American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS American Journal of Food Technology

ISSN 1557-4571
DOI: 10.3923/ajft.2016.240.252

Research Article
Optimization of Ultrasound Pretreatments and Hydrolysis Conditions for Production of Angiotensin-I Converting Enzyme (ACE) Inhibitory Peptides from Sodium Caseinate Protein Using Response Surface Methodology

[1,2]Mohammed Adam Yahya Abdualrahman, [1]Haile Ma, [1,3]Abu ElGasim Ahmed Yagoub and [1]Cunshan Zhou

[1]School of Food and Biological Engineering, Jiangsu University, 212013 Zhenjiangm, China
[2]Department of Food Technology, Nyala Technical College, Nyala, Sudan
[3]Faculty of Agriculture, University of Zalingie, P.O. Box 6, Zalingie, Sudan

Abstract

Objective: The aim of this study was to optimize the alcalse-catalyzed hydrolysis conditions of Single Frequency Counter Current Ultrasound (SFCU) pretreated sodium caseinate (NaCas). **Methodology:** Response Surface Method (RSM) was employed in order to maximize angiotensin-I converting enzyme (ACE)-inhibitory activity (Y_1) and degree of hydrolysis (DH) (Y_2). Sonication parameters were first optimized and then pretreatment of NaCas was performed. Substrate concentration (X_1), enzyme to substrate (E/S) ratio (X_2) and hydrolysis time (X_3) were selected to optimize the response variables (Y_1 and Y_2). Statistical analysis indicated that linear, interaction and quadratic terms of X_1, X_2 and X_3 had significant effects on the response variables. **Results:** The mathematical model has been developed and provided a statistically accurate prediction of Y_1 and Y_2. The optimal hydrolysis conditions for NaCas were substrate concentration of 9.90 g L^{-1}, E/S ratio of 14626 U g^{-1} and hydrolysis time of 60 min. At the optimal conditions, the experimental values for ACE inhibitory activity (83.64%) and the DH (15.75%) were closed to the predicted values. Compared to control, ultrasound pretreatment increased DH and ACE-inhibitory activity by 3.35 and 31.80%, respectively and decreased IC_{50} by 27.94%. **Conclusion:** The optimized conditions would provide important information on proteolysis and bioactive peptides of NaCas to the food industry.

Key words: Sodium caseinate, ultrasonic pretreatment, degree of hydrolysis, ACE-inhibitory activity, response surface methodology

Received: May 14, 2016 Accepted: August 30, 2016 Published: October 15, 2016

Citation: Mohammed Adam Yahya Abdualrahman, Haile Ma, Abu ElGasim Ahmed Yagoub and Cunshan Zhou, 2016. Optimization of ultrasound pretreatments and hydrolysis conditions for production of angiotensin-I converting enzyme (ACE) inhibitory peptides from sodium caseinate protein using response surface methodology. Am. J. Food Technol., 11: 240-252.

Corresponding Author: Haile Ma, School of Food and Biological Engineering, Jiangsu University, 212013 Zhenjiang, China Tel/Fax: +86 511 88790958

Copyright: © 2016 Mohammed Adam Yahya Abdualrahman *et al.* This is an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sodium caseinate (NaCas) is widely used in food industry, as a functional ingredient. The NaCas is used as an emulsifier in a wide range of food applications, such as coffee creamers, infant formulae and soups[1]. Milk proteins potentiality to functioning as inhibitors to angiotensin converting enzyme has been reported in many studies[2-5].

Angiotensin Converting Enzyme (ACE) is a peptidyl di-peptidase enzyme having the capacity to cleave the carboxyl terminal end of the substrate that may regulate an increase in blood pressure by converting angiotensin I to an active peptide hormone angiotensin II. This stimulates the release of aldosterone, thus increasing blood sodium concentration and subsequent high blood pressure[6]. The effect of ACE on blood pressure makes it an attractive target for clinical nutritional intervention in hypertension[7]. The ACE is prepared through enzymolysis processes, which improve the functional properties of the original food without compromising its nutritive value as well as intestinal absorption characteristics. Traditional enzymolysis has many disadvantages such as low enzyme utilization rate, low conversion rate of substrate and long enzymolysis time[8,9]. Therefore, the development of more efficient enzymolysis techniques to overcome these disadvantages is of great demand. Moreover, many researchers have reported on the usage of ultrasound pretreatment to improve the enzymolysis of protein substrates[10-12]. Ultrasound pretreatment has been widely applied in resolving traditional enzymolysis challenges.

Ultrasound frequency is mainly classified into high frequency low-energy ultrasound and low frequency high-energy power ultrasound. The high frequency ultrasound is used as an analytical technique for quality assurance and process control in the food industry[13]. The ultrasonic frequency also influences the yield and intensity of cavitation in liquids. It has been reported that the ultrasound cavitation yield can be enhanced by multi-frequency sonication[9]. The power ultrasound is widely used in protein enzymolysis to produce bioactive peptides[12,14] and to improve the functional properties of the proteins substrate[9,15].

Single Frequency Countercurrent Ultrasound (SFCU) has many advantages such as releasing of uniform energy distribution, avoids the energy waste and generated smaller thermal effect in a pulsed mode with an on-time and off-time cycle[16]. Compared to traditional high energy ultrasound, the countercurrent flow ultrasound affords continuous solution flow with more effective treatment[16]. Response Surface Methodology (RSM) is used to determine the effects of hydrolysis conditions in different protein hydrolysates, as well as optimization process[17-19]. To date, little is known about the effects of ultrasound pretreatment on the enzymolysis of NaCas protein and releasing of ACE-inhibitory peptides. Therefore, the aims of this study is to (1) Investigate on the effects of ultrasound pretreatment parameters of frequency, power density, time and temperature on production of NaCas protein hydrolysates with ACE inhibitory activity and (2) Optimize the hydrolysis conditions (substrate concentration, enzyme to substrate (E/S) ratio and hydrolysis time) by RSM in terms of maximizing release of ACE inhibitory peptides. It is also hoped that our results would provide good information for developing the ultrasound pretreatment technique in the field of proteolysis industry.

MATERIALS AND METHODS

Materials: Sodium caseinate with 90% protein[20] was purchased from Henan Bokang Bio-technology, Ltd., China. Alcalase 2.4 L with an activity of 240,000 U g^{-1} (Folin Phenol method[21] was purchased from Novozymes Co. Ltd., Tianjin, China. The ACE was extracted from a pig lung[22]. Hippuryl-L-histiyl-L-leucine (Hipp-His-Leu) was purchased from Sigma Chemicals Co. Ltd. (St., Louis, MO., USA). All other chemicals and reagents were of analytical grade.

Single Frequency Countercurrent Ultrasound (SFCU) pretreatment: The SFCU pretreatment of NaCas was done in triplicate as described by Ma *et al.*[19]. The NaCas suspensions with different concentrations (5-25 g L^{-1}) were prepared in distilled water. The suspensions were sonicated in a SFCU reactor (Shangjia Biotechnology Co., Wuxi, Jiangsu, China) equipped with a probe of 2.0 cm flat tip (GA92-II DB, Shangjia Biotechnology Co., Wuxi, China). The probe was submerged to a depth of 2.0 cm in the suspension at different temperatures. The suspension was circulated with aid of a pump working at speed of 100 rpm. The pulsed on time and off time were 3 and 2 sec, respectively. Different experiments were performed in terms of optimizing sonication parameters. At first, sonication experiments with different frequencies (0, 20, 28, 35, 40 and 50 kHz) and fixed temperature of 45°C, power density of 450 W L^{-1} and time of 5 min were performed. Secondly, ultrasound power densities (0, 350, 400, 450 and 500 W L^{-1}) were examined at 45°C and 28 kHz for 5 min. A third group of sonication experiments was carried out at different temperatures (0, 35, 40, 45, 50 and 55°C), 28 kHz, 450 W L^{-1} and 5 min. Finally, different ultrasound times (0, 3, 5, 7, 9 and 12 min) were investigated at 28 kHz, 45°C and 450 W L^{-1}.

