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Full Length Research Paper

Optimized enzymatic hydrolysis and pulsed electric field treatment for production of antioxidant peptides from egg white protein

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Egg white protein powder was hydrolyzed by alcalase to produce antioxidant peptides, which were prepared under optimal enzymatic parameters that were obtained from the preliminary one-factor-at-a-time test and response surface methodology (RSM). Thereafter, the hydrolysates were sequentially fractionated by ultra filtration membranes in cut-off molecular weight (MW) of 30, 10 and 1 kDa and tested in terms of their reducing power (RP). The results show that the alcalase hydrolysates possessed strong RP ability, particularly for the fraction within <1 kDa. This fraction was further treated by high-intensity pulsed electric field (PEF) to investigate its effect on the antioxidant activity of the peptide within 6 h. The result demonstrated that the PEF could significantly improve the antioxidant activity of the treated peptide fraction within 4 h, but the effects might be reversible.

Key words: Egg white, hydrolysis, antioxidant, response surface methodology (RSM), high-intensity pulsed electric field (PEF).

INTRODUCTION

Many studies have found that various peptides derived from animals and plants possess strong antioxidant capabilities of scavenging superfluous free radicals and are able to restrain *in vivo* lipid peroxidation, bringing significant health benefits to protect human body from various diseases. As a result, protein hydrolysates, particularly those made from the enzymatic hydrolysis, have attracted extensive and intensive investigations. Sakanaka et al. (2004) found that the egg-yolk protein hydrolysates, when compared with its original protein or amino acids mixture, showed stronger antioxidant activity in a linoleic acid oxidation system. Peng et al. (2009) used alcalase to hydrolyze whey protein isolate (WPI) for 0.5 to 8 h, obtaining a hydrolysate fraction that possessed strong antioxidant activities on scavenging DPPH, hydroxyl and superoxide radicals. Similarly, Li and Liu (2008) reported that chickpea hydrolysates possess antioxidant power of scavenging superoxide anions. Pedroche et al. (2007) applied an immobilized enzyme method to hydrolyze the carina protein from the bird rape seed, yielding two peptides of molecular weight (MW) of 1400 and 1800 Da. Both of them showed desirable antioxidant activities. There are many methods that can be used to evaluate the antioxidant ability, which includes DPPH scavenging ability, reducing power, hydroxyl and superoxide radicals scavenging activity, ORAC, ABTS and so on. The reducing power method is intuitive and easy-handling; therefore, it is extensively used as an antioxidant evaluation method.

High-intensity pulsed electric field (PEF) is a potential complement to or replacement of the traditional thermal pasteurization due to its advantages of short processing time and low energy consumption, PEF has shown remark-

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able superiority compared with other processing methods in food processing.

The PEF processing of food materials can result in an effective inactivation of microorganisms and denaturization of various kinds of enzymes, while keeping some desirable physic-chemical properties of food. Therefore, the PEF treatment has been considered as one of the emerging non-thermal technologies and an alternative to thermal treatments. For example, it cannot only ensure safety and extend shelf-life of fruit juices, but also provide fresh-like products with high antioxidant potential (Oms-Oliu et al., 2009). Elez-Martinez and Martin-Belloso (2007) compared the effects of PEF and thermal pasteurization on the remaining content of the thermal-sensitive chemical vitamin C in orange juice and its antioxidant activity. The study found that the PEF-treated sample could maintain the content of vitamin C in orange juice as high as to 87.5 to 98.2%, which was significantly higher than that in the thermal treated sample. In addition, the DPPH radical inhibition of PEF-treated sample was not significantly influenced. Other studies indicated that field strength, pulse duration, number of pulses and pulse shape are the main variables of PEF (Peña et al., 2010). Li et al. (2007) found that longer pulse duration caused the structural change of soybean protein isolates (SPI), while the short pulse duration had little effect. Regardless of its more and more applications in food processing, the effect of PEF on the antioxidant peptides was scarcely studied yet. According to recent studies, application of low intensity of the PEF treatment might not cause irreversible cell rupture. Angesbach et al. (2000) found that potato cell membrane applied with moderate electric field (1.7 V) resealed again within very short time and the cell membrane recovered its electrically insulating properties. Therefore, intense parameters of PEF were applied on antioxidant peptide to observe the effects of PEF.

