Research Article

Asif Wali, Haile Ma*, Muhammad Tayyab Rashid and Qui Fang Liang

Preparation of rapeseed protein hydrolysates with ACE inhibitory activity by optimization and molecular weight distribution of hydrolysates

Kolza tohumu Protein hidrolizatlarının ACE inhibitör etkinliği tarafından en iyi duruma getirme ve hidrolizatlarının moleküler Ağırlık dağılımı ile hazırlanması

DOI 10.1515/tjb-2017-0044
Received February 8, 2017; accepted March 15, 2017; previously published online June 12, 2017

Abstract

Objective: The main purpose of this study was to screen effective proteolytic enzymes for producing hydrolysates from rapese protein, and to optimize hydrolysis conditions using response surface design to prepare hydrolysates with maximum ACE inhibitor activity.

Methods: RSM design was successfully applied to the hydrolysis conditions on the basis of single factor experiments which further derived a statistical model for experimental validation. The molecular weight distribution of rapeseed protein hydrolysates with different degree of hydrolysis was also investigated.

Results: All the proteolytic enzymes tested produced hydrolysates that possessed ACE inhibitory activity. Aiding RSM design the highest ACE inhibitory activity 56.3% was achieved under optimum hydrolysis conditions at the hydrolysis time, pH, hydrolysis temperature, and enzyme dosage were at 90.11 min, 8.88, 50°C and 3580.36 Ug⁻¹. The mathematical model demonstrated a good fit with experimental results. Furthermore, the molecular weight distribution of rapeseed protein hydrolysates showed remarkable changes, most notably the <500 Da fractions of the rapeseed protein hydrolysates.

Conclusion: Enzymatic hydrolysis and response surface methodology found good techniques in order to achieve hydrolysates with maximum ACE inhibitory activity. The findings of current research suggested that the hydrolysates obtained under optimized conditions could be utilized to formulate nutraceuticals and pharmaceuticals.

Keywords: Rapeseed protein; Enzymatic hydrolysis; ACE inhibitory activity; Response surface methodology; Molecular weight distribution.

Özet

Amaç: Bu çalışmanın temel amacı, kolza tohumundan hidrolizat üretemek için etkili proteolitik enzimlerin taraması ve maksimum ACE inhibitörü aktivitesi ile hidrolizatların hazırlanması için tepki yüzeyi tasarımını kullanarak hidroliz koşullarını optimize etmektir.

 Yöntemler: RSM tasarım, deneySEL doğrulama için istatistiksel bir model oluşturulan tek faktörlü deneyler temelinde hidroliz koşullarına başarıyla uygulanmıştır. Kolza tohum protein hidrolizatlarının farklı derecelerde hidroliz ile moleküler ağrılık dağılımı da araştırılmıştır.

Sonuçlar: Test edilen bütün proteolitik enzimler, ACE inhibe edici aktiviteye sahip olan hidrolizati üretti. RSM
tasarlama yardımı olmak için hidroliz zamanında, pH, hidroliz sıcaklığı ve enzim dozajında optimum hidroliz koşulları altında %56.3'tük en yüksek ACE inhibisyon aktivitesi elde edildi 90.11 dakika, 8.88, 50°C ve 3580.36 Ug⁻¹. Matematiksel model deney sonuçları ile iyi uyum gösterdi. Dahası, kolza tohumu proteini hidrolizatlarının moleküler ağırlık dağılımı, en dikkat çeken kolza tohumu proteini hidrolizatlarının <500 Da fraksiyonları gibi dikkate değer değişiklikler göstermiştir.

Sonuç: Enzimatik hidroliz ve tepki yüzeyi metodolojisi, maksimum ACE inhibitör aktivitesi olan hidrolizatların elde edilmesi için iyi teknikler bundu. Mevcut araştırmaların bulgularını, optimize edilmiş koşullar altında elde edilen hidrolizatların nutrasötikler ve farmasötik maddeleri formülle etmek için kullanılabileceğini önermektedir.