Enzymolysis of NaCas protein: The enzymatic hydrolysis was measured in triplicate as described by Dadzie *et al*.[18]. The enzymolysis apparatus consisted of digital thermostat water bath (DK-S26, JingHong experimental apparatus Co., Shanghai, China), pH-meter (PHS-3C Precision PH/mV Meter, LIDA Instrument, China) and an impeller-agitator (JJ-1, Zhong Da instrument Co., Jiangsu, China) operating at a speed of 100 rpm. Untreated and ultrasound pretreated NaCas solutions were pre-heated at 55°C for 15 min. The pH of each solution was adjusted to 8.5 with 1 M NaOH and then alcalase 2.4 L was added to initiate the hydrolysis reaction. To evaluate the effects of ultrasound frequency, power density, temperature and time on DH and ACE inhibitory peptides, the hydrolysis conditions were set as follows: substrate concentration of 10 g L^{-1}, enzyme/substrate (E/S) ratio of 14400 U g^{-1}, hydrolysis time of 60 min and temperature of 55°C. The pH of the reaction mixture was monitored by continuous addition of 1 M NaOH. The volume of 1 M NaOH needed to keep the pH constant was recorded every 5 min. At the end of hydrolysis, the reaction was stopped by boiling the mixture for 15 min. The hydrolysate was centrifuged (TGL-16, High Speed Tabletop Refrigerated Centrifuge, China) at 10000×g for 10 min at 4°C. The supernatant was stored at 4°C for further analysis.

Single factor hydrolysis experiments: Single-factor experiments of ultrasound pretreated NaCas were conducted to identify the outstanding independent variables in terms of maximizing the response products (DH and ACE-inhibitory peptides). Ultrasound pretreatment of NaCas was performed at frequency of 28 kHz, power density of 450 W L^{-1} and temperature of 45°C and time of 5 min and then hydrolyzed with alcalase. For all experiments, the hydrolysis temperature and pH were fixed at 55°C and 8.5, respectively. In order to evaluate the effect of substrate concentration on ACE-inhibitory activity, E/S ratio of 14400 U g^{-1}, hydrolysis time of 60 min and substrate concentrations ranged between 5 and 25 g L^{-1}, respectively were employed. Another hydrolysis experiment with different E/S ratio (9600, 12000, 14400, 16800 and 19200 U g^{-1} protein), substrate concentration of 10 g L^{-1} and time of 60 min, was conducted. A third experiment with different hydrolysis times (30-150 min), substrate concentration of 10 g L^{-1} and E/S ratio of 14400 U g^{-1} was also done.

Response Surface Methodology (RSM) analysis: The RSM (Box-Behnken) was used to optimize the hydrolysis conditions (interactive variables) of ultrasound pretreated NaCas in order to produce peptides with high ACE-inhibitory activity. For the experimental design, substrate concentration (X_1), E/S ratio (X_2) and hydrolysis time (X_3) were selected as independents variables. The range and central points of independents variables based on the results of single factor tests are displayed in Table 1. All experiments were done in triplicate and the DH and ACE-inhibitory activity were referred as response products (Y_1 and Y_2, respectively). The coded values of the independents variables and the corresponding values of the response products are presented in Table 2. Design-Expert 8 (Stat-Ease, Minneapolis, MN, USA) software was applied to perform the regression analysis for the experimental results and fitted into the empirical second-order polynomial model as follows in Eq. 1:

$$Y_i = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{3}\beta_{ij} X_j \quad (1)$$

where, Y_i is the predicted response variable, β_0, β_i, β_{ii} and β_{ij} are regression coefficients estimated by the model and X_i and X_j are levels of independent variables.

Table 1: Independents variables and their levels used for response surface box-behnken design

		Levels		
Factors	Coded symbol	-1	0	1
Substrate concentration (g L^{-1})	X_1	5	10	15
Enzyme to substrate (E/S) ratio (U g^{-1} protein)	X_2	12000	14400	16800
Hydrolysis time (min)	X_3	30	60	90

Table 2: Box-Behnken arraignment for substrate concentration (X_1), enzyme to substrate (E/S) ratio (X_2) and hydrolysis time (X_3) and their responses; degree of hydrolysis (Y_1) and ACE-inhibitory activity (Y_2)

	Coded levels of independent variables			Responses (%)	
Run order	X_1	X_2	X_3	Y_1	Y_2
1	0	1	-1	14.74	59.27
2	0	-1	1	14.48	55.13
3	1	-1	0	13.04	54.51
4	0	0	0	15.75	84.49
5	-1	0	-1	15.50	50.97
6	-1	1	0	18.04	53.98
7	-1	-1	0	14.72	52.87
8	1	0	1	14.76	66.85
9	0	0	0	15.75	86.92
10	0	0	0	16.01	84.49
11	0	0	0	15.75	86.92
12	-1	0	1	16.77	55.81
13	0	0	0	16.01	85.71
14	1	1	0	15.08	71.72
15	0	-1	-1	12.23	56.49
16	0	1	1	16.77	72.55
17	1	0	-1	13.27	55.48

Degree of hydrolysis (DH): The DH was calculate according to the pH-stat method[23] given in Eq. 2:

$$DH\ (\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times N_b}{\alpha M_p h_{tot}} \times 100 \quad (2)$$

where, B is the volume of NaOH consumed during hydrolysis (mL), N_b is the concentration of NaOH (mol L^{-1}), M_p is the mass of sodium caseinate protein to be hydrolyzed (g), h_{tot} is the total millimoles of peptide bonds per gram of protein substrate (which is 8.20 meq g^{-1} for casein protein) and α is the average degree of dissociation of the α-amino groups released with the pK of the amino groups at particular pH and temperature, which is 0.96 at pH 8.5 and 55°C.

Measurement of ACE-inhibitory peptides: The ACE-inhibitory peptides was measured using HPLC (Shimadzu Inc., Japan) as describe by Dadzie *et al.*[18]. In brief, 10 µL of NaCas supernatant (NaCas in 0.1 M sodium borate buffer, pH 8.3 containing 0.3 M NaCl) were mixed with 25 µL of ACE (ACE in 0.1 M sodium borate buffers, pH 8.3 containing 0.3 M NaCl) and pre-incubated at 37°C for 10 min. The reaction was initiated by adding 40 µL of Hipp-His-Leu (6.5 mM HHL in 0.1 M sodium borate buffer, pH 8.3 containing 0.3 M NaCl) and the reaction was conducted at 37°C for 30 min. The reaction was stopped by adding 85 µL of 1 M HCl to the solution. The reaction mixture obtained was used in the determination of liberated Hippuric Acid (HA) resulting from ACE activity on the substrate. The free HA was separated and quantified by HPLC at 228 nm with a UV-detector. A control was prepared by substituting supernatant with distilled water. The ACE-inhibitory activity was calculated as follows in Eq. 3:

$$ACE\ (\%) = \frac{HA_{control} - HA_{sample}}{HA_{control}} \times 100 \quad (3)$$

IC_{50} of NaCas hydrolysates: The IC_{50} value is defined as the concentration of NaCas hydrolysates that able to inhibit 50% of the original ACE-inhibitory activity. Hydrolysates prepared from different concentrations of NaCas solutions (0.46-1.4 g L^{-1}) were evaluated for their ACE-inhibitory activity. The IC_{50} (expressed as µg mL^{-1}) was determined in triplicate by plotting the different concentrations of the hydrolysate against their ACE-inhibitory activity (%)[24].

