JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Accumulation and Identification of Angiotensin-Converting Enzyme Inhibitory Peptides from Wheat Germ

Runqiang Yang, Yu Zou, Nanjing Yu, and Zhenxin Gu*

College of Food Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, People's Republic of China

ABSTRACT: The incubation conditions of wheat germ for angiotensin I-converting enzyme inhibitory activity (ACEI) elevation and peptide accumulation were investigated, and five ACE inhibitory peptides were obtained. The effect of individual factors such as incubation time, temperature, initial pH, and liquid to solid ratio on ACEI and peptide concentration of incubation medium was evaluated, respectively. The combinations of four factors were further optimized using a Box−Behnken design. Under the best incubation condition (pH 4.4 with a liquid to solid ratio 8.14 mL/g at temperature 47 °C, for 7 h), maximum ACEI (92.16%) and peptide concentration (88.12 mg/g) were obtained, which were 6.2- and 2.4-fold, respectively, as compared to the unincubated wheat germ. After they were purified, five ACE inhibitory peptides, VEV, W, NPPSV, QV, and AMY, were identified by liquid chromatography/tandem mass spectrometry. The IC_{50} were 115.20, 94.87, 40.56, 26.82, and 5.86 μM, respectively.

KEYWORDS: Wheat germ, incubation, ACE, peptide

INTRODUCTION

In recent years, hypertension has become one of the diseases that threatens human health. In human blood, angiotensin II, a hypertensive peptide, can result in a consequence of vasoconstriction, while bradykinin, a hypotensive peptide, can result in a consequence of vasoconstriction. The angiotensin-converting enzyme (E.C. 3.4.15.1; ACE) is a zinc metallopeptidase that participates in the synthesis of angiotensin II and the degradation of bradykinin.[1] Synthesized ACE inhibitors, such as captopril, alacepril, and lisinopril, are currently used in the treatment of hypertensive patients, but these substances may provoke undesirable side effects.[2] Bioactive peptides have been used as the functional compounds in food products. Many ACE inhibitory peptides have been isolated from enzymatic hydrolysates of vegetable proteins,[3−5] animal proteins,[6] egg white proteins,[7,8] milk proteins,[9−11] aquatic product proteins,[12,13] etc. Alcalase hydrolyze isolated soy protein to prepare angiotensin I-converting enzyme inhibitor was studied, and when E/S = 0.01, the hydrolysis temperature = 50 °C, pH = 9.0, and hydrolysis time = 6 h, the IC_{50} value of isolated soy protein was 0.67 mg protein/mL.[14] The two antihypertensive peptides, Lys-Arg-Val-Ile-Gln-Try and Val-Lys-Ala-Gly-Phe, isolated from porcine myosin, embodied a strong ACE inhibitory activity when they were administered orally to spontaneously hypertensive rats at doses of 10 mg/kg, and temporal hypertension was observed after 6 h.[15] Miguel et al.[8] detected that three peptides, IVF, RADHPFL, and YAEERYPIL, derived from egg white proteins, seriously inhibited the blood pressure elevation of male Sprague−Dawley rats. The lowering blood pressure activity of bovine lactoferrin hydrolysate was examined in vitro and in vivo of rabbit, and the results showed that the IC_{50} value was 0.95 mg/mL.[16] Lee et al.[17] elaborated a peptide, GDLGKTTTVSNWSPPKYKDTP, extracted from tuna frame protein hydrolysate, which decreased blood pressure to spontaneously hypertensive rats, and the IC_{50} value was 11.28 μM.

Wheat germ, a byproduct of the flour milling industry, is reported to be one of the potential sources of vegetable proteins. However, its utilization is under development. Defatted wheat germ, rich in protein, is usually obtained after extracting oil. Several studies have shown that defatted wheat germ protein is a potential protein resource for preparing ACE inhibitory peptides.[18,19]

Currently, the method of preparing antihypertensive peptides by adding outer proteases to hydrolyze protein has been commonly used, which has the advantage of conveniently controlling the concentration of enzyme and substrate. Nevertheless, there may be some insecurity factors including microbial toxin because the majority of industrial proteases is derived from microorganism by fermentation.[20] The endogenous proteases, which are derived from plant tissues, can hydrolyze insoluble stored proteins into soluble protein, peptides, and amino acids under appropriate reaction conditions. In this process, some bioactive peptides were generated. The advantages of using this method to produce bioactive peptides are avirulence, harmlessness, and so on.

In the present study, a method for producing hypotensive peptides from wheat germ was developed, which was different from those methods reported previously and without any additive proteases, while using an incubation medium to activate wheat germ endogenous proteases for degrading its own storage protein to hypotensive peptides.

MATERIALS AND METHODS

Materials and Reagents. The wheat germ was supplied by Tong Yuan Wheat Flour Limited Co. (Pizhou City, China). Raw material was collected immediately after milling and then kept at −20 °C until analysis. ACE from rabbit lung, hippuryl-L-histidyl-L-leucine (Hip-His-Leu) and hippuric acid (Hip) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical or chromatographic grade.

Incubation of Wheat Germ. About 2 g of fresh wheat germ, sterilized with O_3 (0.2 mg/L) for 1 min before experiment, was

Received: August 5, 2010
Accepted: March 7, 2011
Revised: March 3, 2011
Published: March 08, 2011