Anahtar Kelimeler: Kolza tohumu proteini; Enzimatik hidroliz; ACE engelleyici aktivite; Tepki yüzeyi metodolojisi; Molekül ağırlığı dağılımı.
Experimental section

Materials and reagents

Rapeseed protein with crude protein content 44.3 g/100 g was determined by Kjeldahl method [16] was kindly provide by (COFCO Eastern Oil & Grains Industries Co. Ltd Zhangjiangang, China ). Alcalase 2.4 L from Bacillus licheniformis (in solution form with an activity 257,564.5 U mL⁻¹) was purchased from Novozymes Biotech, China. Extraction of angiotensin I-converting enzyme was done according to the method [17]. Hippuryl-His-Leu (HHL) substrate was purchased from Sigma Chemicals Co, Ltd (St. Louis, MO, USA). Rest of the chemical and reagents used in this research work was of analytical grade or food gradestands. The mechanical apparatuses during the hydrolysis process are pH-meter (pHS-3C Precision pH/mV Meter, LIDA instruments China), and an impeller-agitator (J-J1, Zhong Da Instruments Co., Jiangsu P.R China) with an operating speed of 100 rpm, Centrifuge (TGL-16, High Speed Tabletop, China) and Digital Thermostat water bath (DK-S26, JingHong Experimental Apparatus Co., Shanghai, P.R China).

Preparation of rapeseed protein hydrolysates and enzymes screening
(Supplementary file S1)

The rapeseed protein was hydrolyzed with five commercial enzymes alcalase, neurase, chymotrypsin, trypsin, and with pepsin in order to select most effective enzyme on the degree of hydrolysis and ACE inhibitory activity using the pH-stat method [18]. Six gram of rapeseed protein was suspended in the distilled water and pre-incubated at 45°C to make the slurry more soluble in electric water bath with continuous stirring for 30 min and adjusted to the desired pH and temperature shown in (Table 1) before the addition of each enzyme. The concentration of individual enzymes was set at 3000 Ug⁻¹. The hydrolysis process was carefully monitored and the desired pH of the mixture was monitored with the pH-stat method with 1 M NaOH and 1 M HCl for pepsin for 2 h. After 2 h of hydrolysis, proteolytic enzymes were deactivated by boiling for 15 min. Hydrolysates were then centrifuged (TGL-16, High-Speed Tabletop, China) at 10,000×g for 10 min with the temperature set at 10°C in order to separate insoluble fractions. The supernatants were collected carefully and stored at 4°C until required for the subsequent analysis.

Table 1: Analysis of variance (ANOVA) for response surface quadratic model.

| Source         | Sum of squares | df  | Mean square | F-value | p-Value |
|----------------|----------------|-----|-------------|---------|---------|
| Model          | 1145.43        | 14  | 81.82       | 51.58   | <0.0001*|
| X₁-Hydrolysis time | 88.02      | 1   | 88.02       | 55.49   | <0.0001*|
| X₂-pH           | 189.21         | 1   | 189.21      | 119.29  | <0.0001*|
| X₃-Temperature  | 8.78           | 1   | 8.78        | 5.53    | 0.0365* |
| X₄-Enzyme dosage | 14.46        | 1   | 14.46       | 9.12    | 0.0107* |
| X₁X₂            | 0.16           | 1   | 0.16        | 0.10    | 0.7533  |
| X₁X₃            | 12.46          | 1   | 12.46       | 7.86    | 0.0160* |
| X₁X₄            | 1.03           | 1   | 1.03        | 0.65    | 0.4360  |
| X₂X₃            | 6.40           | 1   | 6.40        | 4.04    | 0.0676  |
| X₂X₄            | 64.96          | 1   | 64.96       | 40.96   | <0.0001*|
| X₁X₁²           | 2.23           | 1   | 2.23        | 1.40    | 0.2591  |
| X₂²             | 281.74         | 1   | 281.74      | 177.62  | <0.0001*|
| X₃²             | 680.34         | 1   | 680.34      | 428.91  | <0.0001*|
| X₃X₄            | 135.22         | 1   | 135.22      | 85.24   | <0.0001*|
| X₄²             | 88.73          | 1   | 88.73       | 55.94   | <0.0001*|
| Residual        | 19.03          | 12  | 1.59        | 2.96    | 0.2788 not significant |
| Lack of fit     | 17.83          | 10  | 1.78        |         |         |
| Pure error      | 1.20           | 2   | 0.60        |         |         |
| Cor total       | 1164.47        | 26  |             |         |         |