Statistical analysis: All analyses were performed in triplicate. Experimental results were expressed as Mean±Standard deviation. Analysis of variance (ANOVA) was used to compare the significance level at ($p<0.05$). All graphs and calculations were done with Design-Expert 8.00 (Stat Ease, Minneapolis, MN, USA) software.

RESULTS AND DISCUSSION

Effects of ultrasound pretreatment on DH and ACE-inhibitory activity of hydrolysates: The effects of ultrasound pretreatment on the DH and ACE inhibitory activity of NaCas hydrolysate are presented in Fig. 1. Results show that ultrasound pretreatments at all frequency levels (20-50 kHz) increase significantly ($p<0.05$) the ACE inhibitory activity of NaCas hydrolysates compared with control (0 kHz). The ACE inhibitory activity increase steadily ($p<0.05$) with the increase of frequency from 20-28 kHz and thereafter it decreases (Fig. 1a). The maximum ACE-inhibitory activity is achieved at 28 kHz, in which the activity increased by 35.04% compared to untreated hydrolysate. These results agree with some previous studies[14,19]. Ultrasound power density follows similar trends as frequency did, whereas a maximum ACE-inhibitory activity is found at ultrasound power density of 450 W L^{-1} (Fig. 1b). Associated finding has been reported[12,25] in some sonicated proteins. The decrease in ACE-inhibitory activity after power density of 450 W L^{-1} indicates that the hydrophobic amino acids might be accumulated to form a more stable protein structure[26]. These results agree with the findings of previous studies[27,28]. Figure 1c shows NaCas protein pretreated at different sonication times (3-12 min) result in hydrolysates with ACE-inhibitory activity significantly ($p<0.05$) higher than the control. The ACE-inhibitory activity increase significantly ($p<0.05$) with the increase of sonication time, reaching its maxima at 5 min and thereafter it decrease (i.e., 7-12 min). Comparable results have been reported for ultrasound pretreated defatted wheat germ protein[12] and corn gluten meal protein[29]. On the other hand, the highest ACE-inhibitory activity is obtained at ultrasound temperature 45°C. After 45°C, the activity decreased steadily with the increasing temperature (Fig. 1d). Nevertheless, Fig. 1a-d shows that ultrasound pretreatment exerts similar influences on DH of NaCas, which means that the studied conditions have similar effects on the breakage of NaCas peptide bonds. The increase in ACE inhibitory activity may be attributed to the increase of surface hydrophobicity and the change in protein structure of NaCas, which facilitate the release of hydrophobic amino acid during enzymatic hydrolysis[25]. Amino acid C-terminal hydrophobicity has the greatest influence on ACE inhibitory activity and the higher the hydrophobicity the higher the inhibitory activity[30,31]. Thus, ultrasound pretreatment can improve the release of ACE inhibitory of peptides from NaCas.

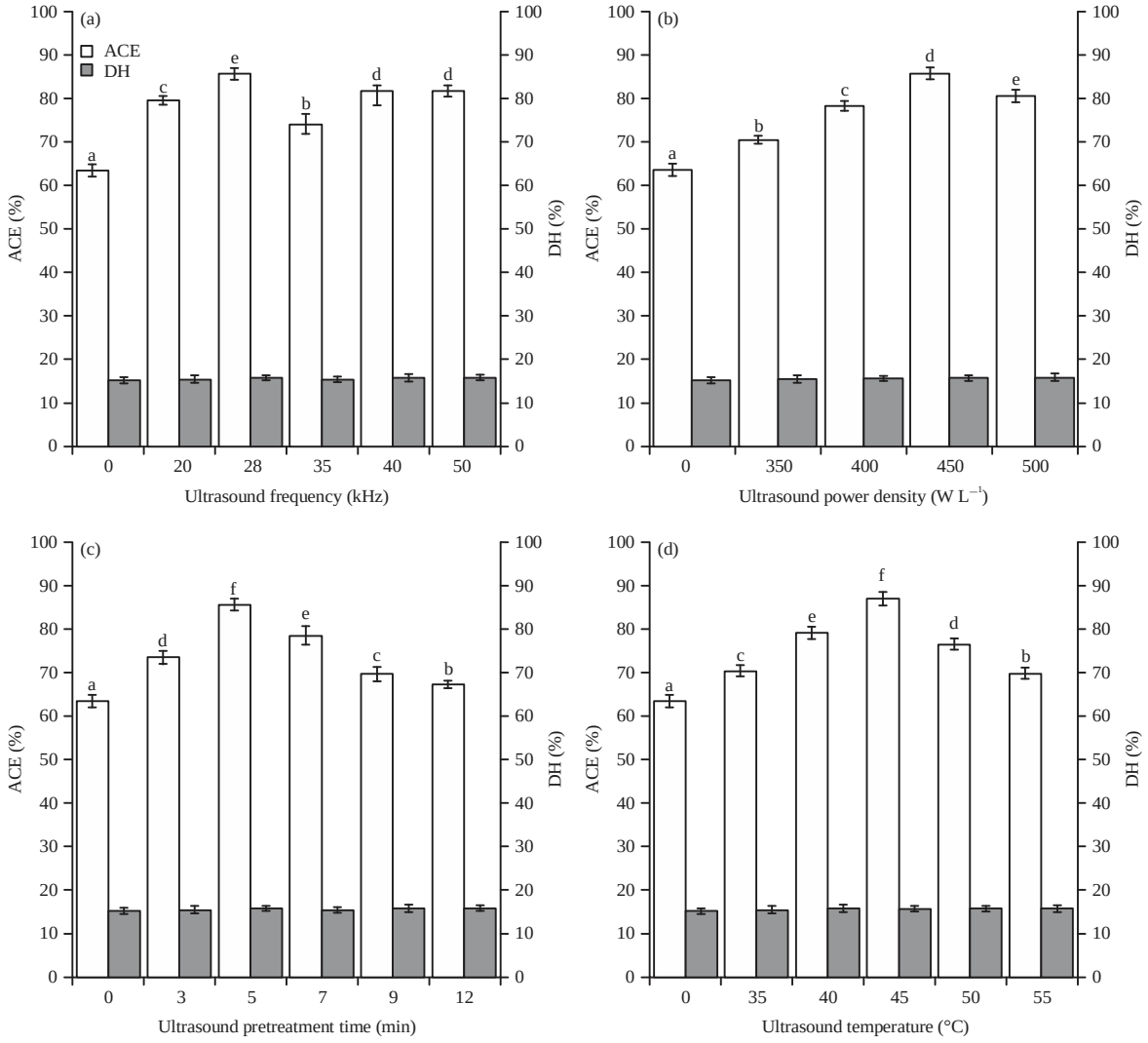

Fig. 1(a-d): Effect of ultrasound (a) Frequency, (b) Power density, (c) Pretreatment time and (d) Temperature on the degree of hydrolysis (DH) and ACE-inhibitory activity of NaCas hydrolysates

In conclusion, ultrasound pretreatment appears to be a useful tool to accelerate the enzymolysis of NaCas and releasing of bioactive peptides.