Table 1. BBD and the Responses for ACE Inhibitory and Peptide Content of Wheat Germ Incubation Medium

trials	X_1-time (h)	X_2-temperature (°C)	X_3-pH	X_4-liquid to solid ratio (mL/g)	ACE inhibitory activity (%)	peptide content (mg/g)
1	−1 (4)	−1 (35)	0 (4.5)	0 (7)	59.12 ± 2.26	59.98 ± 0.71
2	1 (8)	−1 (35)	0 (4.5)	0 (7)	81.71 ± 2.06	74.88 ± 1.06
3	−1 (4)	1 (55)	0 (4.5)	0 (7)	75.93 ± 0.59	65.63 ± 0.56
4	1 (8)	1 (55)	0 (4.5)	0 (7)	77.47 ± 2.71	74.91 ± 0.40
5	0 (6)	0 (45)	−1 (4.0)	−1 (4)	68.90 ± 2.55	64.04 ± 1.76
6	0 (6)	0 (45)	1 (5.0)	−1 (4)	55.63 ± 3.01	55.56 ± 0.35
7	0 (6)	0 (45)	−1 (4.0)	1 (10)	67.03 ± 4.34	87.90 ± 1.76
8	0 (6)	0 (45)	1 (5.0)	1 (10)	59.78 ± 1.79	69.33 ± 0.53
9	−1 (4)	0 (45)	0 (4.5)	−1 (4)	58.01 ± 1.64	55.56 ± 0.35
10	1 (8)	0 (45)	0 (4.5)	−1 (4)	68.30 ± 2.43	70.92 ± 1.06
11	−1 (4)	0 (45)	0 (4.5)	1 (10)	59.87 ± 3.09	67.22 ± 0.35
12	1 (8)	0 (45)	0 (4.5)	1 (10)	75.71 ± 3.96	84.17 ± 0.35
13	0 (6)	−1 (35)	−1 (4.0)	0 (7)	81.18 ± 2.88	70.35 ± 0.71
14	0 (6)	1 (55)	−1 (4.0)	0 (7)	69.91 ± 3.17	78.34 ± 0.35
15	0 (6)	−1 (35)	1 (5.0)	0 (7)	46.87 ± 2.37	56.31 ± 0.71
16	0 (6)	1 (55)	1 (5.0)	0 (7)	77.72 ± 1.56	69.87 ± 0.35
17	−1 (4)	0 (45)	−1 (4.0)	0 (7)	79.78 ± 0.76	59.80 ± 0.53
18	1 (8)	0 (45)	−1 (4.0)	0 (7)	76.56 ± 3.65	84.17 ± 0.35
19	−1 (4)	0 (45)	1 (5.0)	0 (7)	47.75 ± 0.79	56.61 ± 0.53
20	1 (8)	0 (45)	1 (5.0)	0 (7)	74.09 ± 1.89	70.92 ± 1.59
21	0 (6)	−1 (35)	0 (4.5)	−1 (4)	74.64 ± 2.03	48.89 ± 0.35
22	0 (6)	1 (55)	0 (4.5)	−1 (4)	54.42 ± 2.30	59.26 ± 1.73
23	0 (6)	−1 (35)	0 (4.5)	1 (10)	46.77 ± 3.24	80.17 ± 0.71
24	0 (6)	1 (55)	0 (4.5)	1 (10)	86.57 ± 2.32	75.19 ± 1.41
25	0 (6)	0 (45)	0 (4.5)	0 (7)	91.86 ± 1.32	75.70 ± 0.53
26	0 (6)	0 (45)	0 (4.5)	0 (7)	92.22 ± 0.88	74.64 ± 0.88
27	0 (6)	0 (45)	0 (4.5)	0 (7)	89.34 ± 1.07	77.83 ± 0.35
28	0 (6)	0 (45)	0 (4.5)	0 (7)	90.23 ± 1.79	72.51 ± 0.00
29	0 (6)	0 (45)	0 (4.5)	0 (7)	88.64 ± 2.08	74.04 ± 0.40

homogenized in culture solution by grinding (0.1 M citrate buffer solution or Tris-HCl buffer and ice bath) and was incubated in a water bath.

Effects of Incubation Time, Temperature, pH, and Liquid to Solid Ratio on ACEI and Peptide Content. To determine the proper scope of incubation time, temperature, pH, and liquid to solid ratio for ACEI and peptide content in wheat germ during incubation, various incubation times (0, 2, 4, 6, 8, and 10 h), temperatures (30, 40, 45, 50, 55, 60, and 70 °C), pH values (3.0, 3.5, 4.0, 4.5, 5.0, 6.0, and 9.0), and liquid to solid ratios (4, 7, 10, 13, and 16 mL/g) were studied as a single factor first.

Optimization of Incubation Conditions for ACEI and Peptide Accumulation. On the basis of single factor experiments, the incubation conditions, time (X_1), temperature (X_2), pH (X_3), and liquid to solid ratio (X_4) for ACEI (Y_1) or peptide yield (Y_2) in wheat germ incubation medium were optimized using response surface methodology (RSM). The factors and levels investigated in Box−Behnken design (BBD) are shown in Table 1. Twenty-nine combinations, including five replicates of the center points, were employed to evaluate the combined effects of variables on ACEI and peptide yield. The experimental results were analyzed by quadratic stepwise regression to fit the second-order equation:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{i<j}^{4} \beta_{ij} X_i X_j \qquad (1)$$

where Y denotes the response observed for treatment combination $X = (x_1, x_2, ..., x_p)$ for p factors, β^0 represents the intercept, and the parameters of β_i, β_{ii}, and β_{ij} represent the regression coefficients of variables for linear, quadratic, and interaction regression terms, respectively. An analysis of variance (ANOVA) table is generated to determine individual linear, quadratic, and interaction regression coefficients. The significances of polynomial relations were tested using Fisher's F test. The regression coefficients were used for statistical analyses to generate contour maps of the regression models.

Purification and Identification of ACE Inhibitory Peptide. The incubated solution was purified by ethanol precipitation, Sephadex G-25 gel chromatography column (2.5 cm × 30 cm), Sephadex G-10 gel chromatography column (2.5 cm × 30 cm), and DEAE-Sephcrose-FF gel chromatography column (2.5 cm × 30 cm) four steps. The incubation solution was precipitated with 90% (v: v) ethanol. After ethanol precipitation, the supernatant was further treated with Sephadex G-25 column (eluted with distilled water, 1 mL/min), and the fraction with the highest ACE inhibitory was collected. After it was concentrated, the collection was further purified using Sephadex G-10 (eluted with distilled water, 1 mL/min), the fraction with the highest ACE inhibitory was collected and placed in the DEAE-Sephcrose-FF gel column (eluted with 50 mmol/L Tris-HCl buffer solution contained a different concentration of NaCl, pH 8.0, 1 mL/min). The fraction with the highest ACE inhibitory was collected and analyzed with high-performance liquid chromatography (HPLC). The structure of the ACE inhibitory peptide was identified by liquid chromatography/tandem mass spectrometry (LC/MS/MS; Waters). The IC_{50} value was defined as the concentration of peptide required to inhibit 50% of the ACE activity under the assayed condition.

