Angiotensin-I Converting Enzyme Inhibitory Peptides from Sweet Sorghum Grain Protein: Optimisation of Hydrolysis Conditions and Hydrolysate Characterization

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(Received on 1st November 2017, accepted in revised form 13th June 2018)

Summary: In order to utilize sweet sorghum grain protein (SSGP) for food applications, SSGP was hydrolyzed by alcalase to produce the hydrolysates with ACE-inhibitory activity. The Plackett-Burman design (PBD) was used to identify the important factors influencing the ACE-inhibitory activity of SSGP hydrolysates. The result of PBD indicated that hydrolysis temperature, pH and alcalase dosage had significant influence ($P < 0.05$) on the ACE-inhibitory activity of SSGP hydrolysates. Response surface methodology (RSM) was applied to optimize the three significant factors. The optimum hydrolysis conditions obtained from RSM were as follows: hydrolysis temperature 56 ℃, pH 8.0, alcalase dosage 5200U/g. Under the optimum conditions, SSGP hydrolysates contained 24.3% 1–5 kDa (IC_{50}=0.305 mg/ml) and 15.2% <1 kDa (IC_{50}=0.116 mg/ml) peptide fractions having potent *in vitro* ACE inhibitory activities. Moreover, the inhibition pattern against ACE investigated using Lineweaver–Burk plots revealed that the peptides with molecular weights above 5 kDa in the SSGP hydrolysates were non-competitive inhibitors with inhibition constants (K_i) between 1.098 and 1.171 mg/ml, while the peptides with molecular weights below 5 kDa in the hydrolysates were competitive inhibitors with K_i between 0.110 and 0.184 mg/ml. The results of this study suggest that SSGP may be considered as a source of functional ingredients for the prevention of hypertension.

Keywords: Sweet sorghum grain protein (SSGP); Optimisation of hydrolysis conditions; ACE-inhibitory activity; Response surface methodology; Amino acid composition; Lineweaver–Burk.

Introduction

Angiotensin-I converting enzyme (ACE) is a zinc metallopeptidase that catalyzes the synthesis of angiotensin II having hypertensive effect and the degradation of bradykinin having antihypertensive effect [1]. ACE is well known to play a major role in controlling blood pressure by rennin-angiotensin system [2]. Therefore, inhibition of ACE has been studied in order to develop antihypertensive agents. Synthesized ACE inhibitors (e.g. enalapril, perindopril and lisinopril) have been used extensively in the prevention or treatment of high blood pressure in humans [3]. Studies, however, have shown that these chemical molecules may have some undesirable side effects such as taste disturbances, skin rash and coughing [4, 5]. Therefore, it is necessary to find safer and effective ACE inhibitors for the prevention or treatment of hypertension. ACE-inhibitory peptides from food proteins, which can be released from the sequence of parent proteins by enzymolysis, are currently identified as an alternative for the treatment of hypertension [5]. Many ACE-inhibitory peptides have been produced by enzymolysis from animal and plant proteins, such as native collagenous [6], rice bran protein [7], Porcine skin gelatin [8] and bovine caseins [9]. However, there is little information on ACE-inhibitory peptides of sweet sorghum grain protein (SSGP) until now.

Sweet sorghum, an energy crop, which originates from Africa, is considered to be one of the most promising bio-energy crops because it has more tolerance to stress factors such as drought, salt and nutrient deficiencies [10]. The juice from its stalks, which contains sucrose, fructose and glucose, and is mainly used to produce ethanol, biodiesel and hydrogen [11]. Yet, the sweet sorghum grain source has poor utility for human applications. With the knowledge that alcalase-proteolysis leads to the production of large numbers of ACE inhibitory peptides with higher activities [12, 13]. This study, therefore, focused on the use of alcalase in preparing ACE inhibitory peptide from SSGP. Our objective was to optimize the hydrolysis conditions for the preparation of ACE-inhibitory peptides from SSGP with alcalase by Plackett-Burman design (PBD) and response surface methodology (RSM). In addition, The SSGP hydrolysates were separated into different peptide fractions by ultrafiltration membrane. Then ACE inhibition patterns for the peptide fractions were analyzed.

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Experimental

Materials and Chemicals

Sweet sorghum (*Sorghum bicolor* L.) grains were purchased from Xintai Seed Co. (Jiangsu, China). Angiotensin-I converting enzyme (ACE) and Hippuryl-His-Leu (HHL) were purchased from Sigma-Aldrich Trading Co. (Shanghai, China). Alcalase was purchased from Novozymes Biotechnology Co. (Beijing, China). α-amylase was purchased from Solarbio Biotechnology Co. (Beijing, China). All other chemicals and solvents were of analytical grade.

Preparation of Sweet Sorghum Grain Protein (SSGP)

The sweet sorghum grains were crushed to powder (100 mesh particle size) using a WK-150 crusher (Jingcheng machinery manufacture Co., Qingzhou, China). The powder was dispersed in deionized water (1:10, w/v), and the pH of the dispersions was adjusted to 6.5 with 1 mol/L NaOH. Then the suspension was treated with α-amylase at 55°C (enzyme-to-substrate ratio 1:100, w/w) for 4 h. After treatment, the pH of the reaction mixture was adjusted to 11.0 by adding 1 mol/L NaOH to extract the proteins. The reaction mixture was centrifuged using a centrifuge (LR10-24A, Beijing LAB Centrifuge Co., Beijing, China) at 2,500 *g* for 10 min at 20°C. The supernatant was adjusted to pH 5.0 with 1 mol/L HCl to precipitate the proteins. The precipitates obtained by centrifugation at 2500 *g* for 10 min at 20°C were re-dispersed in distilled water, and adjusted to pH 7.0 by using 0.1 mol/L NaOH. The dispersed product was freeze-dried at −80 °C and then stored at −20 °C until further tests.