China is the world's number one egg producer, which produced eggs in more than 27.4 million tons in 2009. Most of them are sold as fresh eggs. Therefore, there is a large room for developing value-added products from eggs. This study aimed to use the egg white protein (EWP) as a raw material to optimize the enzymatic hydrolysis parameters for preparing antioxidant peptides by alcalase. Then, PEF was applied to treat the positive antioxidant fractions in order to improve the antioxidant capacity in terms of the reducing power (RP) activity.

MATERIALS AND METHODS

Materials and reagents

EWP (protein content of 80.96%) was purchased from Jinjiangli Co. (Peking, China). Alcalase was purchased from Fanfuer International Chem. Co. (Tianjin, China). All other chemical reagents required in the experiments were purchased from Peking Chemical Plant (Beijing, China). All of the chemicals and reagents were of analytical grade.

Instruments and equipments

Self-designed PEF system shown in Figure 1A consisted of a high-voltage repetitive pulse generator, a coaxial liquid materials treatment chamber, a fiber-optic temperature sensor and a data acquisition system. The instrument could generate exponentially decaying bipolar triangle pulse waveforms with a pulse duration of 2 μs. The frequency is adjustable, ranging from 1000 to 3000 Hz. The bipolar pulse waveform and input voltage to the treatment chamber can be displayed on a digital oscillograph as shown in Figure 1B. The system can process samples continuously for the mass production. The generator is home-made and was described in our previous report (Yin et al., 2006).

The pulse number (C) can be calculated by the equation of $C= L\pi r^2 f/Q$, while the electric field intensity is expressed in $E=V_{pp}/2L$, where f is the frequency (Hz), L is the length of electrode (cm), r is the radius of electrode (cm), Q is the flow velocity (ml/s) of sample, E is the electric field intensity (kV/cm) and V_{pp} is the input voltage shown on the oscillograph. In this paper, L is 1 mm; r is 0.5 mm; q is 1.6 ml/min.

Parameters for the enzymatic hydrolysis

To produce antioxidant peptides, EWP was dissolved in distilled water, then heated at 90°C in water bath for 10 min to denature the protein before the suspension mixture was cooled down to room temperature. Then, pH and temperature of the solution were adjusted to a desirable condition. Based on our preliminary study, five independent variables were investigated, including the concentration of EWP (that is, substrate) (1, 3, 5 and 7%), ratio of enzyme/ EWP ([E/S]) (1, 3, 5 and 7%), incubation time (1, 2, 3, and 4 h), temperature (40, 45, 50, 55 and 60°C) and pH value (8, 9, 10, 11, 12 and 13). During the hydrolysis, the pH value was adjusted with 1 M NaOH to keep the change within ±0.05. After the enzymatic hydrolysis, the solutions were heated at 90°C in water bath again for 10 min to inactivate the enzymes and centrifuged at $2100 \times g$ for 10 min at 4°C.

Degree of hydrolysis (DH) of hydrolysed EWP was determined using a pH-stat method based on the equation: $DH = (h/h_{tot})\times 100\%$, where $h = B\times N_b\times 1/\alpha\times 1/MP$, B = base consumption (ml), N_b = concentration of base (1 M NaOH), $1/\alpha$ = calibration factors for pH-stat ($1/\alpha$= 1.01 for Alcalase), MP = mass of protein (g) and h = hydrolysis equivalents. For egg white, h_{tot} = 8.38 mmol/g protein.

Determination of reducing power (RP)

The reducing power (RP) assay was modified from the method described by Oyaizu (1986). 1 ml hydrolysate was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated in the 50°C water bath for 20 min before 2.5 ml of 10% trichloroacetic acid solution was added. The mixture was vortexed and then centrifuged for 10 min under the rotate rate of 3000 r/min. 2.5 ml of the supernatant was mixed with 0.1 ml of ferric chloride solution (0.1%) and measured at 700 nm after shaking. The higher the absorbance means the better the RP ability of the hydrolysates performed.