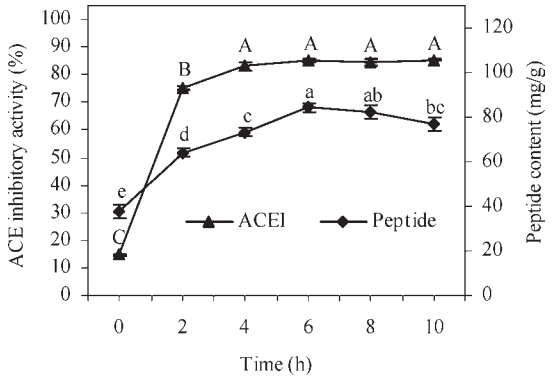

Figure 1. Effect of incubation time on ACEI and peptide content. Two grams of frozen wheat germ was mulled with 20 mL of distilled water (pH 6.8) and then incubated in a 50 °C water bath. Values are the means of triplicate analyses. Error bars show the standard deviation. Capital letters reflect the significance of ACEI, and lower case letters reflect the significance of peptide content.

ACEI Assay. ACEI was analyzed by reversed-phase (RP) HPLC using hippury-L-histidyl-L-leucine (HHL) as the substrate, according to the method of Cushman and Cheung[21] with some modifications. A 5 mM concentration of HHL was prepared with 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl. ACE extracted from rabbit lung was dissolved in the same buffer at a concentration of 50 mU/mL. A mixture containing 30 μL of HHL solution and 30 μL of incubation solution or the buffer (control) was incubated at 37 °C for 5 min, 20 μL of ACE solution was then added, and the mixture was incubated for 60 min. The reaction was stopped with 200 μL of 1 M HCl. Hippuric acid (Hip) liberated by ACE was determined by RP-HPLC on an Agilent SB-C_{18} column (4.6 mm × 250 mm, 5 μM). The elution system involved 75% of mobile phase A (0.5% acetic acid solution) and 25% of mobile phase B (acetonitrile) at a flow rate of 0.6 mL/min during the entire run. Twenty microliters of each sample was injected, at a column temperature of 25 °C. The effluent was monitored with an ultraviolet detector at 228 nm. ACEI was expressed as

$$\text{ACEI}\ (\%) = \frac{H_0 - H_i}{H_0} \times 100 \qquad (2)$$

where H_0 is the peak area of the Hip peak without incubation solution; H_i is the peak area of the Hip peak with incubation solution. IC_{50} is defined as the concentration of inhibiting half the activity of ACE.

Peptide Content Determination. About 3 mL of incubated solution was centrifuged at 3000*g* for 15 min. The supernatant was used for peptides assay according to the biuret method.[22] Macromolecule protein in the supernatant was deposited by an equal volume of 10 g/100 mL trichloroacetic acid (TCA) and then centrifuged at 6000*g* for 15 min. Two milliliter aliquots of biuret reagent were added into 3 mL of the supernatant. After an incubation period of 10 min at 25 °C and a centrifugation at 6000*g* for 15 min, and the peptides were determined by the absorbance at 540 nm using L-glutathione (reduced) as the standard.

Statistical Analysis. Average values and standard deviations were computed according to experimental data. Statistical analysis was performed using Fisher's *F* test. $p < 0.1$, 0.05, or 0.01 was taken as significant.

RESULTS AND ANALYSIS

Effect of Incubation Time on ACEI and Peptide Content. The effects of incubation time on ACEI and peptide yield of wheat germ are shown in Figure1. Within the incubation time range of 0−10 h with every 2 h interval, there was a gradual increase in ACEI and peptide yield with the increase of incubation time, but it had no obvious change ($p < 0.05$) after 4 and 6 h of incubation, respectively. When wheat germ was incubated for 4 h, ACEI was 82.96%, increased 5.61-fold as compared with the control (14.78%). When the incubation time reached 6 h, the peptide content was 84.24 mg/g DW, which was 1.25 times higher as compared with the control (37.52 mg/g DW).

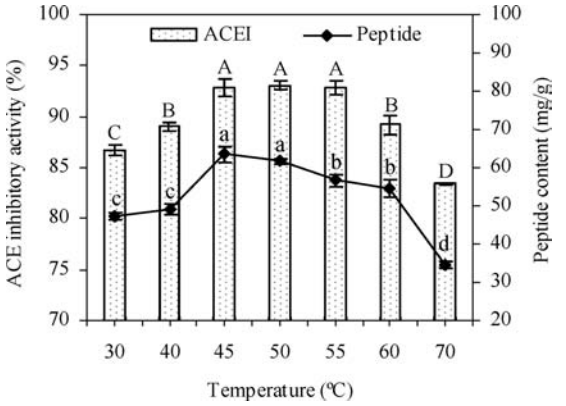

Figure 2. Effect of incubation temperature on ACEI and peptide content. Two grams of frozen wheat germ was mulled with 20 mL of distilled water (pH 6.8) and then incubated in a water bath with different temperature for 6 h. Values are the means of triplicate analyses. Error bars show the standard deviation. Capital letters reflect the significance of ACEI, and lower case letters reflect the significance of peptide content.