Effects of substrate concentration, E/S ratio and hydrolysis time on DH and ACE-inhibitory activity of hydrolysates: To study the effect of different substrate concentrations (5-25 g L^{-1}) on DH and ACE-inhibitory activity, the SFCU pretreated NaCas was prepared first at optimum processing conditions (28 kHz, 450 W L^{-1}, 45°C and 5 min) and then hydrolyzed by alcalase 2.4 L at E/S ratio of 14400 U g^{-1} for 60 min. Figure 2a shows the ACE-inhibitory activity increases ($p<0.5$) with the increase of substrate concentration from 5-10 g L^{-1} and beyond that it decreases. In contrast, the DH decreases ($p<0.5$) with the increase of substrate concentration. It is obvious that the highest values for the ACE-inhibitory activity and DH are obtained at substrate concentrations of 10 and 5 g L^{-1}, respectively. Hence, 10 g L^{-1} was selected as center point (Table 1). Similarly, previous studies reported a decrease in DH with increasing of substrate concentration[18,32]. The decrease in DH at higher substrate concentration than the optimal, which is attributed to an inhibitory effect on enzyme activity[33,34] could inhibit the release of ACE inhibitory peptides. Consequently, the ACE inhibitory activity decrease with the increase of substrate concentration. Similar findings has been reported earlier[12,18].

The effect of different E/S ratios (9600-19200 U g^{-1}) on the DH and ACE-inhibitory activity of SFCU pretreated NaCas was investigated at substrate concentration of 10 g L^{-1} and time of 60 min and results are displayed in Fig. 2b. It can be noticed

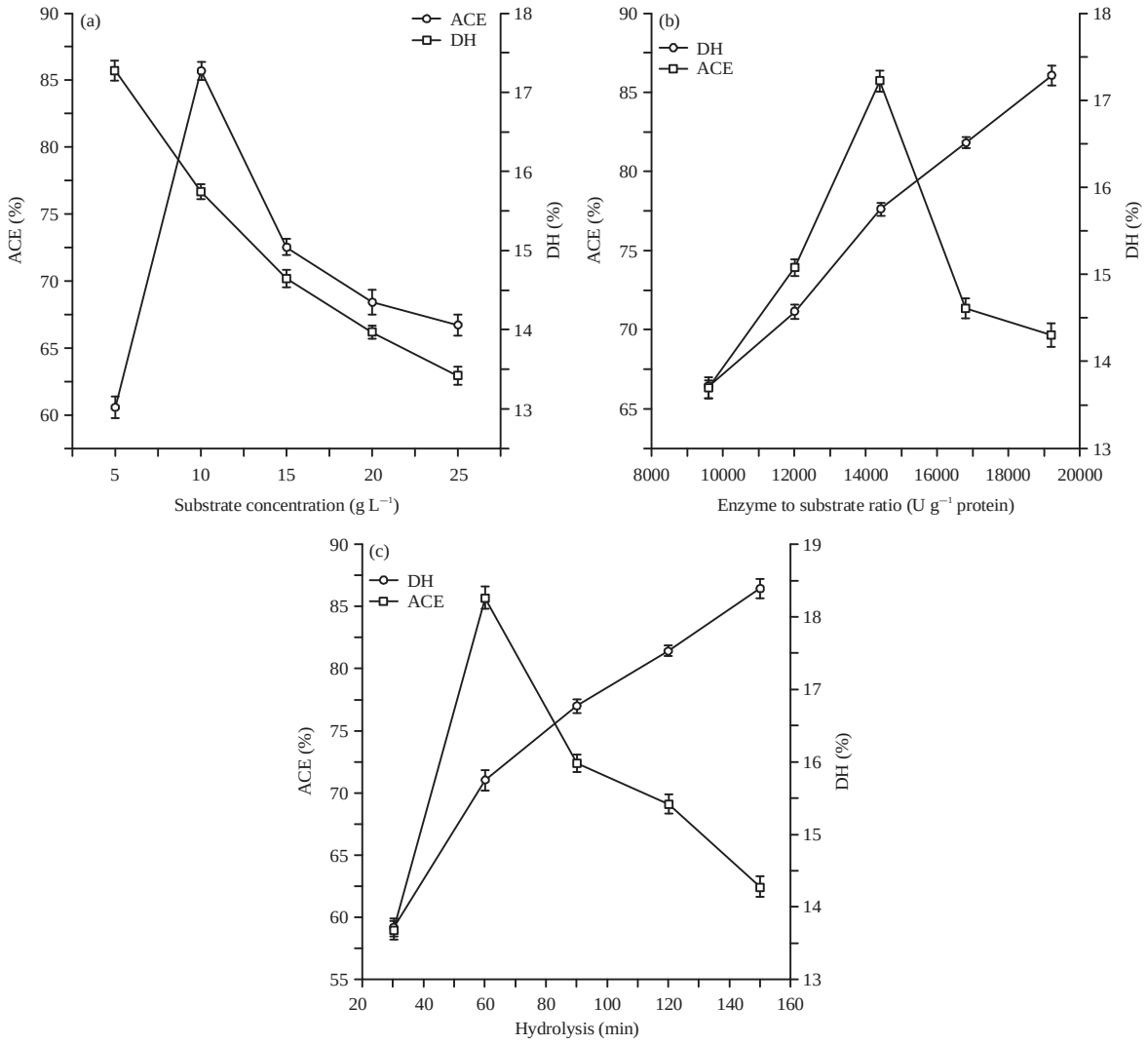

Fig. 2(a-c): Effect of (a) Substrate concentration, (b) E/S ratio and (c) Hydrolysis time on the DH and ACE-inhibitory activity of NaCas hydrolysates

that the DH increases steadily ($p<0.5$) with the increase of E/S ratio. This increasing trend in DH agrees with a previous study[35]. In contrast, the ACE inhibitory activity of the hydrolysates increases gradually with the increase of E/S, reaching its maximal value at 14400 U g^{-1} (85.71%) and then decreases with further increase in the E/S ratio. This increment is supposed arising from the exposure of more active sites on the protein molecule due to sonication and hence providing suitable conditions for more enzyme attack[18]. Similar behavior of the ACE inhibitory activity in relation to the E/S has been reported in the literature[18]. Accordingly, the E/S ratio of 14400 U g^{-1} is chosen as a centre point (Table 1).

The effect of hydrolysis time from 30-150 min on the DH and ACE-inhibitory activity of the ultrasound pretreated NaCas was investigated and results are shown in Fig. 2c. It is clear that the DH increases ($p<0.05$) sharply at the first 30 min of hydrolysis and then continues the increase, but with slow rate, to the end of hydrolysis time. On the other hand, the ACE inhibitory activity increases sharply after 30 min of hydrolysis, reaching its maxima at 60 min and thereafter the ACE inhibitory activity decreases as the hydrolysis time is lengthened. Hence, 60 min is chosen as a center point (Table 1). The decrease of ACE inhibitory activity after 60 min might be due to the higher degradation of bioactive peptide than formation of new peptides[12,35].

Fitting the response surface model: To optimize the independent variables in terms to maximize the release of ACE inhibitory activity RSM box-behnken design is employed to investigate the effects of substrate concentrations, E/S ratio

and hydrolysis time on the DH and ACE-inhibitory activity of NaCas hydrolysis. Results from the response surface experiments are listed in Table 2. The regression coefficient of linear, quadratic and interaction terms of DH (Eq. 4) and ACE inhibitory activity (Eq. 5) are used to fit a response surface model. The response products and the independent variables are empirically related by the following second-order polynomial given in Eq. 4 and 5:

$$Y_1 = 15.85-1.11\ X_1+1.27\ X_2+0.88\ X_3-0.32\ X_1X_2+ 0.055X_1X_3-0.055\ X_2X_3-0.057\ X_1^2-0.58\ X_2^2-0.72\ X_3^2 \quad (4)$$

$$Y_2 = 85.71+4.37\ X_1+4.82\ X_2+3.52\ X_3+4.02\ X_1X_2+ 1.63X_1X_3+3.66\ X_2X_3-15.51\ X_1^2-11.93\ X_2^2-12.92\ X_3^2 \quad (5)$$

where, Y is the response value of DH or ACE inhibitory activity and X_1, X_2 and X_3 are the coded variables for substrate concentration, E/S ratio and hydrolysis time, respectively.

