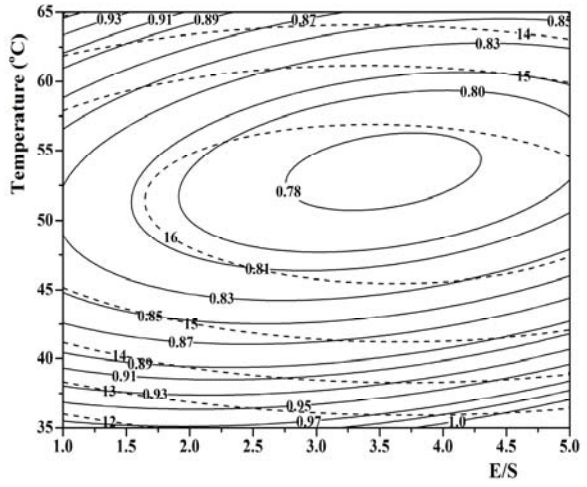

Fig. 4: Contour plot for the relationship between DH and IC_{50} Dash line: DH value; Solid line: IC_{50}; The time and pH are fixed at 2.95 h and 8.43

Relationship between DH and ACE inhibitory activity: For obtaining the most powerful ACE inhibitory hydrolysate, it was useful and necessary for exploring the relationship between DH of peanut protein and ACE inhibitory activity of hydrolysate to prepare the ACE inhibitory peptide. To illustrate the link between DH and ACE inhibitory activity, the variation of ACE inhibitory activity and DH under the Optimum conditions for ACE inhibitory activity was shown in Fig. 4. It was clear that properly increasing DH of peanut protein favored the enhancement of ACE inhibitory activity. This is consistent with the results of Jamdar *et al*. (2010) who found that the ACE inhibitory activity of hydrolysates of peanut protein was improved with increasing DH. Since DH is defined as the percentage of peptide bonds cleaved by protease, the hydrolysate with high DH is believed to contain more low-molecular peptide than the hydrolysate with low DH. Nakai *et al*. (2006) reported short peptide showed stronger antihypertensive activities than polypeptides. Therefore, the hydrolysate with high DH always had high ACE inhibitory activity.

However, it was strange that the IC_{50} for ACE inhibitory activity whose DH belonged to between 15 and 16% of 0.83 was obtained at the E/S ratio from 1 to 4 and the same IC_{50} value was also obtained at the E/S ratio more than 4.5, but its DH was over 16% (Fig. 4). It could be explained by the generation and degradation of ACE inhibitory peptide. Initially, peanut protein was the main reactant of hydrolysis, which contributed more to the increase of DH. Meanwhile ACE inhibitory activity increased due to the generation of ACE inhibitory peptides by hydrolyzing peanut protein. Thus ACE inhibitory activity was consistent to the change of DH. With the development of hydrolysis, more ACE inhibitory peptides produced and less peanut protein remained in the reaction system. More ACE inhibitory peptides took part in the hydrolytic reaction, which also resulted in the increase of DH. At contrary, the ACE inhibitory activity fell. Therefore, high DH was adverse to the enhancement of ACE inhibitory activity.

CONCLUSION

This study was demonstrated the application of Trypsin covalently immobilized on chemically modified chitosan-coated Fe_3O_4 particles to prepare ACE inhibitory peptide by hydrolyzing peanut protein and analyzed the significant factors of hydrolysis by RSM. The results showed that Immobilization increased the thermal and pH stability of trypsin. To gain optimum ACE inhibitory activity, the parameters of hydrolytic factors were E/S ratio of 3.52, temperature of 54.65°C, time of 2.95 h and pH of 8.43. Moreover, further hydrolysis would reduce the ACE inhibitory activity of hydrolysate. So it was feasible to produce high ACE inhibitory peptide from peanut protein by trypsin immobilized on chemically modified chitosan-coated Fe_3O_4 particles.

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