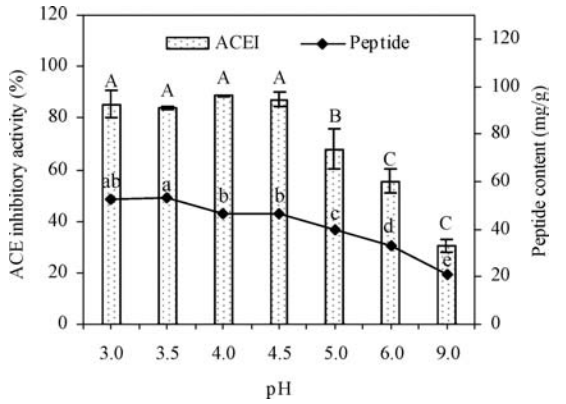

Figure 3. Effect of incubation medium pH on ACEI and peptide content. Two grams of frozen wheat germ was mulled with 20 mL of buffer solution and then incubated in 50 °C water bath for 6 h. Values are the means of triplicate analyses. Error bars show the standard deviation. Capital letters reflect the significance of ACEI, and lower case letters reflect the significance of peptide content.

Effect of Incubation Temperature on ACEI and Peptide Content. The temperature seriously influenced ACEI and peptide content in the incubation solution of wheat germ (Figure 2). With the temperature increasing from 30 to 45 °C, the ACEI and peptide content increased, and a sharp decrease occurred when the temperature was beyond 60 °C. With the temperature ranging from 45 to 55 °C, ACEI of incubation solution had no obvious change ($p > 0.05$), but the peptide content decreased when the temperature was higher than 50 °C. It could be conjectured that there were other ACE inhibitory substances in the incubation solution except for peptide.

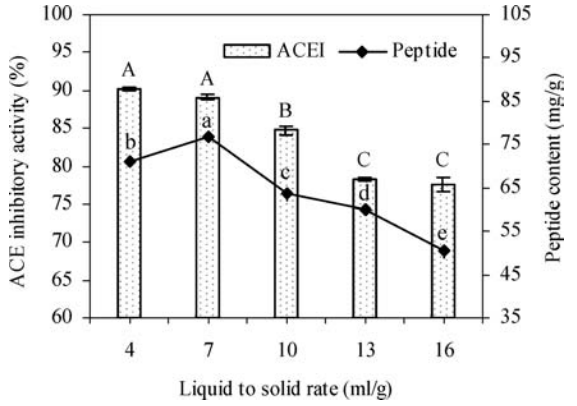

Figure 4. Effect of liquid to solid ratio on ACEI and peptide content. Two grams of frozen wheat germ was mulled with 8, 14, 20, 26, and 32 mL of buffer solution (pH 3.5), respectively, and then incubated in 50 °C water bath for 6 h. Values are the means of triplicate analyses. Error bars show the standard deviation. Capital letters reflect the significance of ACEI, and lower case letters reflect the significance of peptide content.

Effect of pH Value on ACEI and Peptide Content. As shown in Figure 3, a higher ACEI was shown at acidic conditions, but the ACEI decreased rapidly when the pH value was higher than 4.5. With the increase of pH, the peptide content decreased gradually. It was demonstrated that the lower pH values (3.0−4.5) were helpful to the accumulation of peptide. At pH 3.5, the ACEI and peptide contents were 2.77- and 2.54-fold of that at pH 9.0.

Effect of Liquid to Solid Ratio on ACEI and Peptide Content. With the increase of the liquid to solid ratio, the ACEI of incubation solution was descending. When wheat germ was dispersed in 7 times incubation solution and incubated at 50 °C, the peptide content was 76.57 mg/g, higher than that of incubating under other solids concentration (Figure 4). The enzyme reaction was usually carried out in aqueous solution. The enzyme and substrate concentration influenced the extent of the reaction of all reaction processes. The liquid to solid ratio affected ACEI and the peptide content by influencing the contact extent of the enzyme and substrate. A suitable enzyme concentration and substrate content was very important to the enzyme reaction.

Analysis of Box−Behnken Experiment. The optimal levels of the significant factors (time, temperature, pH, and liquid to solid ratio) and their interaction effects on ACEI and peptide yield were further explored by the BBD of RSM. By applying multiple regression analysis, the following two second-order polynomial equations were established to explain the ACEI (eq 3) and peptide content (eq 4), respectively:

$$\begin{aligned} Y_1 = & -510.84 + 7.43X_1 - 3.01X_2 \\ & + 287.73X_3 + 2.71X_4 - 0.26X_1X_2 + 7.39X_1X_3 \\ & + 2.11X_2X_3 + 0.50X_2X_4 - 2.15X_1^2 - 0.09X_2^2 \\ & - 48.94X_3^2 - 1.77X_4^2 \end{aligned} \quad (3)$$

$$\begin{aligned} Y_2 = & -331.57 + 13.09X_1 + 5.17X_2 \\ & + 87.56X_3 + 14.03X_4 - 0.13X_2X_4 - 0.76X_1^2 \\ & - 0.04X_2^2 - 10.95X_3^2 - 0.37X_4^2 \end{aligned} \quad (4)$$

The ANOVA for the BBD experiments gave relatively high F values (100.55 for ACEI and 235.54 for peptide content), very low probability values (<0.0001 for both ACEI and peptide content), and fairly large coefficients of determination (R^2 = 0.9869 for ACEI and 0.9063 for peptide content) as shown in Tables 2 and 3. A good agreement between experimental and predicted values implied that the mathematical model was very reliable for ACEI and peptide content in the present study.

Table 2. ANOVA for ACEI from Box−Behnken Experiment[a]

source	sum of squares	df	mean square	F value	p value prob > F	significance
model	5356.27	12	446.36	100.55	<0.0001	***
X_1	448.76	1	448.76	101.10	<0.0001	***
X_2	223.01	1	223.01	50.24	<0.0001	***
X_3	553.82	1	553.82	124.76	<0.0001	***
X_4	20.88	1	20.88	4.70	0.0455	**
X_1X_2	110.75	1	110.75	24.95	0.0001	***
X_1X_3	218.44	1	218.44	49.21	<0.0001	***
X_2X_3	443.73	1	443.73	99.96	<0.0001	***
X_2X_4	900.46	1	900.46	202.85	<0.0001	***
X_1^2	479.13	1	479.13	107.94	<0.0001	***
X_2^2	507.46	1	507.46	114.32	<0.0001	***
X_3^2	970.96	1	970.96	218.74	<0.0001	***
X_4^2	1646.07	1	1646.07	370.82	<0.0001	***
residual	71.02	16	4.44			
lack of fit	61.35	12	5.11	2.11	0.2453	NS
pure error	9.68	4	2.42			
cor total	5427.29	28				

[a] R^2 = 0.9869, Adeq precision = 30.32, coefficients of variation = 2.94%. *Significant at p < 0.1, **significant at p < 0.05, ***significant at p < 0.01, and NS, not significant.