Optimizing preparation of antioxidant peptide by RSM

According to the results of the independent variable experiments, three independent variables at three levels (3^3) were adopted for the response surface methodology (RSM) by the Design Expert Software (Trial Version 7.0.0, Stat-Ease Inc., Minneapolis, MN, USA). These three independent variables were the concentration of egg white solution [S] (A), ratio of enzyme to substrate [E/S] (B),

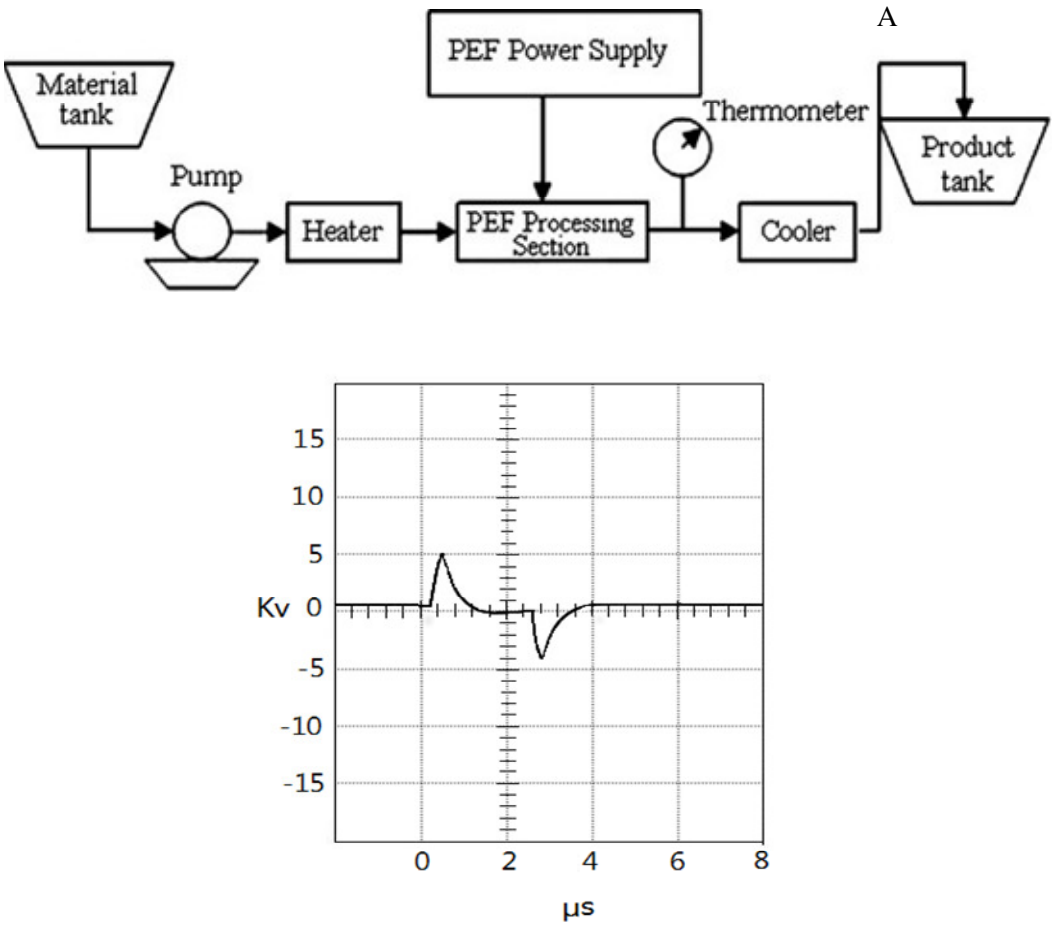


Figure 1. Schematic diagram of high intensity pulsed electric field processing apparatus (A) and the form of pulsed wave (B).

Table 1. Independent variables and their levels in the Box-Behnken design.

Independent variable	Code	Variable level		
		-1	0	1
[S] (%)	X_1	4	5	6
[E/S] (%)	X_2	1	2	3
pH value	X_3	10.0	10.5	11.0