The statistical significance of Eq. 4 and 5 is checked by F-test and the results of analysis of variance (ANOVA) are displayed in Table 3 and 4 for DH and ACE inhibitory activity, respectively. The fitness of the models was evaluated through the coefficients of determination (R^2), probability values (p) and lack of-fit. The estimated regression coefficients of the polynomial response surface model with the corresponding R^2 values and lack of fit are also listed in Table 3 and 4. The F-values of 60.27 and 233.60 for the DH and ACE inhibitory activity, respectively and the very low p-value ($p<0.0001$) imply that the model fitness is highly significant. For DH and

Table 3: ANOVA analysis for the degree of hydrolysis (DH) of SFCU pretreated NaCas

Source	DF	Coefficient	SS	MS	F-value	p-value	Significance
Model	9	15.85	33.25	3.69	60.27	<0.0001**	Significant
X_1	1	-1.11	-	-	160.79	<0.0001**	
X_2	1	1.27	-	-	210.48	<0.0001**	
X_3	1	0.88	-	-	101.06	<0.0001**	
X_1X_2	1	-0.32	-	-	6.68	0.0362*	
X_1X_3	1	0.055	-	-	0.20	0.6703*	
X_2X_3	1	-0.055	-		0.20	0.6703*	
X_1^2	1	-0.057	-	-	0.22	0.6510*	
X_2^2	1	-0.58	-	-	22.87	0.0020*	
X_3^2	1	-0.72	-	-	35.80	0.0006*	
Residual	7		0.43	0.061			
Lack of fit	3		0.35	0.12	5.72	0.0626	Not significant
Pure error	4		0.081	0.020			
Total	16		33.68				
R^2			0.9873				
Adj-R^2			0.9709				
Pred-R^2			0.8309				
Adeq precision			29.332				
CV (%)			1.63				

**Significant within a 99% confidence interval, *Significant within a 95% confidence interval

Table 4: ANOVA analysis for the ACE-inhibitory activity of SFCU pretreated NaCas

Source	DF	Coefficient	SS	MS	F-value	p-value	Significance
Model	9	85.71	3147.90	349.77	233.60	<0.0001**	Significant
X_1	1	4.37	-	-	101.86	0.0001**	
X_2	1	4.82	-	-	123.88	0.0001**	
X_3	1	3.52	-	-	66.06	0.0001**	
X_1X_2	1	4.02	-	-	43.28	0.0003**	
X_1X_3	1	1.63	-	-	7.12	0.0321*	
X_2X_3	1	3.66	-	-	35.79	0.0006**	
X_1^2	1	-15.51	-	-	676.43	0.0001**	
X_2^2	1	-11.93	-	-	400.02	0.0001**	
X_3^2	1	-12.92	-	-	469.37	0.0001**	
Residual	7		10.48	1.50			
Lack of fit	3		4.58	1.53	1.03	0.4677	Not significant
Pure error	4		5.90	1.48			
Total	16		3158.38				
R^2			0.9967				
Adj-R^2			0.9924				
Pred-R^2			0.9739				
Adeq precision			36.952				
CV (%)			1.83				

**Significant within a 99% confidence interval, *Significant within a 95% confidence interval

ACE inhibitory activity, the lack of fit is not significant (Prob>F, 0.0626 and 0.4677, respectively). This indicates that the model equations are adequate for predicting the values of DH and ACE inhibitory activity under any combination of the independent variables. Therefore, the lack of fit is used to assess the reliability of the equation[36]. However, model terms coefficients of X_1, X_2 and X_3 (Table 3, 4) are highly significant ($p<0.0001$). The coefficients of interaction and the quadratic terms of the model indicate the significant effects on DH ($p<0.05$) and ACE inhibitory activity ($p<0.0001$). Coefficient of determination (R^2) is defined as the ratio of the explained variation to the total variation; it measures the fitness[37]. The model can fit well with the actual data when R^2 approaches unity[33]. It has been reported that the regression model is well defined, if R^2 value is higher than 0.80. A small value of R^2 indicates a poor relevance of the response in the model[38,39]. In this study, R^2 values for DH and ACE are 0.9873 and 0.9967, respectively, indicating the reasonable fit of the model to the experimental data. A large value of R^2 does not always imply that the model is good. For a good statistical model, Adj-R^2 should be close to R^2. In this model, Adj-R^2 values for DH and ACE inhibitory activity are 0.9709 and 0.9924, implying that the model does not explain only 1-3.0% of the total variations. The Adeq precision is used to measure the signal to noise ratio and that the ratio greater than 4 is desirable[39,40]. In this study, the Adeq precision ratios for the DH and ACE inhibitory activity models are 29.332 and 36.952, respectively, indicating an adequate signal-to-noise ratio. The coefficient of variation (CV) is an indication of the degree of precision to which the treatments are compared and is a good index of the reliability of the experiment[41]. The CV values for DH and ACE inhibitory activity are 1.63 and 1.83%, respectively, indicating a good accuracy of the model.

Response surface and contour plots: Three-dimensional response surface graphs and their corresponding contour plots are drawn to illustrate the interactive effects of independent variables on DH and ACE inhibitory activity (Fig. 3, 4). In general, an increase in DH is observed with interactions between the substrate concentration and E/S ratio, substrate concentration and hydrolysis time and E/S ratio and hydrolysis time (Fig. 3). The interactive effect between substrate concentration (X_1) and E/S ratio (X_2) on the DH is illustrated in Fig. 3a and b. The elliptical shape of contour plot in Fig. 3b illustrates the mutual interactions of substrate concentration and E/S ratio. The DH decreases with the increase of substrate concentration and increases with the increase in E/S ratio to a limited extent and then dropping down (Fig. 3a, b). This behavior might be attributed either to saturation of enzyme with the substrate, or to enzyme inhibition by the formed peptides[18,42]. However, NaCas concentration of 9.90 g L^{-1} and E/S ratio of 14626 U g^{-1} have interactive effects that result in the maximal DH. The interactive behavior of substrate concentration (X_1) and hydrolysis time (X_3) on the DH (Fig. 3c, d) follows similar trends as X_1X_2. Whereas, the predicted values for X_1 and X_3 that maximizes the DH are 9.90 g L^{-1} and 60 min, respectively. Moreover, DH also decreased with association of interactions between substrate concentration and hydrolysis time. For the combined effect of E/S ratio (X_2) and hydrolysis time (X_3), the slight decrease in DH could be due to a progressive loss of catalytic activity due to enzyme denaturation with lengthening of the hydrolysis time (Fig. 3e, f). The enzymatic hydrolysis of NaCas at different time agrees with previous reports in the literature[12,19]. As displayed in Fig. 4, the response surface plots generated for the interactive effects of X_1, X_2 and X_3 on the ACE inhibitory activity show convex shapes, indicating presence of well defined optimum conditions[43]. In Fig. 1a-c, the ACE inhibitory activity increases with the increase of substrate concentration from 5-9.9 g L^{-1} and thereafter decreases. At the same time the ACE inhibitory activity increases with the increase in E/S to 14626 U g^{-1} and then decreases. Similar interactive effects is found on ACE inhibitory activity for X_1X_3 (Fig. 4b) X_2X_3 (Fig. 4c).