Table 3. ANOVA for Peptide Content from Box−Behnken Experiment[a]

source	sum of squares	df	mean square	F value	p value prob > F	significance
model	2459.35	9	273.26	20.43	<0.0001	***
X_1	754.78	1	754.78	56.43	<0.0001	***
X_2	88.67	1	88.67	6.63	0.0186	**
X_3	363.00	1	363.00	27.14	<0.0001	***
X_4	1003.76	1	1003.76	75.04	<0.0001	***
X_2X_4	58.91	1	58.91	4.40	0.0495	**
X_1^2	47.58	1	47.58	3.56	0.0747	*
X_2^2	108.08	1	108.08	8.08	0.0104	**
X_3^2	54.72	1	54.72	4.09	0.0574	*
X_4^2	80.75	1	80.75	6.04	0.0238	**
residual	254.14	19	13.38			
lack of fit	238.41	15	15.89	4.04	0.0933	*
pure error	15.73	4	3.93			
cor total	2713.49	28				

[a] R^2 = 0.9063, Adeq precision = 15.90, coefficients of variation = 5.26%. *Significant at p < 0.1, **significant at p < 0.05, ***significant at p < 0.01, and NS, not significant.

As shown in Tables 2 and 3, the linear effects of incubation time, temperature, pH, and liquid to solid ratio were significant (p < 0.05) for both ACEI and peptide content. Furthermore, the interactive terms between incubation time and temperature, incubation time and pH, temperature and pH, and temperature and liquid to solid ratio were very significant model terms for

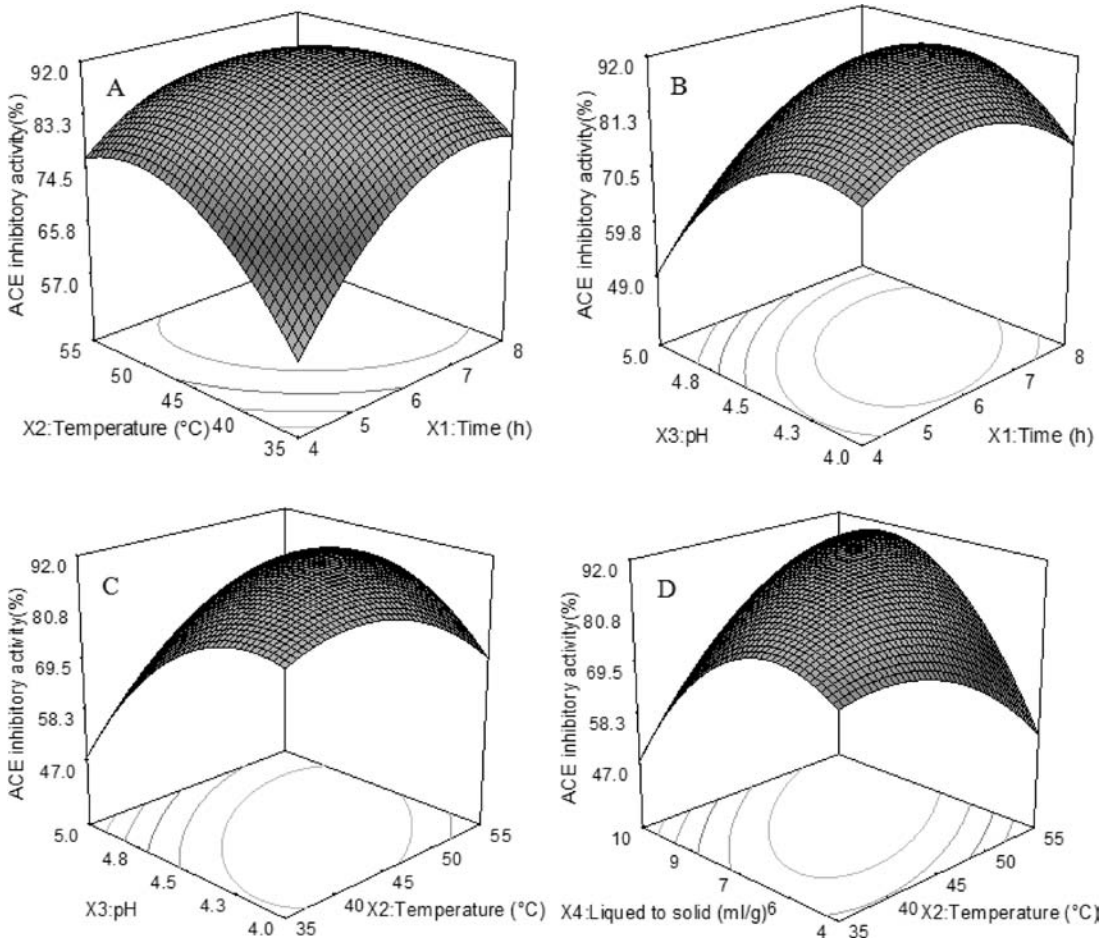

Figure 5. Response surface plots showing the interactions of variables on ACEI. (A) Interaction on ACEI between time and temperature, (B) interaction on ACEI between time and pH, (C) interaction on ACEI between temperature and pH, and (D) interaction on ACEI between temperature and liquid to solid ratio.

ACEI ($p < 0.01$), while only the temperature and liquid to solid ratio was significant ($p < 0.05$) for peptide content.

Figures 5 and 6 showed the response surface plots for the present study and depicted the pairwise interaction of the four variables, which had a significant effect ($p < 0.05$) on ACEI and peptide content, when other variables were kept at their zero levels. The change trend of ACEI or peptide content corresponded with the results of the single factor experiment.