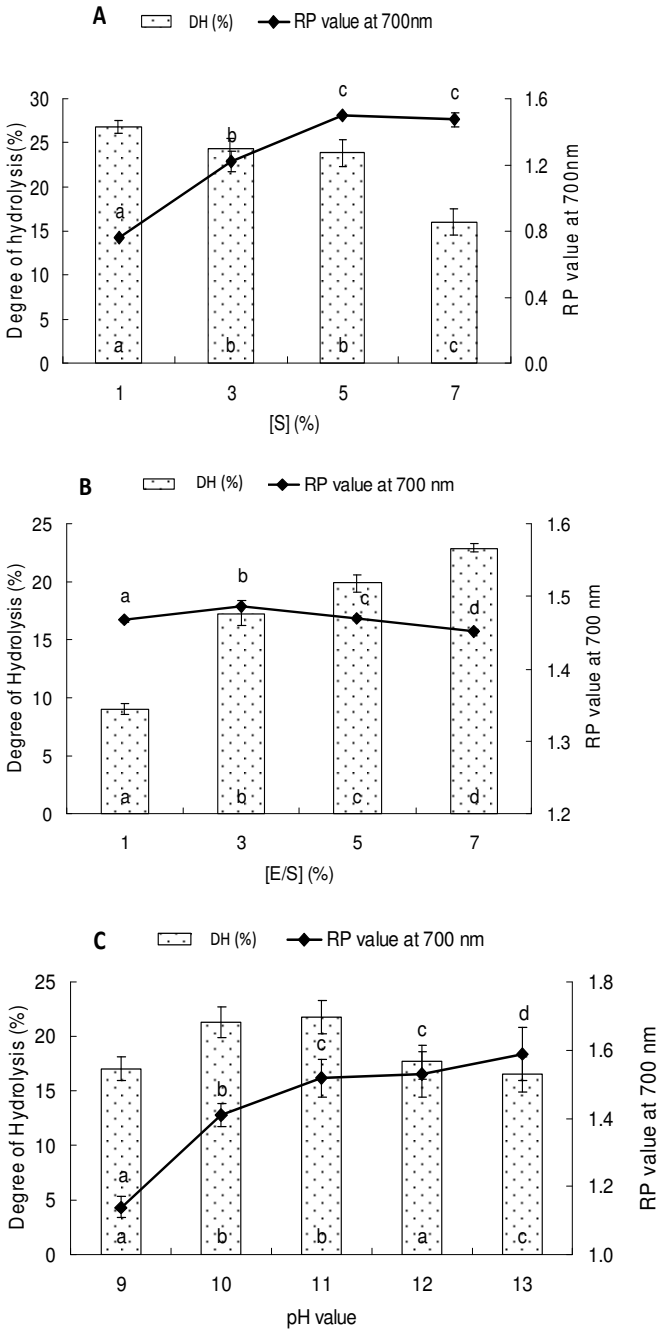
and pH value (C), which were labeled as X_1, X_2 and X_3, respectively, as shown in Table 1. The dependent variable Y was the RP activity. Each variable was coded at three levels: –1, 0 and +1. Triplicates at the center (0, 0 and 0) of the design were conducted to allow the estimation of the pure error sum of squares. All experiments were carried out in a randomized order to minimize the effects of unexplained variability in the observed responses, which might be caused by extraneous independent variables. A full quadratic equation for this model is shown as:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j}^{k} \beta_{ij} X_i X_j$$

Where, Y is the estimated response, β_0, β_j, β_{jj}, and β_{ij}, represent regression coefficients for intercept, linearity, square and interaction of the equation, respectively, while X_i and X_j are the independent coded variables. The coefficient of determination R_2 and analysis of variance (ANOVA) were used to evaluate the fitness of the model.

Effect of PEF on improving the antioxidant activities of peptides

Ultrafiltration equipment (UF) (Millipore Minitan system, Millipore, Bedford, MA) was used to fractionate enzymatic hydrolysates prepared under the optimal RSM conditions. The hydrolysates were sequentially separated by the UF membranes in the cut-off MW of 30, 10 and 1 kDa. The hydrolysates were first UF treated through the 30 kDa membrane. The retentate and permeate fractions were



collected separately and then the permeates were further UF treated through the 10 kDa membrane. The retantates and permeates were once more collected and a final UF was applied to permeates by using a 1 kDa membrane to obtain the other fractions. Each fraction was freeze-dried and stored in the desiccators. All fractions were assayed for their antioxidant activity.

In order to observe the conspicuous influence of antioxidant peptide cause by PEF, the hydrolysate fractions within the range of 1< kDa which showed strongest antioxidant capacity, was further treated by the PEF technique with intense electric field strength at 10 kV/cm, pulsed number at 300 and the frequency at 3000 Hz. The treated hydrolysates were measured in their RP values every hour within 6 h.