R² 0.9837
Adjusted R² 0.9646
Predicted R² 0.9095
Adequate Precision 26.673
CV % 2.88

*Significant within a 99% confidence interval, ^Significant within a 95% confidence interval.
**Single-factor experiments**

Rapeseed protein was hydrolysed with alcalase was chosen from experimental observations of Section “Preparation of rapeseed protein hydrolysates and enzymes screening (Supplementary file S1)”. For all the experiments substrate concentration value was fixed. The Hydrolysis factors for the single-factor experiments included hydrolysis time, pH, temperature, and enzyme dosage. In order to see the effect different hydrolysis time on the DH and ACE inhibitory activity, the hydrolysis conditions were pH value 8.5, hydrolysis temperature 50°C, enzyme dosage 3000 Ug⁻¹ for the different length of time i.e. 30, 60, 90, and 120 min. The effect of different pH levels (7.0, 7.5, 8.0, 8.5, 9.0, and 9.5, respectively on the DH and ACE inhibitory activity was measured. Hydrolysis conditions were time 120 min, hydrolysis temperature 50°C, and enzyme dosage was 3000 Ug⁻¹. The effect different hydrolysis temperatures (30°C, 35°C, 40°C, 45°C, 50°C, 55°C, and 60°C) on the DH and ACE inhibitory activity was also conducted at following hydrolysis conditions pH value 8.5, enzyme dosage 3000 Ug⁻¹, hydrolysis time 120 min, respectively. Effect of different enzyme dosages (1200 Ug⁻¹, 1800 Ug⁻¹, 2400 Ug⁻¹, 3000 Ug⁻¹, and 3600 Ug⁻¹) on the DH and the ACE inhibitory activity of rapeseed protein was also conducted with following processing conditions hydrolysis time, pH 8.5, and temperature 50°C, respectively.

**Assessment of degree of hydrolysis**

DH was calculated during the hydrolysis according to the equation (1) described by the [18].

$$\text{DH} = \frac{B \times N \times a}{\alpha \times M_p \times h_{\text{tot}}} \times 100\%$$

where B is the amount of base (NaOH) consumed to keep the pH, N is the concentration of base (mol/L), a is average degree of dissociation of the α-NH groups in rapeseed proteins, M_p is mass of hydrolysed protein (g), h_{tot} is the total number of peptide bonds in the protein substrate (7.8 mmol/g rapeseed protein).

**Determination of ACE inhibitory activity by HPLC**

According to the method described by [19]. In brief 10 µL of the sample was prepared in sodium borate buffer 0.1 M pH buffer containing 0.3 M NaCl were mixed with 25 µL ACE in sodium borate buffer containing 0.3 M NaCl (ACE in sodium borate buffer 0.1 M pH 8.3 containing 0.3 M NaCl) and incubated at 37°C for 10 min. The reaction was initiated by adding Hipp-His-leu (6.5 mM HHL in the sodium borate buffer 0.1 M pH 8.3 containing 0.3 M NaCl) and the reaction was conducted at 37°C for 30 min. The reaction was terminated using 85 µL of 1 M HCL and the mixture was passed through 0.22 µ filter. The filtrate was used to determine the liberation of HA (Hippuric acid) resulting from ACE inhibitory activity on the substrate. The free HA was separated and quantified by HPLC at 228 nm with UV-detector. A blank was also prepared by replacing the actual sample with distilled water. The ACE inhibitory activity was calculated from equation (2) given below.