Optimization of enzymatic hydrolysis conditions: According to RSM analysis, the substrate concentration of 9.90 g L^{-1}, E/S ratio of 14626 U g^{-1} and hydrolysis time of 60 min are determined as the optimal hydrolysis conditions for maximal ACE inhibitory activity. Under the optimum conditions, the predicted values for ACE-inhibitory activity and DH are 85.71 and 15.85%, respectively. In order to confirm the validity of the model, three experiments were run under the optimum conditions. The experimental ACE-inhibitory activity and the DH are 83.64 and 15.75%, respectively, which are closed to the predicted values (Table 5). These result is in-line with previous

Table 5: Effect of ultrasound pretreatment on DH, ACE-inhibitory peptides and IC_{50} of NaCas hydrolysates

Parameters	DH (%)	ACE inhibitory activity (%)	IC_{50} (μg mL^{-1})
Control	15.24±0.04[a]	63.46±0.51[a]	0.68±0.31[a]
SFCU-pretreated	15.75%±0.03[b]	83.64±1.20[b]	0.49±0.14[b]
Level of change (%)	+3.35	+31.80	-27.94

Mean±SD, (n = 3). Within the column, means with different superscript letters are significantly different ($p<0.05$). Ultrasound conditions: Frequency of 28 kHz, power density of 450 W L^{-1}, temperature of the solution at 45°C, pulsed on-time 3 sec and off time 2 sec, pretreatment time 5 min and circulation pump speed of 100 rpm. Hydrolysis conditions: E/S ratio of 14626 U g^{-1}, substrate concentration of 9.90 g L^{-1} and reaction time of 60 min

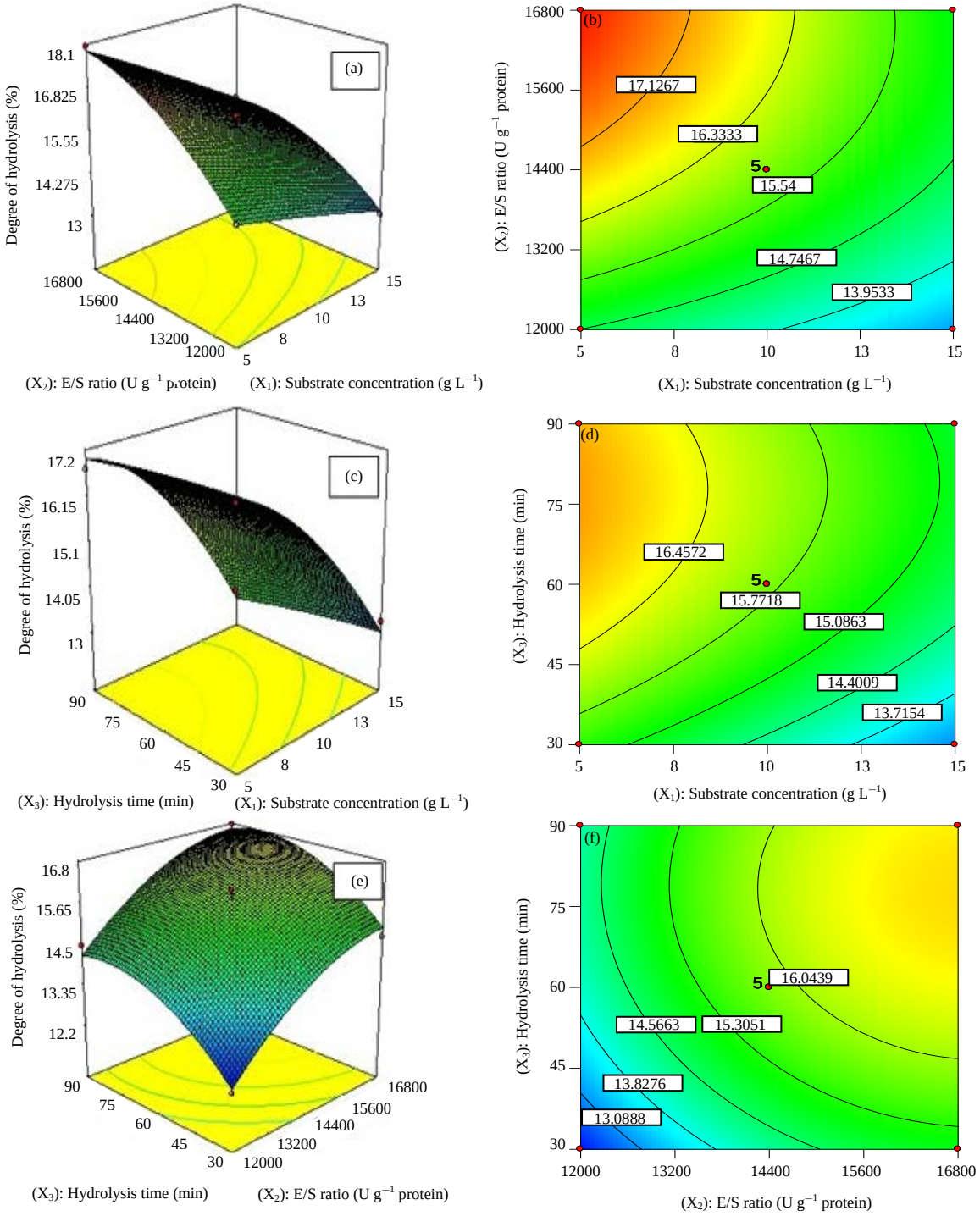

Fig. 3(a-f): Response surface plots (a, c and e) and contour plots (b, d and f) for the interactive effects of the substrate concentrations (X_1), E/S ratio (X_2) and hydrolysis time (X_3) on the degree of hydrolysis (DH%)

studies of response surface methodology[17-19]. Moreover, SFCU pretreatment significantly ($p<0.05$) improved the enzymolysis of NaCas and release more ACE-inhibitory peptides compared to control (Table 5). Similar results have been reported for ultrasound pretreated casein protein[27], milk protein concentrate[2], oat-isolated protein[28] and wheat gluten[44]. Table 5 shows the improvement in ACE-inhibitory activity after sonication is probably resulting from the