When the pH and liquid to solid ratio were fixed at pH 4.5 and 7.0, respectively, with the incubation time ranging from 4 to 8 h (Figure 5A,B), the change of ACEI was not similar at different temperature. With the increase of incubation time, ACEI increased at 35 °C, but at 50 °C, it increased first and then decreased. When temperature and liquid to solid ratio were fixed (Figure 5B), ACEI increased first and then decreased at pH 4.0 and showed an increased trend at pH 5.0. With the temperature ranging from 35 to 55 °C (Figure 5C,D), when incubation time and liquid to solid ratio were fixed at 6 h and 7.0, respectively, ACEI was decreased rapidly at pH 4.0, while it increased at pH 5.0 with the increase of temperature (Figure 5C). However, when the incubation time and pH were fixed at 6 h and 4.5, respectively, ACEI decreased seriously when liquid to solid was 4 and increased when liquid to solid was 10 (Figure 5D), which indicated that the interactions of X_1X_2, X_1X_3, X_2X_3, and X_2X_4 were significant ($p < 0.05$).

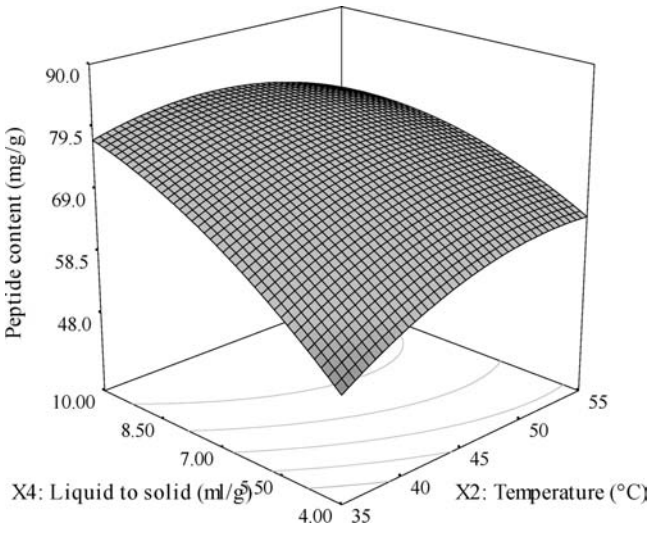

Figure 6. Response surface plots showing the interactions of variables on peptide content.

As shown in Figure 6, the interaction between temperature and liquid to solid ratio on peptide content was significant ($p < 0.05$). When the incubation time and pH were fixed at 6 h and

pH 4.5 respectively, with the temperature ranging from 35 to 55 °C, the peptide content was increased seriously when the liquid to solid ratio was 4 and increased first and then reduced when the liquid to solid ratio was 10 with the increase of temperature.

Optimum Conditions and Model Verification. According to the RSM test results, the optimal incubation conditions for ACEI and peptide accumulation were 7 h of time, 47 °C, pH 4.4, and 8.14 of liquid to solid ratio. Under the optimal conditions, the maximal ACEI and peptide content detected were 92.16% and 88.12 mg/g DW, respectively. The ACEI and peptide content, under the optimal conditions, were 6.23 times and 2.35-fold higher than that of the control (Table 4). Verification of the model eqs 3 and 4 were performed under the optimum conditions for ACEI and peptide accumulation. The observed ACEI and peptide content of wheat germ incubation solution under the optimal conditions agreed with the predicted value in the model. The experimental results proved that the models were valid.

Purification and Structure Identification. The incubated solution was purified using methods of ethanol precipitation and chromatography. As shown in Table 5, after the four steps of purification, the peptide content decreased, and ACEI was up to 80%. The IC_{50} decreased significantly, and the purity was 15.86 times as compared to that of unpurified incubated solution. It was indicated that precipitation and chromatography can efficiently purify bioactive peptide. After purification, the 12 peptides were identified from the purified sample by LC/MS/MS. Thereafter, each peptide was synthesized by the solid phase procedure peptide using FMOC protected amino acids synthesis methods (Shanghai TASH Biotechnology Co., Ltd.), and the ACE inhibitory activity was verified. The results showed that five new peptides were identified as the ACE inhibitory peptides. Within five peptides, AMY presented the highest ACE inhibitory activity, and the amino acid Trp also showed the ACE inhibitory activity. The IC_{50} values of the peptides are shown in Table 6.

Table 4. Arrangement and Results of Validation Trials

	ACEI (%)			peptide content (mg/g)		
index	control[a]	random	optional	control	random	optional
observed value	14.79	79.86	92.16	37.52	55.63	88.12
predicted value		76.34	92.24		56.55	88.46

[a] Not being incubated, wheat germ was taken as the control.

Table 5. Purification of ACE Inhibitory Peptide

purification procedure	peptide content (mg/mL)	ACEI (%)	IC_{50} (mg/mL)	purification fold[a]
crude extract	7.03	86.24	1.11	1.00
ethanol precipitation	4.36	97.09	0.61	1.82
Sephadex G-25	1.46	83.46	0.27	4.16
Sephadex G-10	0.91	88.53	0.14	8.00
DEAE-Sepharose-FF	0.99	84.56	0.07	15.86

[a] Purification fold was defined as the ratio of IC_{50} (incubated solution) to IC_{50} (purified by each step).

DISCUSSION

Many researchers have prepared bioactive peptides with the method of adding outer proteases. Udenigwea et al.[5] have prepared ACE inhibitory peptides using pepsin, ficin, trypsin, papain, thermolysin, pancreatin, and alcalase to hydrolyze flaxseed protein. The protein hydrolysates showed a concentration-dependent ACE inhibition (IC_{50}, 0.0275−0.151 mg/mL) with thermolysin hydrolysate and alcalase cationic peptide showing the most potent activity. Jia et al.[23] obtained protein hydrolysate with ACEI by hydrolyzing wheat germ protein using alcalase and studied the effect of ultrasonic power on ACE inhibitory activity of protein hydrolysate. In present study, using wheat germ as the material, incubation conditions were adjusted, and endogenous proteases from wheat germ were activated to degrade stored protein for antihypertensive peptides. As compared with the previous research, the methods developed in our study not only avoided the unsafe factors such as microbial toxin but also reduced the cost of preparing bioactive peptides.