$$\text{ACE(\%)} = \frac{\text{HA}_{\text{control}} - \text{HA}_{\text{sample}}}{\text{HA}_{\text{control}}} \times 100$$

**Experimental design (RSM)**

For the modeling, data analysis and experimental design, the software (Design Expert trail version 8.0.6) was applied. Response surface analysis Box-Behnken Design (BBD) with four independent variables hydrolysis time (X1), pH (X2), temperature (X3), and an enzyme dosage (X4) by setting the maximum response value Y (ACE inhibitory activity %) as the primary goal. Center points for RSM design were obtained from previously studied single-factor experiments. The center point value was set according to results of the single-factor experiments studied previously expressed in the (S.2). The proposed model was expressed in the form of 2D and 3D counter plots. Furthermore, the model was validated by performing an additional experiment (n = 3) was conducted to check the model validity and significance, respectively.

**Analysis of molecular weight distribution (MW)**

The molecular weight distribution of alcalase rapeseed protein hydrolysates was measured by HPLC equipped with a TSKgel-G2000 SWXL molecular exclusion column (7.8 mm×30 cm Tosoh) according to the procedure described by [20]. During the hydrolysis process samples were drawn at different DH levels to determine the influence of DH on the molecular weight distribution. The hydrolysed samples were filtered with 0.22 µ filter and analysed in the HPLC molecular exclusion column. Phosphate
buffer (0.1 mol L\(^{-1}\), pH 6.7) was used as mobile phase at a flow rate of 0.5 mL min\(^{-1}\). Ten microlitre of a hydrolyzed sample were injected into the column (30°C) and detection was performed at 220 nm. Bovine serum albumin (67,000 Da), cytochrome (12,500 Da), bacitracin (1450 Da) and L-tryptophan (204 Da) were used to prepare the calibration curve. The total surface area of the chromatogram was designated into five fractions with molecular weight \( MW > 5000, 3000–5000, 1000–3000, 500–1000, \) and \(< 500\) Da. The relative content of each range was expressed as a percentage of the total area. Results were processed using Breeze software (Waters, MA, USA).

**Statistical analysis**

All the experiments were conducted in triplicates and the data was expressed as a mean ± standard deviation. Analysis of variance (ANOVA) was employed to compare the level of significance at \((p < 0.05)\). Graphs were drawn in the Origin Pro 9.0 (OriginLab Corporation, MA, USA). Response surface analysis was done in the software (Design Expert trial version 8.0.6 State Ease, Minneapolis, MN, USA).

**Results and discussion**

**Enzymes screening**

It can be seen from the (Figure 1) the hydrolysates obtained by different kind of commercial enzymes showed positive effects on the DH and ACE inhibitory activity. However, slight differences were observed in the DH and the rate of inhibition among all five enzymes tested. It is probably due to the hydrophobic nature of amino acids in the sequence and the specificity of each enzyme to cleave peptide bonds, are responsible for maintaining the activity. The rapeseed protein hydrolyzed with alcalase was highest in terms of DH (15.35%) and the ACE inhibitory activity (55.07%) the five enzymes were screened followed by neutrase and chymotrypsin (13.81%, 50.43%, and 13.52%, 46.45%) after 2 h of hydrolysis time. The results concluded that enzyme type and specificity pattern is more considerable factors to generate hydrolysate with ACE inhibitory activity. The maximum ACE inhibitory activity from alcalase was probably the result of increased dissolution of rapeseed proteins under alkaline conditions, which lead to the substrate more utilisable during the enzymatic hydrolysis. Subsequently, the pH values of other enzymes were either acidic or neutral, which resulted in the low ACE inhibitory activities. In this study minimum, DH and ACE inhibitory activity were obtained (11.73%, 41.14%) in the pepsin generated rapeseed protein hydrolysates after 2 h. The lowest DH and ACE inhibitory activity in pepsin generated hydrolysates as compare to alcalase by a decreased margin of (−23%) and (−25%), probably due to specificity of pepsin enzyme in the formation peptides bonds by aromatic amino acids and low availability enzyme molecules to react with protein molecules [21]. Alcalase has been widely used as a potent enzyme for the production of hydrolysates and bioactive peptides. Many researchers found alcalase has great potential to produce potent hydrolysates with ACE inhibitory activity.