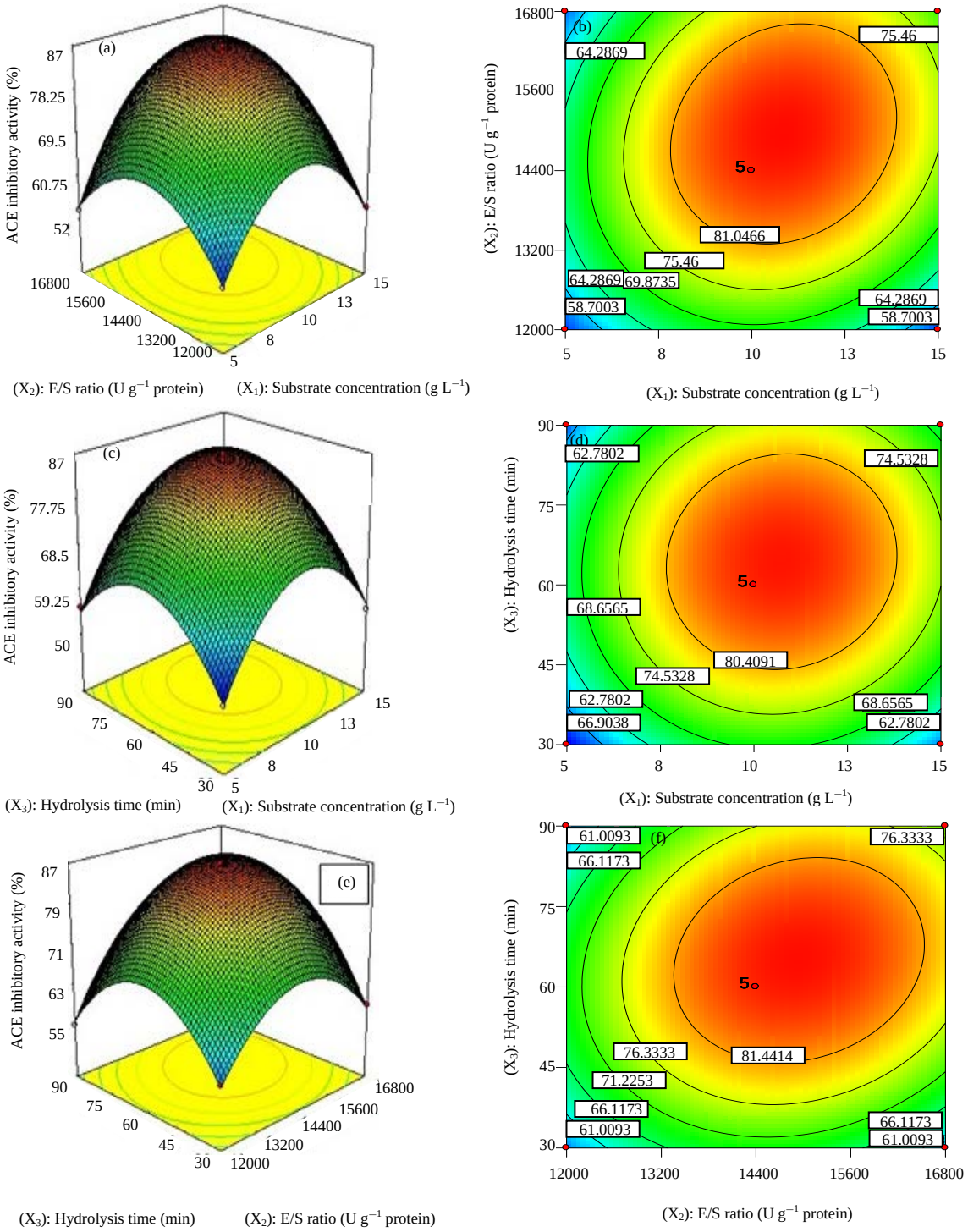

Fig. 4(a-f): Response surface plots (a, c and e) and contour plots (b, d and f) for the interactive effects of the substrate concentrations (X_1), E/S ratio (X_2) and hydrolysis time (X_3) on the ACE inhibitory activity (%)

improvement in DH. Jin *et al.*[45] have attributed the improvement in enzymatic hydrolysis to the mechanical effect induced by power ultrasound, breaking the starch-protein cross-linkage, releasing more protein molecules into solution and hence becoming more accessible to enzymes.

CONCLUSION

In conclusion, RSM was successfully employed to optimize the hydrolysis parameters of SFCU pretreated NaCas protein in terms to maximize release of ACE inhibitory peptides. The developed model could adequately represent the real relationship among the parameters chosen. The optimum extraction conditions were obtained (substrate concentration of 9.90 g L^{-1}, E/S ratio of 14626 U g^{-1} and hydrolysis time of 60 min) and the experimental values for ACE-inhibitory activity and DH were in close agreement with the predicted values. The SFCU pretreatment of NaCas improved ACE-inhibitory activity and DH over the control.

ACKNOWLEDGMENTS

The authors wish to extend their appreciation to the National Nature Science Foundation of China (No. 31471698) and the National Publicsector Special Science and Technology (No. 201303071) for their financial support toward this study.

REFERENCES

1. O'Sullivan, J., M. Arellano, R. Pichot and I. Norton, 2014. The effect of ultrasound treatment on the structural, physical and emulsifying properties of dairy proteins. Food Hydrocolloids, 42: 386-396.
2. Uluko, H., S. Zhang, L. Liu, J. Chen and Y. Sun *et al*., 2013. Effects of microwave and ultrasound pretreatments on enzymolysis of milk protein concentrate with different enzymes. Int. J. Food Sci. Technol., 48: 2250-2257.
3. Uluko, H., L. Liu, H. Li, W. Cui and S. Zhang *et al*., 2014. Effect of power ultrasound pretreatment on peptidic profiles and angiotensin converting enzyme inhibition of milk protein concentrate hydrolysates. J. Sci. Food Agric., 94: 2420-2428.
4. Uluko, H., S. Zhang, L. Liu, H. Li and W. Cui *et al*., 2014. Pilot-scale membrane fractionation of ACE inhibitory and antioxidative peptides from ultrasound pretreated milk protein concentrate hydrolysates. J. Funct. Foods, 7: 350-361.
5. Wu, W., P.P. Yu, F.Y. Zhang, H.X. Che and Z.M. Jiang, 2014. Stability and cytotoxicity of angiotensin-I-converting enzyme inhibitory peptides derived from bovine casein. J. Zhejiang Univ. Sci., 15: 143-152.
6. Mohanty, D.P., S. Mohapatra, S. Misra and P.S. Sahu, 2015. Milk derived bioactive peptides and their impact on human health-a review. Saudi J. Biol. Sci., (In Press). 10.1016/j.sjbs.2015.06.005.
7. Shamloo, M., P. Eck and T. Beta, 2015. Angiotensin converting enzyme inhibitory peptides derived from cereals. J. Human Nutr. Food Sci., Vol. 3, No. 1.
8. Qu, W., H. Ma, B. Liu, R. He, Z. Pan and E.E. Abano, 2013. Enzymolysis reaction kinetics and thermodynamics of defatted wheat germ protein with ultrasonic pretreatment. Ultrason. Sonochem., 20: 1408-1413.
9. Jin, J., H. Ma, K. Wang, A.E.G.A. Yagoub and J. Owusu *et al*., 2015. Effects of multi-frequency power ultrasound on the enzymolysis and structural characteristics of corn gluten meal. Ultrason. Sonochem., 24: 55-64.
10. Qu, W., H. Ma, J. Jia, R. He, L. Luo and Z. Pan, 2012. Enzymolysis kinetics and activities of ACE inhibitory peptides from wheat germ protein prepared with SFP ultrasound-assisted processing. Ultrason. Sonochem., 19: 1021-1026.
11. Zhou, C., H. Ma, X. Yu, B. Liu, A.E.G.A. Yagoub and Z. Pan, 2013. Pretreatment of defatted wheat germ proteins (by-products of flour mill industry) using ultrasonic horn and bath reactors: Effect on structure and preparation of ACE-inhibitory peptides. Ultrason. Sonochem., 20: 1390-1400.
12. Huang, L., B. Liu, H. Ma and X. Zhang, 2014. Combined effect of ultrasound and enzymatic treatments on production of ACE inhibitory peptides from wheat germ protein. J. Food Process. Preserv., 38: 1632-1640.
13. Jambrak, A.R., T.J. Mason, V. Lelas, L. Paniwnyk and Z. Herceg, 2014. Effect of ultrasound treatment on particle size and molecular weight of whey proteins. J. Food Eng., 121: 15-23.
14. Ren, X., H. Ma, S. Mao and H. Zhou, 2014. Effects of sweeping frequency ultrasound treatment on enzymatic preparations of ACE-inhibitory peptides from zein. Eur. Food Res. Technol., 238: 435-442.
15. Shanmugam, A., J. Chandrapala and M. Ashokkumar, 2012. The effect of ultrasound on the physical and functional properties of skim milk. Innov. Food Sci. Emerg. Technol., 16: 251-258.
16. Zou, Y., Y. Ding, W. Feng, W. Wang and Q. Li *et al*., 2016. Enzymolysis kinetics, thermodynamics and model of porcine cerebral protein with single-frequency countercurrent and pulsed ultrasound-assisted processing. Ultrason. Sonochem., 28: 294-301.
17. Goudarzi, M., A. Madadlou, M.E. Mousavi and Z. Emam-Djomeh, 2012. Optimized preparation of ACE-inhibitory and antioxidative whey protein hydrolysate using response surface method. Dairy Sci. Technol., 92: 641-653.
18. Dadzie, R.G., H. Ma, E.E. Abano, W.J. Qu and S.Y. Mao, 2013. Optimization of process conditions for production of Angiotensin I-Converting Enzyme (ACE) inhibitory peptides from vital wheat gluten using response surface methodology. Food Sci. Biotechnol., 22: 1531-1537.