Hydrolysis of Sweet Sorghum Grain Protein (SSGP)

SSGP were hydrolyzed with alcalase according to the hydrolysis conditions defined by the experimental design. During the enzymatic reaction process, the pH was maintained at the required values (the values are 6.4, 7.0, 8.0 and 9.0, respectively) by the addition of 1 mol/L NaOH and the reaction temperature was controlled at the required values (the values are 44°C , 50°C, 55°C and 60°C, respectively) using a water bath. At the end of the incubation period, the enzymatic hydrolysis was stopped by boiling for 10 min. Subsequently, the reaction mixture was centrifuged using a centrifuge (LR10-24A, Beijing LAB Centrifuge Co., Beijing, China) at 2500 *g* and 20°C for 10 min, and the supernatant was collected and stored at 4°C for subsequent analysis of ACE inhibition activity.

Measurement of ACE-Inhibitory Activity

ACE-inhibitory activity was measured by our previously described method [14]. Briefly, 10 μl of sample solution was mixed with 45 μl sodium borate buffer (pH 8.3) containing 6.5 mmol/L Hippuryl-His-Leu (HHL) and 0.3 mol/L NaCl, and the mixture was pre-incubated for 5 min at 37°C. The reaction was initiated by adding 10 μl ACE (ACE in 0.1 mol/L borate buffers containing the 0.3 mol/L NaCl, pH 8.3). Finally, the reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of 85 μl of 1 mol/L HCl to the reaction mixture except for the blank (the HCl solution was added before the pre-incubation). Then 1000 μl of ethyl acetate was used to extract the hippuric acid formed from the reaction mixture. Then 800 μl of the ethyl acetate layer was collected by centrifugation using a high-speed refrigerated centrifuge (H205DR-1, Changsha Xiangyi Centrifuge Co., Hunan, China) at 4000 *g* for 10 min, dried at 100°C for 20 min. Subsequently, the hippuric acid was re-dissolved in 800 μl of distilled water. The absorbance was determined at 228 nm with a spectrophotometer (UV-2100, Unico Instrument Co., Shanghai, China). ACE inhibition activity was calculated as follows:

$$\text{ACE - inhibition activity } (\%) = \frac{C - S}{C - B} \times 100 \qquad (1)$$

where *C* is the absorbance without sample (buffer for samples), and *S* is the absorbance in the presence of both ACE and sample. *B* is the absorbance of blank (hydrochloric acid was added before ACE).

Experimental Design

Plackett-Burman Design (PBD)

Table-1: Factors levels in Plackett-Burman design.

Variables	Code	Experimental value	
		Low level (-1)	High level (+1)
Hydrolysis temperature (°C)	X_1	44	55
pH	X_2	6.4	8.0
Dummy 1	N_1	0	0
Hydrolysis time (min)	X_3	70	90
Alcalase dosage (U/g)	X_4	4000	5000
Dummy 2	N_2	0	0
Substrate concentration (g/L)	X_5	8	10

Table-2: Plackett–Burman design and response values.

Run	Code levels							ACE-inhibitory activity Y (%)
	X_1	X_2	N_1	X_3	X_4	N_2	X_5	
1	-1	1	-1	-1	-1	1	1	58.87
2	-1	-1	1	1	1	-1	1	46.86
3	1	1	-1	1	-1	-1	-1	57.14
4	1	-1	1	1	-1	1	-1	37.14
5	1	-1	1	-1	-1	-1	1	42.27
6	1	1	1	-1	1	1	-1	73.01
7	-1	-1	-1	1	1	1	-1	39.56
8	-1	-1	-1	-1	-1	-1	-1	30.31
9	-1	1	1	1	-1	1	1	45.45
10	1	-1	-1	-1	1	1	1	50.24
11	-1	1	1	-1	1	-1	-1	64.15
12	1	1	-1	1	1	-1	1	85.42

Plackett-Burman experimental design was applied to identify the factors that have significant effect on the ACE-inhibitory activity of SSGP hydrolysates [15]. In this study, the seven factors (including two dummy variables) were investigated using the PBD with a first-order polynomial equation. Each factor was examined as low and a high level, coded as (-1) and (+1), respectively. Table-1 shows the variables and their corresponding levels used in the experimental design. The PBD and the response value of ACE-inhibitory activity are shown in Table-2. The fitted first-order model was obtained by the equation [16] :

$$Y = \beta_0 + \sum \beta_i X_i \qquad (2)$$

where Y is the predicted response; β_0 is the intercept; β_i is the regression coefficient and X_i is the coded independent factor.

Path of Steepest Ascent

The path of steepest ascent is a procedure for moving along the path of steepest ascent to the maximum increase in the response [16]. The direction of steepest ascent was the direction in which response (ACE-inhibitory activity) increased rapidly. The zero level of the Plackett–Burman design was identified as the base point of the steepest ascent path. Experiments were performed along the steepest ascent path till no further increase in response was observed [16, 17].

Central Composite Design (CCD) and Response Surface Methodology (RSM)

Table-3: Factors levels in central composite design.

Factors	Code	Levels of factors		
		-1	0	+1
Hydrolysis temperature (°C)	*A*	50	55	60
pH	*B*	7.0	8.0	9.0
Alcalase dosage (U/g)	*C*	4000	5000	6000

Table-4: Central composite design and response values [a].