**Figure 1**: Effect of different enzymes on DH and ACE inhibitory activity of rapeseed protein. The data was expressed as mean ± standard deviation of three observations, and those with different lower case letters are significantly different at \((p < 0.05)\).
inhibitory activity from food sources [8, 22, 23]. However, previous reports on the enzymatic hydrolysis of rapeseed protein with different enzymes [8, 12, 24], the results are differing from our current observations, may be due to the different analytical techniques, hydrolysis conditions, and materials used. So, therefore, alcalase was chosen because of its great potency to ACE inhibitory activity, readily availability, ease of handling, and less expensive.

Single-factor experimental results

The single factor experimental results are illustrated in (Figure 2A–D). It can be seen from the (Figure 2A) the hydrolysis curve showed a significant increase in the DH value up to 90 min, during this phase the rate of hydrolysis was fast, and degradation of rapeseed proteins by alcalase enzyme was high which indicated that there were more peptides are available to act on. Subsequently, after an initial rapid phase of the rate of hydrolysis entered into the stationary phase. With extending the hydrolysis time can reduce enzymatic hydrolysis of peptide bonds, at the same time new material and substrate exist which might be a reason for the stationary phase after 90 min. The rapeseed protein hydrolyzed with alcalase showed a rapid increase in the ACE inhibitory in the first hour and then gradually increased up to 90 min reached maximum ACE inhibitory activity (56.87%) at 90 min of hydrolysis. But after that decreasing trend was observed up to 120 min. Hydrolysis for a long time may results in the degradation of peptides subsequently.

Figure 2: Effect of hydrolysis variables on DH and ACE inhibitory activity of alcalase generated rapeseed protein hydrolysates (A) hydrolysis time, hydrolysis parameters: rapeseed protein concentration 6 g/100 mL, enzyme dosage 3000 Ug⁻¹, pH 8.5, and hydrolysis temperature 50°C (B) pH, hydrolysis parameters: rapeseed protein concentration 6 g/100 mL, enzyme dosage 3000 Ug⁻¹, hydrolysis temperature 50°C, and hydrolysis time 2 h, (C) hydrolysis temperature °C, hydrolysis parameters: rapeseed protein concentration 6 g/100 mL, pH 8.5, enzyme dosage 3000 Ug⁻¹, and hydrolysis time 2 h, (D) enzyme dosage Ug⁻¹, hydrolysis conditions: rapeseed protein concentration 6 g/100 mL, pH 8.5, hydrolysis temperature 50°C, and hydrolysis time 2 h.
point, the degradation of bioactive peptides surpasses and formation of the new bioactive peptides similar trend was also observed by [14, 25]. The declining trend after a certain time of hydrolysis was also reported when the potato and soybean proteins were hydrolyzed with alcalase [26, 27]. It is evident from these results that there might be an optimal point exists for the ACE inhibitory activity so, therefore, hydrolysis time 90 min was chosen as the center point for further optimization.

Each enzyme has an optimum pH value at which it works best. High or low pH of hydrolysis process will have an impact on the desired responses. It can be observed from (Figure 2B) that the hydrolysis curves for both responses DH and ACE inhibitory activity increased up to pH 8.5 to 9.0 demonstrating that between this range an optimum condition exist, it is an evident from the maximum values 16.14% DH while the ACE inhibitory activity 54.50% were observed. This tendency was probably due to the increasing pH values caused the structure of rapeseed protein became loose and aided the hydrolysis process. Therefore, DH was inclined during the pH range. Later, the DH started to decrease as pH value exceeded 9.0 (Figure 2B) obviously, alcalase enzyme was effective within the pH range 8.5–9.0 at this point the binding capacity of the enzyme and its substrate decreased, which affected adversely the hydrolysis process. Furthermore, at extreme pH, the spatial characteristics of enzyme molecules changed, led to the enzyme inactivation. The DH and ACE inhibitory activity showed declined trend beyond the optimum pH, this is probably due to the degradation of bioactive peptides under strong alkaline conditions. Hence, pH 9.0 was chosen as the center point for further optimization of rapeseed protein hydrolysis.