19. Ma, H., L. Huang, L. Peng, Z. Wang and Q. Yang, 2015. Pretreatment of garlic powder using sweep frequency ultrasound and single frequency countercurrent ultrasound: Optimization and comparison for ACE inhibitory activities. Ultrason. Sonochem., 23: 109-115.
20. AOAC., 2005. Official Methods of Analysis of the Association of Official Analytical Chemist. 18th Edn., Horwitz William Publication, Washington, DC., USA.
21. Zhou, X., 1995. Enzyme Technology. Petroleum Industry Press, Beijing, China, pp: 284-286.
22. Guillen-Casla, V., N. Rosales-Conrado, M.E. Leon-Gonzalez, L.V. Perez-Arribas and L.M. Polo-Diez, 2011. Principal Component Analysis (PCA) and Multiple Linear Regression (MLR) statistical tools to evaluate the effect of E-beam irradiation on ready-to-eat food. J. Food Compos. Anal., 24: 456-464.
23. Adler-Nissen, J., 1986. Enzymic Hydrolysis of Food Proteins. Elsevier Applied Science Publishers, London, UK., ISBN: 9780853343868, Pages: 427.
24. Rawendra, R.D.S., Aisha, C.I. Chang, Aulanni'am, H.H. Chen, T.C. Huang and J.L. Hsu, 2013. A novel angiotensin converting enzyme inhibitory peptide derived from proteolytic digest of chinese soft-shelled turtle egg white proteins. J. Proteomics, 94: 359-369.
25. Jambrak, A.R., T.J. Mason, V. Lelas, Z. Herceg and I.L. Herceg, 2008. Effect of ultrasound treatment on solubility and foaming properties of whey protein suspensions. J. Food Eng., 86: 281-287.
26. Jia, J., H. Ma, W. Zhao, Z. Wang, W. Tian, L. Luo and R. He, 2010. The use of ultrasound for enzymatic preparation of ACE-inhibitory peptides from wheat germ protein. Food Chem., 119: 336-342.
27. Madadlou, A., D. Sheehan, Z. Emam-Djomeh and M.E. Mousavi, 2011. Ultrasound-assisted generation of ACE-inhibitory peptides from casein hydrolyzed with nanoencapsulated protease. J. Sci. Food Agric., 91: 2112-2116.
28. Wang, B., G.G. Atungulu, R. Khir, J. Geng, H. Ma, Y. Li and B. Wu, 2015. Ultrasonic treatment effect on enzymolysis kinetics and activities of ACE-inhibitory peptides from oat-isolated protein. Food Biophys., 10: 244-252.
29. Zhou, C., H. Ma, Q. Ding, L. Lin and X. Yu *et al*., 2013. Ultrasonic pretreatment of corn gluten meal proteins and neutrase: Effect on protein conformation and preparation of ACE (angiotensin converting enzyme) inhibitory peptides. Food Bioprod. Process., 91: 665-671.
30. Wu, J., R.E. Aluko and S. Nakai, 2006. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship study of Di- and tripeptides. J. Agric. Food Chem., 54: 732-738.
31. Campos, M.R.S., F.P. Gonzalez, L.C. Guerrero and D.B. Ancona, 2013. Angiotensin I-converting enzyme inhibitory peptides of chia (*Salvia hispanica*) produced by enzymatic hydrolysis. Int. J. Food Sci., Vol. 2013. 10.1155/2013/158482
32. Qu, W., H. Ma, W. Zhao and Z. Pan, 2013. ACE-inhibitory peptides production from defatted wheat germ protein by continuous coupling of enzymatic hydrolysis and membrane separation: Modeling and experimental studies. Chem. Eng. J., 226: 139-145.
33. Roslan, J., S.M.M. Kamal, M.K.F. Yunos and N. Abdullah, 2015. Optimization of enzymatic hydrolysis of tilapia (*Oreochromis niloticus*) by-product using response surface methodology. Int. Food Res. J., 22: 1117-1123.
34. Zhou, C., X. Yu, X. Qin, H. Ma, A.E.G.A. Yagoub and J. Hu, 2016. Hydrolysis of rapeseed meal protein under simulated duodenum digestion: Kinetic modeling and antioxidant activity. LWT-Food Sci. Technol., 68: 523-531.
35. Guo, Y., D. Pan and M. Tanokura, 2009. 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., 114: 328-333.
36. Cui, X., J. Sun, X. Li, J. Wang and H. Wang, 2014. Optimization for ultrasound-assisted calcium hydroxide extraction of protein from shrimp waste using response surface methodology. Adv. J. Food Sci. Technol., 6: 228-232.
37. Wang, L.Z., C.H. Xue, Y. Xue, Y.M. Wang and Z.J. Li, 2015. Optimization and evaluation of a novel technique for hydrolyzing Antarctic krill (*Euphausia superba*) proteins. Food Bioprod. Process., 94: 629-636.
38. Koochecki, A., A.R. Taherian, S.M.A. Razavi and A. Bostan, 2009. Response surface methodology for optimization of extraction yield, viscosity, hue and emulsion stability of mucilage extracted from *Lepidium perfoliatum* seeds. Food Hydrocholloids, 23: 2369-2379.
39. Oladapo, S.O. and O.G. Akanbi, 2015. Models for predicting body dimensions needed for furniture design of junior secondary school one to two students. Int. J. Eng. Sci., 4: 23-36.
40. Kumar, M., D. Kumar and B. Singh, 2014. Utilization of agro residue corncob for production of acetone-butanol using clostridium acetobutylicum and process optimization through RSM. J. Microb. Biochem. Technol., Vol. S8. 10.4172/1948-5948.S8-005
41. Nyatuame, M., V. Owusu-Gyimah and F. Ampiaw, 2014. Statistical analysis of rainfall trend for volta region in Ghana. Int. J. Atmos. Sci. 10.1155/2014/203245
42. Kamau, S.M. and R.R. Lu, 2011. The effect of enzymes and hydrolysis conditions on degree of hydrolysis and DPPH radical scavenging activity of whey protein hydrolysates. Curr. Res. Dairy Sci., 3: 25-35.

43. Shao, P., S.T. Jiang and Y.J. Ying, 2007. Optimization of molecular distillation for recovery of tocopherol from rapeseed oil deodorizer distillate using response surface and artificial neural network models. Food Bioprod. Process., 85: 85-92.
44. Zhang, Y., H. Ma, B. Wang, W. Qu, Y. Li, R. He and A. Wali, 2015. Effects of ultrasound pretreatment on the enzymolysis and structural characterization of wheat gluten. Food Biophys., 10: 385-395.
45. Jin, J., H. Ma, W. Qu, K. Wang and C. Zhou *et al*., 2015. Effects of multi-frequency power ultrasound on the enzymolysis of corn gluten meal: Kinetics and thermodynamics study. Ultrason. Sonochem., 27: 46-53.