Determining peptide distribution by HPLC

The enzymatic hydrolysates of egg white protein are composed of different peptides that may exert different bioactivities. In order to explore the relationship between the antioxidant activity and MW Figure 2 distribution of the hydrolysates, the antioxidant hydrolysates with and without (control) the PEF-treatment were determined by HPLC after a micropore membrane filtration with a pore size of 0.45 μm. The HPLC system was equipped with a TSK-G2000 gel column (TOSOH Company, Japan) and a UV detector to determine the peptide distribution at 220 nm. 1 μl sample was injected and eluted at a flow rate of 0.5 ml/min with eluent of acetonitrile: water: trifluoroacetic= 10: 90: 0.01 (v/v/v).

Statistical analysis

ANOVA was performed using the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). The significances of the regression coefficients were also tested by F-test. The quality of the fitness of the polynomial model equation was expressed by the coefficient of determination R^2. All experiments were in triplicates and the means of three data sets were presented. The significant difference was determined with $P<0.05$.

RESULTS AND DISCUSSION

Effects of hydrolytic variables of alcalase on egg white protein

Enzymatic hydrolysis of proteins is commonly affected by factors such as temperature, pH value and [E/S]. Teng et al. (2010) found that changing these independent variables could significantly affect the degree of hydrolysis and antioxidant activity of peptides, for example, goat placenta hydrolysates. In our preliminary one-factor-at-a-time (OFAT) study, the effects of the concentration of egg white solution [S] (%), [E/S] (%), pH, hydrolysis temperature and time on the RP values were investigated when four variables were fixed at a certain value, while changing only one variable value. The OFAT tests fixed the variable values at [S] (1 to 7%), [E/S] (1 to 7%), pH (9 to 13), hydrolysis temperature (40 to 60°C) and time (1 to 4 h). The results are profiled in Figure 2A to E.

According to Figure 2, increasing the [S] from 1 to 5% significantly increased the RP values ($P < 0.05$), of which the best value was obtained from 5% egg white hydrolysates prepared by the alcalase (AH) (Figure 2A). When the [E/S] ratio was set at 5%, the AH gave a significantly high RP activity at 1.474. Meanwhile, within the experimental pH range (9 to 13) (Figure 2C), the RP value of AH increased along with the increasing pH value significantly ($P < 0.05$). However, regarding the problems that may occur in real enzymatic practice using extreme caustic solutions and the significant decrease of DH from pH 11 to 12, pH 11 was recommended to be used as a desirable parameter for AH. Moreover, the AH showed the significantly higher RP value at 50°C ($P < 0.05$). Regarding the processing time, 3 h was selected as the best incubation time for the AH.