Run	Code levels			ACE-inhibitory activity (*Y*) (%)
	Hydrolysis temperature (*A*)	pH (*B*)	Alcalase dosage (*C*)	
1	0	0	0	80.33
2	0	-1	-1	40.74
3	-1	0	1	62.25
4	1	0	1	68.41
5	1	1	0	60.44
6	-1	1	0	44.84
7	-1	-1	0	48.08
8	0	0	0	82.12
9	-1	0	-1	49.06
10	0	-1	1	63.37
11	1	0	-1	58.89
12	0	0	0	82.26
13	0	1	-1	59.07
14	0	1	1	48.13
15	1	-1	0	54.79

[a] In this study, the substrate concentration and hydrolysis time was 10 g/L and 90 min, respectively.

The CCD was used to further investigate the three most significant factors (Hydrolysis temperature, pH and alcalase dosage) for increasing the ACE-inhibitory activity of SSGP hydrolysates, screened by Plackett-Burman design (PBD). The three independent factors were investigated at three different levels (-1, 0, +1) (Table-3), and a 15-run CCD used for study was shown in Table-4. The behavior of the system was explained by the second-order polynomial equation[17]:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \qquad (3)$$

where Y is the predicted response, β_0 is the intercept, X_i and X_j are the coded independent factors, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

Fractionation of SSGP Hydrolysate

The SSGP hydrolysate was ultrafiltered sequentially using an ultrafiltration unit (Pellicon, Millipore, USA) through three ultrafiltration membranes with molecular weight cut-off of 10, 5 and 1 kDa, respectively. Four peptide fractions with molecular weights of <1 kDa (represented hydrolysates <1 kDa), 1-5 kDa (represented hydrolysates between 1 and 5 kDa), 5-10 kDa (represented hydrolysates between 5 and 10 kDa), and >10 kDa (represented hydrolysates >10 kDa) were obtained from SSGP hydrolysates. The Contents of peptide fractions were determined by micro-Kjeldahl method.

Determination of ACE Inhibition Pattern

To investigate the inhibition pattern on ACE, different concentrations of peptide fraction from SSGP hydrolysate were added to each reaction mixture according to the method of Barbana and Boye [18]. The enzyme activities (absorbance at 228 nm) were measured with different concentrations of the HHL (0.8125, 1.625, 3.25, 6.5 mmol/L). Lineweaver–Burk plots of 1/absorbance versus 1/HHL were used to determine the ACE inhibitory pattern.

Statistical Analysis

All experiments were done in triplicate. Design-Expert statistical software package (Version 7.1.3, Stat-Ease Inc., Minneapolis, USA) was used for the experimental designs and statistical analysis of the experimental data. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Results and Discussion

Optimization of Hydrolysis Conditions for the Production of ACE-Inhibitory Peptides from SSGP

Plackett–Burman Design (PBD)

Table-5: Analysis of variance (ANOVA) of Plackett-Burman test.

Variable	Degrees of freedom	Sum of squares	*F*-value	*P*-value
X_1	1	0.030011	8.49	0.044[a]
X_2	1	0.157949	44.68	0.003[b]
N_1	1	0.001339	0.38	0.572
X_3	1	0.000442	0.12	0.742
X_4	1	0.064617	18.28	0.013[a]
N_2	1	0.003992	1.13	0.348
X_5	1	0.006446	1.82	0.248

[a] Statistically significant at 95% of confidence level ($P < 0.05$); [b] Statistically significant at 99% of confidence level ($P < 0.01$).

PBD has been widely used by many researchers as a screening method to determine significant factors in experiment design [15, 16]. A PBD in twelve runs was used to study the effect of seven factors (including two dummy variables) on the ACE-inhibitory activity of protein hydrolysates from SSGP and the result was shown in Table-2. The analysis of variance (ANOVA) of Plackett-Burman test in this study (Table-5) was obtained using Design-Expert version 7.1.3 (Stat-Ease Inc., USA). Table-5 showed that hydrolysis temperature, pH and alcalase dosage in the tested range had more than 95% of confidence level, and other variables had a confidence level less than 76%. The results implied that hydrolysis temperature, pH and alcalase dosage had significant influence on ACE-inhibitory activity of the hydrolysates ($P < 0.05$), while hydrolysis time and substrate concentration played a minor role on the ACE-inhibitory activity of the hydrolysates. Therefore, hydrolysis temperature, pH and alcalase dosage were selected for optimization. Here, substrate concentration 10 g/L and hydrolysis time 90min were used for follow-up tests.

Path of Steepest Ascent

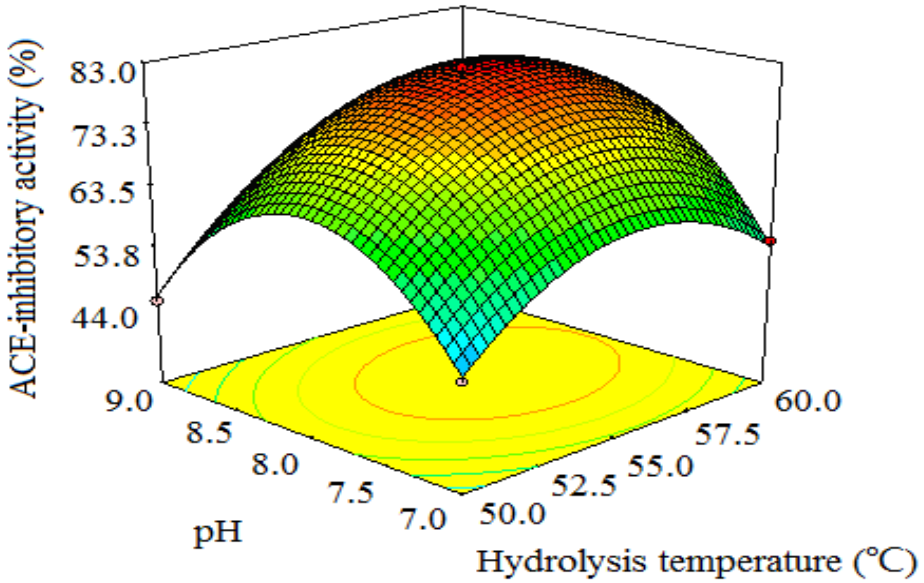
Table-6: Experiment design of the steepest ascent path and response values [a].