The pH value significantly influenced the ACEI and peptide content of the incubated wheat germ according to the result in this study (Figure 3). The reason may be that the protein solubility was different at various pH values. It has been reported that the solvent pH significantly impacts the rice protein extraction yield.[24] When at pH−isoelectric point, little protein was dissolved, and the enzyme reaction was slowed down.

The degree of hydrolysis is one of the most important factors for effecting ACEI of protein hydrolysate. It has reported that the higher hydrolysis degree is, the higher ACE inhibitory activity is.[25] In this study, the initial ratio of enzyme and substrate was fixed, and the variation of enzyme and substrate concentration was implemented by regulating the liquid to solid ratio. It is obvious that the liquid to solid ratio was very important for the elevation of ACEI and peptide yield of wheat germ protein hydrolysate (Figure 4). When other factors were fixed, the ACE inhibitory activity was the same when the liquid to solid ratio was at 7 and 4. It is possible that other substances with the antihypertensive function such as γ-aminobutyric acid were generated in the process of incubation.[26]

The reaction condition of preparing ACE inhibitory peptide by adding outer proteases was usually the optimum reaction condition of the enzyme added. The E/S ratio at 0.60, pH at 9.18, temperature at 38.9 °C, and a reaction time of 8 h were the optimal conditions for whey protein hydrolysis to obtain high ACE inhibitory activity close to 92.2%, which were optimized by Guo et al.[10] using RSM. However, there is no detailed research on the reaction characteristics of the endogenous proteases. In

Table 6. Peptide Information

serial number	time (min)	sequence	prec m/z	theo MS	prec MS	b ion	y ion	IC_{50} (μM)
1	0.919	VEV	346.7	345.43	345.61	3	3	115.20
2	0.936	W	205.08	204.22	204.05			94.87
3	1.123	NPPSV	513	512.57	512.548	5	5	40.56
4	1.702	QV	246.1	245.3	245.263	2	2	26.82
5	1.839	AMY	384.5	383.49	383.65	3	3	5.86

this study, the reaction condition of wheat germ protease was investigated with RSM. The two prediction models were significant ($p < 0.05$), the R^2 values were 0.9869 and 0.9063, and the coefficients of variation (CV) were 2.94 and 5.26%, respectively, indicating that the models can predict the change of ACEI and peptide content excellently.

The amino acid sequences of five ACE inhibitory peptides were different from those derived from wheat germ[19] or other sources,[27,28] indicating that the peptides isolated in this study were new. Usually, the ACE inhibitory activity is related to the amino acids sequence of peptides. Three of the peptides in this study had Val at the C-terminal. It showed that C or N-Val was very important for an ACE inhibitory peptide. Muguruma et al.[29] isolated an ACE peptide Val-Lys-Ala-Gly-Phe from porcine myosin, and Sheih et al.[12] obtained Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe as an ACE inhibitory peptide, with an IC_{50} value of 66 μM in vitro. Moreover, it was found that the amino acid Trp presented ACE inhibitory activity. In previous study, the ACE inhibitory activity of Met was detected,[30] indicating that some amino acids also have the function of inhibiting ACE activity.

AUTHOR INFORMATION

Corresponding Author

*Tel/Fax: 86-25-84396293. E-mail: guzx@njau.edu.cn.

Funding Sources

We are grateful for the financial support from the Jiangsu Science and Technology Department under the Science and Technology Supporting Program (BE2009303).

REFERENCES

(1) Cushman, D. W.; Cheung, H. S.; Sabo, E. F.; Ondetti, M. A. Development and design of specific inhibitors of angiotensin-converting enzyme. *Am. J. Cardiol.* **1982**, *49* (6), 1390–1394.

(2) Kapel, R.; Rahhou, E.; Lecouturier, D.; Guillochon, D.; Dhulster, P. Characterization of an antihypertensive peptide from an Alfalfa white protein hydrolysate produced by a continuous enzymatic membrane reactor. *Process Biochem.* **2006**, *453* (41), 1961–1966.

(3) Kapel, R.; Chabeau, A.; Lesage, J.; Riviere, G.; Ravallec-Ple, R.; Lecouturier, D.; Wartelle, M.; Guillochon, D.; Dhulster, P. Production, in continuous enzymatic membrane reactor, of an anti-hypertensive hydrolysate from an industrial alfalfa white protein concentrate exhibiting ACE inhibitory and opioid activities. *Food Chem.* **2006**, *98*, 120–126.

(4) Hajji, M.; Masmoudi, O.; Souissi, N.; Triki, Y.; Kammoun, S.; Nasri, M. Chemical composition, angiotensin I-converting enzyme (ACE) inhibitory, antioxidant and antimicrobial activities of the essential oil from *Periploca laevigata* root barks. *Food Chem.* **2010**, *121*, 724–731.

(5) Udenigwe, C. C.; Lin, Y. S.; Hou, W. C.; Aluko, R. E. Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *J. Funct. Foods* **2009**, *1*, 199–207.

(6) Kong, Q.; Chen, F.; Wang, X.; Li, J.; Guan, B.; Lou, X. Optimization of Conditions for Enzymatic Production of ACE Inhibitory Peptides from Collagen. *Food Bioprocess Technol.* **2010**, DOI: 10.1007/s11947-009-0198-7.

(7) Miguel, M.; Alonso, M. J.; Salaices, M.; Aleixandre, A.; López-Fandiño, R. Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion. *Food Chem.* **2007**, *104*, 163–168.

(8) Miguel, M.; Alvarez, Y.; López-Fandiño, R.; Alonso, M. J.; Salaices, M. Vasodilator effects of peptides derived from egg white proteins. *Regul. Pept.* **2007**, *140*, 131–135.