The changes occurred in the reaction rate is the result of variations in the hydrolysis temperature. The high DH was observed at high temperature 50°C and 55°C (15.69%, 15.54%). From (Figure 2C) it can be observed further increase in the hydrolysis temperature up to 60°C led to the decline in DH value (13.33%) this decrease probably due to the inactivation of the enzyme during the 2 h of hydrolysis [28] reported that the optimum range 32°C to 49°C is more efficient shown in practice and enzymes are inactivated at high temperature which caused by the unfolding of their structure. Similar results were also observed by [29]. From the (Figure 2C) it can be seen the ACE inhibitory activity increased significantly from 30°C to 45°C (37.50%, 53.21%). Subsequently, when the temperature increased beyond the optimum range the ACE inhibitory activity of rapeseed protein hydrolysate tends to declined. From these results it is suggested that increase in the temperature may help in the increase of the DH, but it would not promote the ACE inhibitory activity to further increase. So, the temperature ranges 45°C to 55°C was chosen for the response surface analysis.

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A linear relationship between enzyme dosage and DH was observed in the (Figure 2D). Increasing the enzyme dose increased the rate of hydrolysis, but when the substrate is saturated, the effect of enzyme concentration is no more obvious. DH of rapeseed protein increased substantially from (11.32%–17.15%) when enzyme dose was in triplicate 3600 Ug⁻¹. Further increase in the enzyme dosage did not increase the DH value significantly (Figure 2D). The minimum DH was observed at 1200 Ug⁻¹ was probably due to the presence of soluble peptides, inhibited the enzyme proportionally more than at a high dose of an enzyme was given. A similar trend was also reported by [29, 30]. The higher DH value observed in this study was possibly due to the release of smaller peptides present in the hydrolysate as some of the peptides released with greater hydrolysis rate when the alcalase dose was higher. A remarkable increased in the ACE inhibitory activity was observed (Figure 2D) as the enzyme dosage increased from 1200 Ug⁻¹ to 3600 Ug⁻¹. This indicates that the substrate has been nearly saturated with the enzyme. Though, there was no significant increase in the ACE inhibitory activity was observed up to 4200 Ug⁻¹. The increment rate was slower than it was at the beginning. The cost of an enzyme is very considerable factor for a commercial production. Hence, considering the effectiveness and economy it is advisable to choose optimum dosage. For further optimization of hydrolysis 3600 Ug⁻¹ was chosen as the center point.

**Optimization of rapeseed protein hydrolysis conditions and validation of response surface analysis (RSM) design.**

Regression coefficients and the aggregate performance in the regression analysis were achieved with all regression variables are demonstrated as Eq (3). The parameters in the Eq (3) were obtained by multiple regression analysis of the experimental observations. The subsequent quadratic model describes the experimental facts:
Y = 55.97 + 2.71X1 − 3.97X2 + 0.86X3 − 1.10X4 − 0.20XX2
− 1.76XX3 + 0.51XX4 − 1.26XX1 + 4.03XX4
+ 0.75XX4 − 7.27X12 + 11.29X22 + 5.04X32 + 4.08X42 (3)

where Y is the predicted response value in real terms. X1 is the coded value of hydrolysis time, X2 is the coded value of hydrolysis pH, X3 is the coded value of hydrolysis temperature, and X4 is the coded variable value of enzyme dosage.