Run	Hydrolysis temperature (°C)	pH	Alcalase dosage (U/g)	ACE-inhibitory activity(%)
Origin	40	6.5	3500	39.82
1	45	7.0	4000	40.31
2	50	7.5	4500	68.86
3	55	8.0	5000	82.88
4	60	8.5	5500	74.13
5	65	9.0	6000	68.70

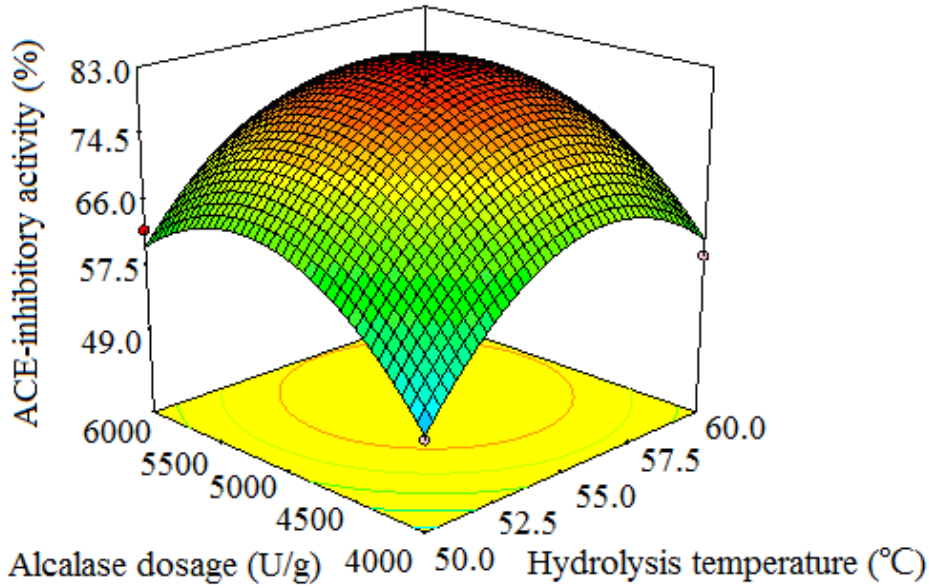
[a] In this study, substrate concentration and hydrolysis time were 10 g/L and 90min, respectively.

According to the result of PBD experiment, the path of steepest ascent was used to approach the optimal region of hydrolysis conditions subsequently [19]. In this study, the path of steepest ascent was designed on the basis of the zero level of PBD, and then was moved along the path of increase of hydrolysis temperature, pH and alcalase dosage. The path of steepest ascent result is presented in Table-6. The highest response (82.88%) was obtained under the hydrolysis conditions of the temperature 55°C, pH 8.0 and alcalase dosage 5000 U/g. Then the hydrolysis conditions were chosen for further optimization.

Central Composite Design (CCD) and Response Surface Methodology (RSM)



(a)



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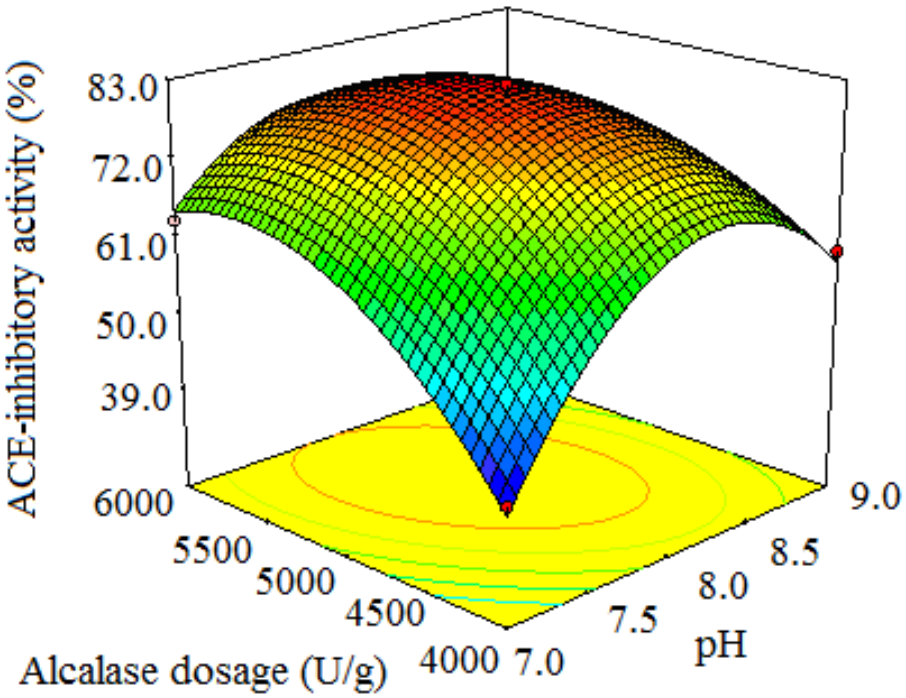


(c)

Fig. 1: Three dimensional response surface plots for ACE-inhibitory activity of hydrolysate from sweet sorghum grain protein(SSGP) showing variable interactions of: (a) hydrolysis temperature and pH; (b) hydrolysis temperature and alcalase dosage; (c) alcalase dosage and pH.