(9) Hernández-Ledesma, B.; Recio, I.; Ramos, M.; Amigo, L. Preparation of ovine and caprine β-lactoglobulin hydrolysates with ACE-inhibitory activity. Identification of active peptides from caprine β-lactoglobulin hydrolysed with thermolysin. *Int. Dairy J.* **2002**, *12*, 805–812.

(10) Guo, Y.; Pan, D.; Tanokura, M. Optimisation of hydrolysis conditions for the production of the angiotensin-I converting enzyme (ACE) inhibitory peptides from whey protein using response surface methodology. *Food Chem.* **2009**, *114*, 328–333.

(11) Pan, D.; Luo, Y.; Tanokura, M. Antihypertensive peptides from skimmed milk hydrolysate digested by cell-free extract of Lactobacillus helveticus JCM1004. *Food Chem.* **2005**, *91*, 123–129.

(12) Sheih, I. C.; Fang, T. J.; Wub, T. K. Isolation and characterisation of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste. *Food Chem.* **2009**, *115*, 279–284.

(13) Raghavan, S.; Kristinsson, H. G. ACE-inhibitory activity of tilapia protein hydrolysates. *Food Chem.* **2009**, *117*, 582–588.

(14) Chiang, W. D.; Tsou, M. J.; Tsai, Z. Y.; Tsai, T. C. Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. *Food Chem.* **2006**, *98*, 725–732.

(15) Muguruma, M.; Ahhmed, A. M.; Katayama, K.; Kawahara, S.; Maruyama, M.; Nakamura, T. Identification of pro-drug type ACE inhibitory peptide sourced from porcine myosin B: Evaluation of its antihypertensive effects in vivo. *Food Chem.* **2009**, *114*, 516–522.

(16) Ruiz-Giménez, P.; Burguete, M. C.; Castelló−Ruiz, M.; Marcos, J. F.; Salom, J. B.; Vallés, S.; Torregrosa, G.; Alborch, E.; Manzanares, P. Bovine lactoferrin pepsin hydrolysate exerts inhibitory effect on angiotensin I-converting enzyme-dependent vasoconstriction. *Int. Dairy J.* **2007**, *17*, 1212–1215.

(17) Lee, S. H.; Qian, Z. J.; Kim, S. K. A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. *Food Chem.* **2010**, *118* (1), 96–102.

(18) Matsui, T.; Li, C. H.; Osajima, Y. Preparation and Characterization of Novel Bioactive Peptides Responsible for Angiotensin I-Converting Enzyme Inhibition from Wheat Germ. *J. Pept. Sci.* **1999**, *5*, 289–297.

(19) Matsui, T.; Li, C. H.; Tanaka, T.; Maki, T.; Osajima, Y.; Matsumoto, K. Depressor Effect of Wheat Germ Hydrolysate and Its novel angiotensin I-Converting Enzyme Inhibitory Peptide, LIe-Val-Tyr, and the Metabolism in Rat and Human Plasma. *Biol. Pharm. Bull.* **2000**, *23* (4), 427–431.

(20) Finn, E. S.; Pursifull, A. C.; Limardi, L. C.; Chapoval, S.; Sarlo, K.; Kirchner, B. A.; Rubingh, D. N. Complexity of the immune response to a protease and implications for the safety assessment of novel enzyme-containing products. *Toxicol. Lett.* **2003**, *144* (28), 33.

(21) Cushman, D. W.; Cheung, H. S. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* **1971**, *20*, 1637–1648.

(22) Wen, H. B.; Cao, X. H.; Gu, Z. X.; Tang, J. T.; Han, Y. B. Effects of components in the culture solution on peptides accumulation during germination of brown rice. *Eur. Food Res. Technol.* **2009**, *228*, 959–967.

(23) Jia, J.; Ma, H.; Zhao, W.; Wang, Z.; Tian, W.; Luo, L.; He, R. The use of ultrasound for enzymatic preparation of ACE-inhibitory peptides from wheat germ protein. *Food Chem.* **2010**, *119*, 336–342.

(24) Cao, X.; Li, C.; Wen, H.; Gu, Z. Extraction technique and characteristics of soluble protein in germinated brown rice. *Int. J. Food Prop.* **2010**, *13*, 810–820.

(25) Jamdar, S. N.; Rajalakshmi, V.; Pednekar, M. D.; Juan, F.; Yardi, V.; Sharma, A. Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chem.* **2010**, *121*, 178–184.

(26) Bai, Q.; Chai, M.; Gu, Z.; Cao, X.; Li, Y.; Liu, K. Effects of components in culture medium on glutamate decarboxylase activity and γ-aminobutyric acid accumulation in foxtail millet (*Setaria italica* L.) during germination. *Food Chem.* **2009**, *116*, 152–157.

(27) Rizzello, C. G.; Cassone, A.; Di Cagno, R.; Gobbetti, M. Synthesis of Angiotensin I-Converting Enzyme (ACE)-Inhibitory Peptides and γ-Aminobutyric Acid (GABA) during Sourdough

Fermentation by Selected Lactic Acid Bacteria. *J. Agric. Food Chem.* **2008**, *56*, 6936–6943.

(28) Liu, J. B.; Yu, Z. P.; Zhao, W. Z.; Lin, S. Y.; Wang, E. L.; Zhang, Y.; Hao, H.; Wang, Z. Z.; Chen, F. Isolation and identification of angiotensin-converting enzyme inhibitory peptides from egg white protein hydrolysates. *Food Chem.* **2010**, *122*, 1159–1163.

(29) Muguruma, M.; Ahhmed, A. M.; Katayama, K.; Kawahara, S.; Maruyama, M.; Nakamura, T. Identification of pro-drug type ACE inhibitory peptide sourced from porcine myosin B: Evaluation of its antihypertensive effects in vivo. *Food Chem.* **2009**, *114*, 516–522.

(30) Yust, M. M.; Pedroche, J.; Girón-Calle, J.; Alaiz, M.; Millán, F.; Vioque, J. Production of ace inhibitory peptides by digestion of chickpea legumin with alcalase. *Food Chem.* **2003**, *81*, 363–369.