The analysis of variance (ANOVA) was performed in order to investigate the model fitness, significance, and the effects of the individual variables and interactions to the desired response are summarized in (Table 1). F-test was conducted to assess the statistical significance of the Eq (3) indicated that the F-value of 51.58 (p-value < 0.0001) confirmed that the proposed model was highly significant. Furthermore, the lack of was not significant which further confirmed the model adaptability. The F-value and p-value of lack of fit were 2.96 and 0.2788, respectively. The linear coefficient X1, X2, X3, and X4, interactive coefficient X1X2, X1X3, and the quadratic term coefficient X12, X22, X32, and X42 (Table 1) were found to be significant (p < 0.05). While the quadratic term X1X2 was least significant (p > 0.05). During the hydrolysis process X1 (pH) played a very dominant role as it has highest F-value (Table 1). The higher F value, the more influential in the course of hydrolysis. The coefficient of multiple determination (R2 = 0.9837) revealed that 98.37% of the variation in the response (ACE inhibitory activity) was due to the independent variables. The total variation of 1.63% only was not defined by the proposed model. Adjusted coefficient of determination (Ra2 = 0.9646) further, affirmed the small variation among the predicted values and experimental values. The low coefficient of variance (CV% = 2.88) evident of the model accuracy and consistency. Lower CV values endorsed a good precision and stability of the model [31]. Adequate precision measure the signal to noise ratio, a ratio higher than four is considered to be desirable [32]. In this proposed model the adequate precisions 26.67 (Table 1) indicates the adequacy to use this model for predictions.

Additionally, 3D and 2D response surface and contour plots were drawn from Box-Bhenken design in order to visualize the reciprocal effects of independent variables and their interactions between the test variable on the response (ACE inhibitory activity).

Figure 3A and B horizontal plots and 3D response surfaces plots represents the effect of most significant variables in terms of interaction on the ACE inhibitory activity and each Figure presents the effect of two variables on the ACE inhibitory activity. Figure 3A and B illustrates the effect of two variables on the ACE inhibitory activity while the effect of other two variables on the ACE inhibitory activity was kept at zero level. Figure 3A showed the 3D response was in convex shape in the 3D plot indicated that the ACE inhibitory activity has maximum predicted value over here. While in the counter plot there was an ellipse indicated that the interaction between the variable was significant. It is clear from the (Figure 3A) prolonging the hydrolysis time and at a higher temperature, the ACE inhibitory activity followed a declined trend, suggesting maximal ACE inhibitory activity could be within the levels of processing conditions studied. (Figure 3B) shows the interactive effect of hydrolysis pH and enzyme dosage on the ACE inhibitory activity. It can be observed from the (Figure 3B) the pH from 8.5 to 8.94 showed a linear effect on ACE inhibitory activity and further increased in the pH the ACE inhibitory reached equilibrium. In this model both the high and low pH values tends to decrease the ACE inhibitory activity. Correspondingly, with the increasing enzyme dosage to 3580.36 Ug−1 the ACE inhibitory activity reached to its maximum value 56.3%. The ACE inhibitory activity dropped off as a further increase in enzyme dosage (>3593.45 U g−1). There was an ellipse in the counter plot which expressed the interaction of pH and enzyme dosage, which indicates the interaction between these two variables are highly significant (Table 1).

According to the regression equation (Eq. 3), use the Design Expert for the response surface analysis between factors. The results are shown in (Figure 3A and B). The most significant variables in terms of interaction between the hydrolysis time and temperature, and the interaction between pH and dosage were directly reflected on the desired response. In the view of above results and discussion the optimized values of the each parameter for the preparation of rapeseed protein hydrolysate with maximum ACE inhibitory activity 56.3% with an overall desirability value 1.00 were at (90.11 min), at pH (8.88), at temperature (50°C) and at an enzyme dosage (3580.36 Ug−1). The optimal conditions obtained from RSM model for the production of hydrolysates from rapeseed protein with a maximum ACE inhibitory activity were further validated experimentally. Under the optimized conditions an experiment with three replicates was conducted. The average actual ACE inhibitory activity was recorded as 55.87% which was in the agreement with fitted model predicted value 56.3% and the average actual ACE inhibitory activity was 55.87% which was in the agreement with the predicted values of 56.3% and these two values are close enough to validate the model.
Effect of degree of hydrolysis on molecular weight distribution (MW)