Table-7: Analysis of variance (ANOVA) of central composite design of RSM [a].

Source	Sum of squares	Degree of freedom	Mean Square	*F*-value	*P*-value
Model	**2495.07**	**9**	**277.23**	**61.48**	**0.0001** [b]
A	**183.36**	**1**	**183.36**	**40.66**	**0.0014** [b]
B	**3.78**	**1**	**3.78**	**0.84**	**0.4018**
C	**147.92**	**1**	**147.92**	**32.80**	**0.0023** [b]
AB	**19.76**	**1**	**19.76**	**4.38**	**0.0905**
AC	**3.37**	**1**	**3.37**	**0.75**	**0.4270**
BC	**281.74**	**1**	**281.74**	**62.48**	**0.0005** [b]
A^2	**475.97**	**1**	**475.97**	**105.55**	**0.0002** [b]
B^2	**1220.19**	**1**	**1220.19**	**270.59**	**< 0.0001** [b]
C^2	**412.04**	**1**	**412.04**	**91.37**	**0.0002** [b]
Residual	**22.55**	**5**	**4.51**		
Lack of Fit	**20.23**	**3**	**6.74**	**5.82**	**0.1501**
Pure Error	**2.32**	**2**	**1.16**		
Total	**2517.62**	**14**			

[a] R^2 = 0.9910, adjusted R^2 = 0.9749; [b] Significant within a 99% confidence interval.

The three selected variables (hydrolysis temperature, pH and alcalase dosage) were further explored using CCD of RSM. The matrix for CCD and the experimental results were shown in Table-4. Here, substrate concentration and hydrolysis time were 10 g/L and 90min, respectively. A second-order polynomial equation was obtained to explain the ACE-inhibitory activity of SSGP hydrolysates by applying multiple regression analysis on the CCD data:

$$Y = 81.57 + 4.79\,A + 0.69\,B + 4.3\,C + 2.22\,AB - 0.92\,AC - 8.39\,BC - 11.35\,A^2 - 18.18\,B^2 - 10.56\,C^2 \quad (4)$$

where Y is predicted ACE-inhibitory activity of SSGP hydrolysates (%), A-coded values of hydrolysis temperature, B-coded values of pH, C-coded values of alcalase dosage.

The result of ANOVA for second-order polynomial equation was shown in Table-7. The value of ''*P*-value" of the model and lack of fit were 0.0001 and 0.1501, respectively, suggesting that the model was highly significant. Linear terms of A and C, interaction term of BC, and all the quadratic terms (A^2, B^2 and C^2) were all significant within a 99% confidence interval ($P < 0.01$). The coefficient of determination (R^2) and the adjusted R^2 for ACE-inhibitory activity of SSGP hydrolysates were 0.9910 and 0.9749, respectively, showing a strong agreement between the experimental and predicted values [19]. The lack of fit indicated no significance as well as with *P*-values of 0.1505, suggesting there is a 15.01% chance that the lack of fit *F*-value that large could occur due to noise [16, 20]. Thus the regression model was highly reliable and could be used to optimize the hydrolysis conditions for the preparation of ACE-inhibitory peptides from SSGP.

The interaction effects of hydrolysis temperature (A), pH (B) and alcalase dosage (C) on ACE-inhibitory activity of SSGP hydrolysates were depicted in Fig. 1(a, b and c). For each response surface plot, two factors were considered while one factor was kept constant at zero level. As shown in Fig. 1, the response surface plots showed the clear peaks, indicating that the optimum point was inside the design boundary well [20]. Thus the optimum parameters of hydrolysis conditions can be calculated from the model equation (4) by first-order partial derivatives [21]. The equation (4) was derived as follow:

$$\partial A / \partial Y = 4.79 + 2.22\,B - 0.92C - 22.7\,A = 0 \quad (5)$$

$$\partial B / \partial Y = 0.69 + 2.22\,A - 8.39C - 36.36\,B = 0 \quad (6)$$

$$\partial C / \partial Y = 4.3 - 0.92\,A - 8.39\,B - 21.12C = 0 \quad (7)$$

The linear equations (5), (6) and (7) were solved and the values of obtained A, B and C were 0.2014, -0.0151 and 0.2008, respectively. Here, the calculated values (A, B and C) were the coded values of the corresponding variables. Thus, the optimum conditions in real values were hydrolysis temperature 56 °C, pH 8.0 and alcalase dosage 5200U/g.

Validation of the model

To validate the statistical model, three verification experiments were carried out at the predicted optimal conditions. Under the optimized conditions, the average of actual response for ACE-inhibitory activity was 82.12% (The actual response values of triplicate trials were 82.99%, 80.12% and 83.26%, respectively), which agreed well with the predicted response for ACE-inhibitory activity (82.48%). This result demonstrated the validity of the model. Therefore, the above model was suitable for optimizing the hydrolysis condition in the production of the protein hydrolysates with ACE-inhibitory activity from sweet sorghum grain by alcalase.