Molecular weight distribution of alcalase generated rapeseed protein hydrolysates under optimized hydrolysis conditions obtained from response surface analysis was analyzed by HPLC. The molecular weight distribution has greatly influenced by the degree of hydrolysis and the specificity of alcalase enzyme. The molecular weight distribution in this study showed variations among the hydrolysates generated with different DH values. As it can be seen from the (Table 2) during the hydrolysis of rapeseed protein by alcalase as the DH increased the intact proteins of higher molecular size >5000 Da were gradually disappeared to form smaller size peptides. This indicated that at low DH fewer peptides bonds were cleaved during rapeseed protein hydrolysis thus, larger peptides of low molecular weight were found. The average molecular weight of peptides were found in the fraction. It can also be seen that the average molecular weight of peptides <3000 Da. The molecular size of peptides >3000–5000 Da were 7.20%, 5.64%, 1.55%, and 3.49% with respect to their corresponding DH values (5.3%, 9.72%, 14.71% and 18.24%, respectively. The size of peptides >500–1000 Da progressively decreased from 27.75%–5.04%
Table 2: Effect of degree of hydrolysis (DH) on molecular weight distribution.

| Molecular weight ranges Da | DH 5.34% | DH 9.72% | DH 14.71% | DH 18.24% |
|---------------------------|-----------|-----------|-----------|-----------|
| >5000                     | 4.75      | 3.93      | 0.21      |           |
| 3000–5000                 | 7.20      | 5.64      | 1.55      | 3.49      |
| 1000–3000                 | 27.75     | 28.34     | 22.87     | 15.09     |
| 500–1000                  | 22.14     | 21.93     | 5.04      |           |
| <500                      | 38.16     | 40.15     | 70.33     | 81.43     |

(DH 5.9%, 14.71%) while at maximum DH 18.24% peptides with this size were disappeared to form more molecular weight peptides. An increase of low molecular weights peptide size <500 was observed as the DH of rapeseed protein hydrolysate was increased 38.16%–81.43%. The increase in the small size peptide fractions might be due to the exposure of hydrophobic residues and the considerable factor specificity of alcalase enzyme which is endoprotease having hydrophobic residues. These results indicated that alcalase enzyme has cleaved peptide bonds during the course of hydrolysis of rapeseed protein. The decrease in high molecular weight peptides with the increase of DH was also observed in salmon by-products [28].

Conclusion

In general conclusion, enzymatic hydrolysis can be employed to prepare hydrolysates with ACE inhibitory activities under optimum conditions. In this study the alcalase enzyme was good choice for the preparation of rapeseed protein hydrolysates with maximum ACE inhibitory activity. The DH and ACE inhibitory activity of rapeseed protein hydrolysates were significantly affected by the hydrolysis processing conditions including hydrolysis time, pH, temperature, and enzyme dosage, respectively. Furthermore, RSM model was found statistically significant at 95% confidence level to the response (ACE inhibitory activity). The predicted value of ACE inhibitory activity 56.3% and the experimentally observed value 55.87% were in good agreement suggesting a good fit between predicted model and experimental results. The molecular weight of rapeseed protein hydrolysates suggesting the biological functions are correlated with the degree of hydrolysis. It is recommended that this information may be useful and could be utilized to prepare ACE inhibitory peptides from food sources for the food industry and pharmaceuticals.

Acknowledgments: The authors wish to express their deep gratitude and appreciation for the support obtained from the National High Technology Research and Development Program 863 (No. 2013AA100203), and the Jiangsu provincial major project on natural science for universities (No. 12KJA550001).

Author contributions: The authors entitled in this manuscript contributed equally.

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Supplemental Material: The online version of this article (DOI: 10.1515/tjb-2017-0044) offers supplementary material, available to authorized users.