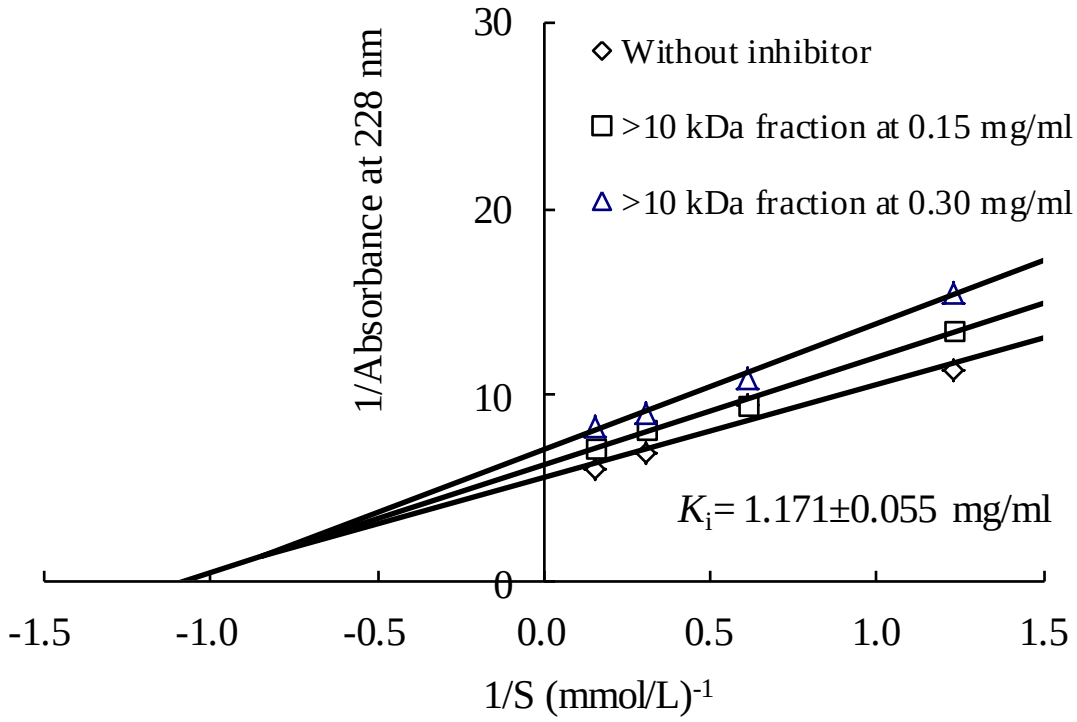
Contents of the Peptide Fractions and Their ACE-Inhibitory Activities

Table-8: The contents and ACE-inhibitory activities of the peptide fractions derived from SSGP hydrolysates.

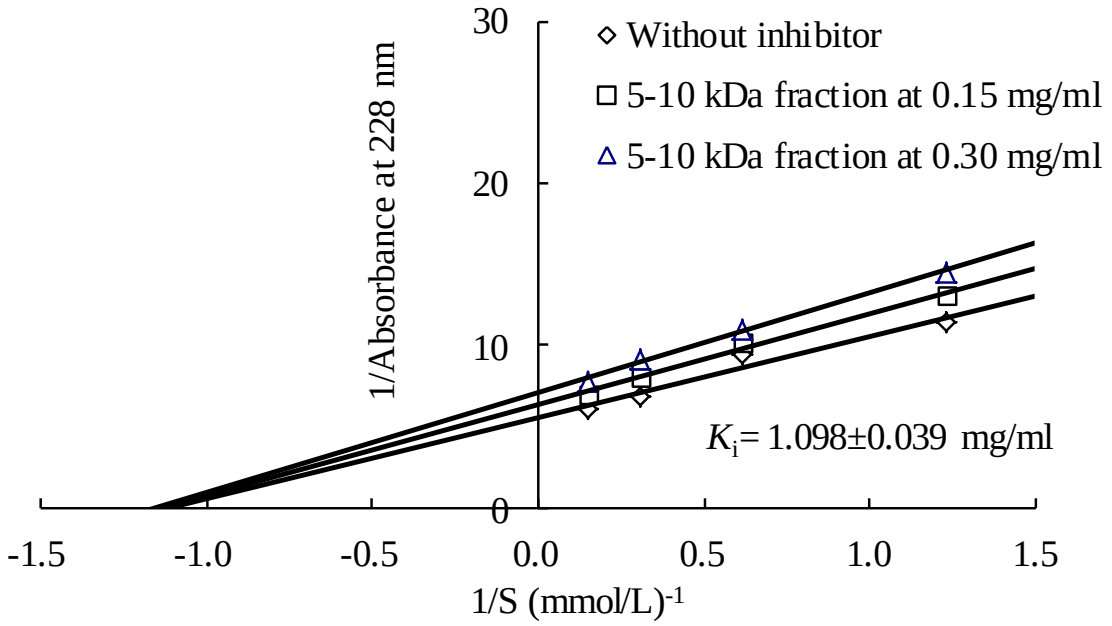
Fractions	IC_{50} (mg/ml)	Content [a] (%)
>10 kDa	1.225	31.1±1.2
5–10 kDa	1.012	26.4±0.9
1–5 kDa	0.305	24.3±0.5
<1 kDa	0.116	15.2±0.1

[a] Values are expressed as means ± standard deviation based on three repetitions.

SSGP was hydrolyzed using alcalase at above optimized hydrolysis condition. its hydrolysates were then fractionated into four fractions with molecular weights of >10 kDa, 5–10 kDa, 1–5 kDa, and <1 kDa by ultrafiltration method. Their contents and ACE-inhibitory activities were shown in Table-8. Among these fractions, the >10 kDa fraction (31.1%) had the maximum content, followed by 5–10 kDa (26.4%), 1–5 kDa (24.3%), and <1 kDa (15.2%). The change in ACE inhibitory activity with various molecular weight ranges was widely observed. The IC_{50} values of >10 kDa, 5–10 kDa, 1–5 kDa and <1 kDa fractions were 1.225, 1.012, 0.305 and 0.116 mg/ml, respectively. The <1 kDa and 1–5 kDa fractions showed more potent ACE-inhibitory activities, indicating that the ACE inhibition could be attributed mainly to the mixture of short peptides in SSGP hydrolysates. The results were in accordance with Ko *et al.* [22] who reported that <5 kDa fraction of Protamex hydrolysate from flesh tissue had more potent ACE-inhibitory activity than the >5 kDa fraction. Similar results were also reported by Lin *et al.* [23] for squid skin gelatin hydrolysates and by Jung *et al.* [24] for yellowfin sole frame protein hydrolysates. Thus, the product, enriched in ACE inhibitory peptides, could be obtained by the ultrafiltration membrane with <5 kDa molecular weight cut-off.



(a)


30
20
10
0
1/Absorbance at 228 nm
Without inhibitor
5-10 kDa fraction at 0.15 mg/ml
5-10 kDa fraction at 0.30 mg/ml
K_i= 1.098±0.039 mg/ml
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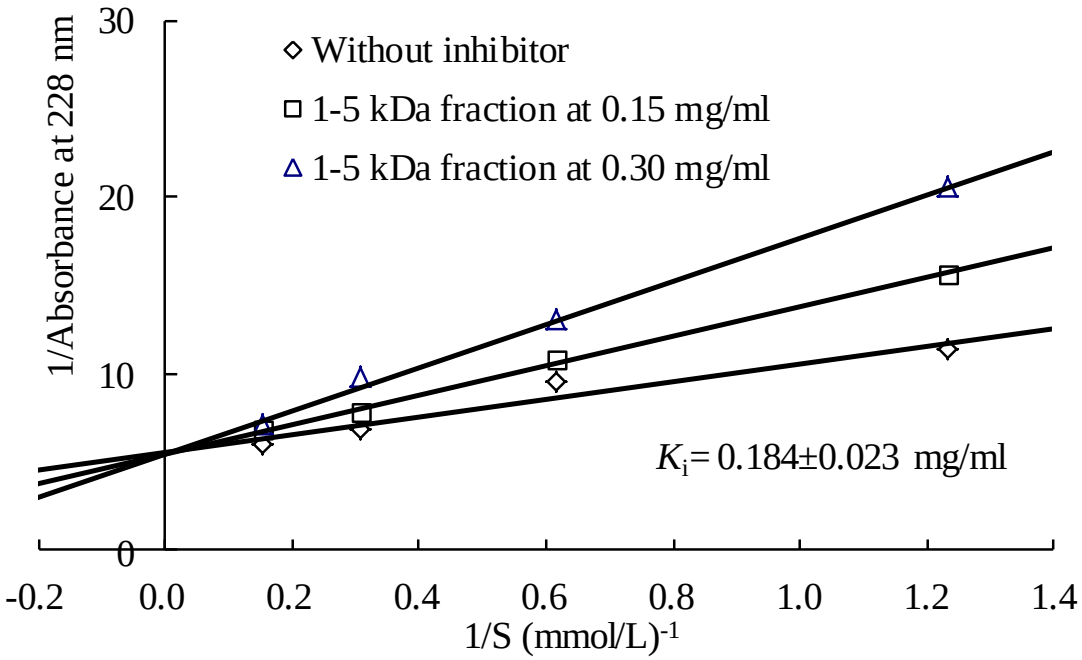
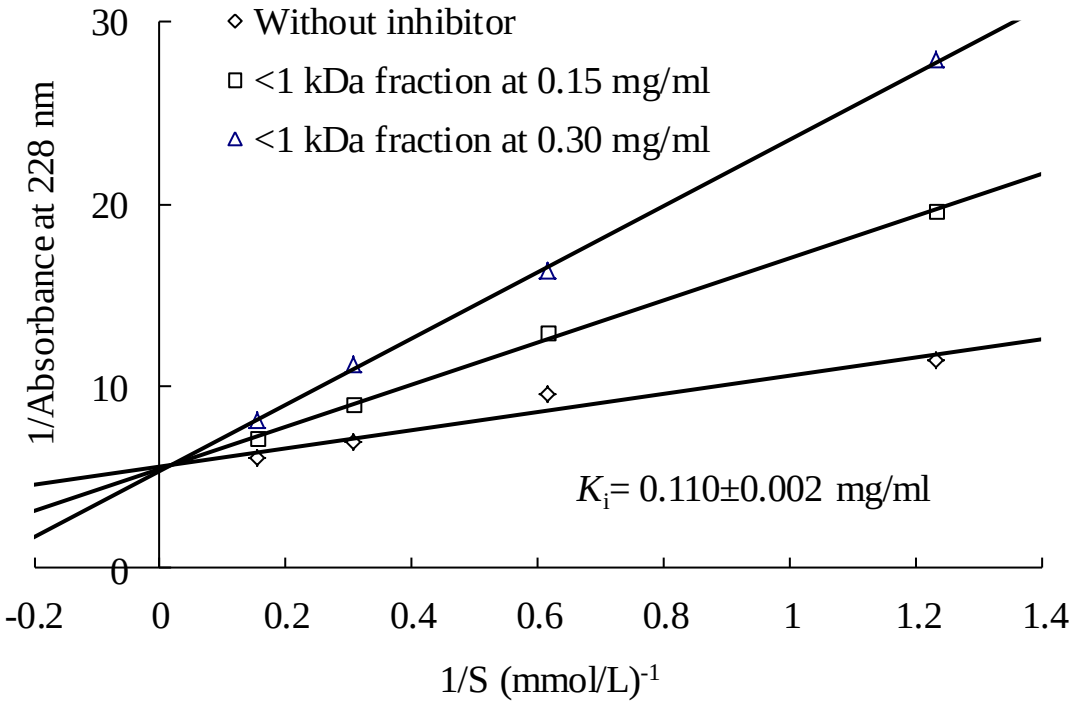
-1.0
-0.5
0.0
0.5
1.0
1.5
1/S (mmol/L)$^{-1}$

(b)


30
20
10
0
1/Absorbance at 228 nm
Without inhibitor
1-5 kDa fraction at 0.15 mg/ml
1-5 kDa fraction at 0.30 mg/ml
K_i= 0.184±0.023 mg/ml
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1/S (mmol/L)$^{-1}$

(c)



(d)

Fig. 2: ACE inhibition pattern of the peptide fractions from SSGP hydrolysates, estimated using Lineweaver-Burk plots. (a) >10 kDa fraction; (b) 5-10 kDa fraction; (c) 1-5 kDa fraction; (d) <1 kDa fraction. Inhibition constants (K_i) are expressed as the mean of three determinations ± standard deviation.

ACE Inhibition Pattern

To clarify the pattern of ACE inhibition by SSGP hydrolysates, the isolated peptide fraction was co-incubated with ACE solution and HHL with different concentrations (0.8125, 1.625, 3.25, 6.5 mmol/L). The Lineweaver–Burk plots for the inhibition of ACE by SSGP hydrolysates fractions were shown in Fig.2. According to the theory of enzymatic inhibition pattern, the Lineweaver-Burk plots at different concentrations of inhibitor with intersecting at the same point on the y-axis show that the type of inhibition is competitive [25], and these plots with coinciding intercept on the x-axis indicate that the type of inhibition is non-competitive [18]. Fig.2 showed that the inhibition patterns of >10 kDa and 5–10 kDa fractions were non-competitive, suggesting that the ACE-inhibitory peptides in those fractions did not bind to the active site of ACE but could bind to other sites on the ACE molecule to produce an inactive complex, regardless of substrate binding[18, 26]. The 1–5 kDa and <1 kDa fractions were shown to be competitive inhibition patterns, indicating that the ACE-inhibitory peptides in those fractions could bind competitively at the active site of ACE to produce an inactive complex [25, 27].

The inhibition constant (K_i) values of the four peptide fractions were obtained according to the linear regression fit of Lineweaver–Burk (Fig.2). The K_i values of >10 kDa, 5–10 kDa, 1–5 kDa and <1 kDa fractions were 1.171, 1.098, 0.184 and 0.110 mg/ml, respectively. In general, the lowest K_i value of inhibitor indicates that its inhibition is the strongest. The K_i values of 1–5 kDa and <1 kDa fractions were markedly lower than >10 kDa and 5–10 kDa fractions, indicating the peptides with molecular weights below 5 kDa have much stronger affinity to the ACE active sites than the peptides with molecular weights above 5 kDa [18]. In this study, although the K_i values of 1–5 kDa and <1 kDa fractions were almost 27500 fold higher than that of captopril (6.70 $\times 10^{-6}$ mg/ml) [28], their K_i values were lower than that of green lentil protein hydrolysate (0.31

mg/ml)[18], red lentil protein hydrolysate (0.46 mg/ml)[18], milk whey fermented with lactic acid bacteria (0.188 mg/ml) [28] and rapeseed hydrolysates (0.2–0.3 mg/ml) [29]. Thus, our study results showed that the 1–5 kDa and <1 kDa fractions derived from SSGP hydrolysates were promising source of peptides with ACE-inhibitory activity.

Conclusions

PBD, path of steepest ascent and CCD of RSM have been proved to be effective in optimizing hydrolysis conditions for enzymatic production of sweet sorghum grain protein hydrolysates with ACE-inhibitory activity using alcalase. The optimized enzymolysis conditions were as follows: hydrolysis temperature 56 °C, pH 8.0, alcalase dosage 5200U/g, substrate concentration 10 g/L, and hydrolysis time 90min. The optimum SSGP hydrolysates contained 31.1% >10 kDa, 26.4% 5–10 kDa, 24.3% 1–5 kDa and 15.2% <1 kDa fractions. The 1–5 kDa and <1 kDa fractions had potent ACE inhibitory activity *in vitro* which IC_{50} values were 0.305 and 0.116 mg/ml, respectively. Lineweaver–Burk plots revealed a non-competitive pattern of ACE inhibition for >10 kDa and 5–10 kDa peptide fractions and a competitive pattern of ACE inhibition for 1–5 kDa and <1 kDa peptide fractions. In conclusion, SSGP hydrolysates can be considered as a potential source of natural antihypertensive agents for use in functional foods. Further studies on the bioavailability of SSGP hydrolysates using an animal model of hypertension (spontaneously hypertensive rats) would be necessary to evaluate the efficacy of the hydrolysates for lowering blood pressure.

Acknowledgement

This work was supported by Natural Science Foundation of Jiangsu Province (No. BK2012693).

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