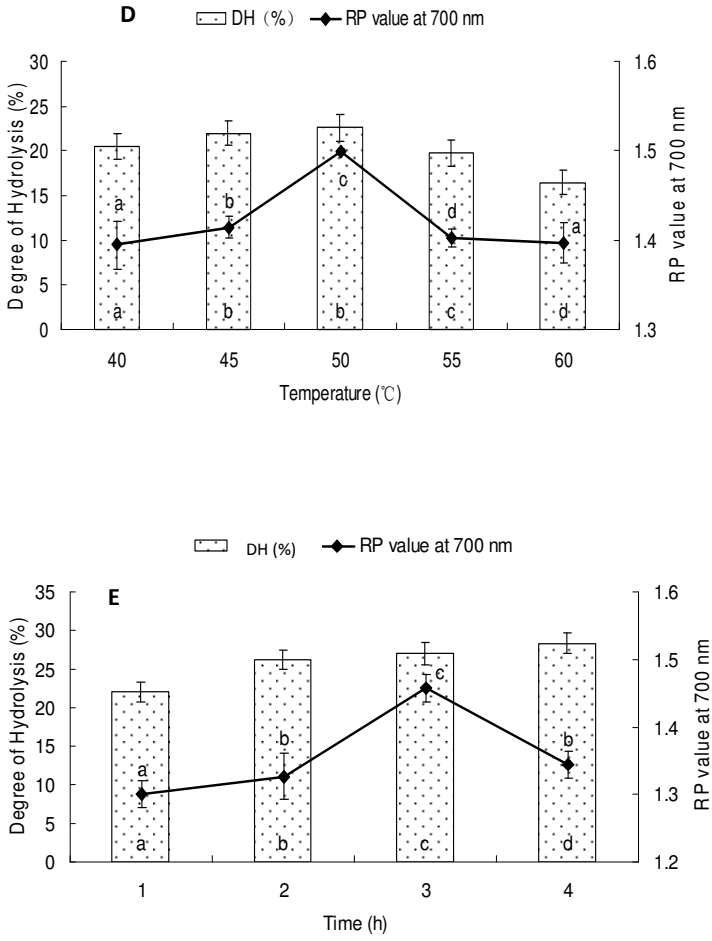


Figure 2. Effects of the concentration of egg white solution [S] (%), [E/S] (%), pH, hydrolysis temperature and time on the RP values. (A) To evaluate the effect of [S] of EWP on producing antioxidant peptide, four different concentrations of 1, 3, 5 and 7% were investigated at E/S of 3%, pH of 9, temperature of 50℃ and time of 4 h, respectively; (B) to evaluate the effect of [E/S] on producing antioxidant peptide, four different concentrations of 1, 3, 5 and 7% were investigated at [S] of 5%, pH of 9, temperature of 50℃ and time of 4 h, respectively; (C) to evaluate the effect of pH value on producing antioxidant peptide, five different values of 9, 10, 11, 12 and 13 were investigated at [S] of 5%, E/S of 3%, temperature of 50℃ and time of 4 h, respectively. (D) to evaluate the effect of temperature on producing antioxidant peptide, five different values of 40, 45, 50, 55 and 60℃ were investigated at [S] of 5%, E/S of 3%, pH value of 11 and time of 4 h, respectively; (E) to evaluate the effect of hydrolysis time on producing antioxidant peptide, four different values of 1, 2, 3 and 4 h were investigated at [S] of 5%, E/S of 3%, pH value of 11 and temperature of 60℃, respectively.

From the variance of DH (Figure 2A to E), it was observed that the increase of DH did not necessarily result in a higher RP value. Therefore, extensive enzymatic hydrolysis of proteins is not suggested since it caused loss of functional properties and formation of bitter peptides in the hydrolysates (Chabanon et al., 2007).

Based on the results of one-factor-at-a-time experiments, the further desirable hydrolytical parameters for the preparation of antioxidant peptides were suggested as follows: the recommended condition was at 5% concentration of egg white solution, 3% [E/S], pH 11, hydrolysis temperature of 50℃ and time (3 h). Under those aforementioned respective best conditions, the RP activity of AH approached to 1.533.

Table 2. Box-Behnken design and response of the RP value of antioxidant peptide.

Experiment	Coded level			Response	
	X_1	X_2	X_3	RP value at 700 nm	
	[S] (%)	[E/S] (%)	pH	Experimental	Predicted
1	0	1	-1	1.302	1.304
2	0	0	0	1.507	1.510
3	1	0	1	1.433	1.442
4	-1	-1	0	1.286	1.269
5	1	1	0	1.441	1.458
6	-1	1	0	1.441	1.448
7	0	0	0	1.513	1.510
8	0	0	0	1.509	1.510
9	0	-1	1	1.281	1.269
10	0	1	1	1.497	1.471
11	-1	0	-1	1.288	1.279
12	-1	0	1	1.435	1.454
13	0	-1	-1	1.202	1.228
14	1	-1	0	1.376	1.369
15	1	0	-1	1.418	1.399

Table 3. Design matrix evaluation for the response surface quadratic model.

Source	Sum of squares	Degrees of freedom	Mean square	F Value	Prob > F
Model	0.137	9	0.0152	25.87	0.0011
Residual	0.003	5	0.0006		
Lack of fit	0.110	3	0.0340	4.63	0.086
Pure error	0.039	4	9.77×10^{-3}		
Cor. Total	0.140	16			

R= 0.989; R^2 = 0.9790; adjusted. R^2 = 0.9411, adequate.; precision = 14.226.

Generally, the AH possessed good RP activity. Similarresults was obtained from Qian et al. (2008) who tested alcalase, neutrase, pepsin, papain, α-chymotrypsin and trypsin to hydrolyze bullfrog skin protein and found that the alcalase hydrolysate exhibited the highest antioxidant activities among those enzymatic hydrolysates. Ren et al. (2008) investigated antioxidant peptides from the grass carp muscles that were hydrolyzed by various proteases (for example, papain, bovine pancreatin, bromelain, neutrase and alcalase). It was found that the hydrolysate prepared by the alcalase provided the strongest antioxidant activity. Therefore, alcalase was chosen as a favorable proteinase for the antioxidant peptide preparation.

The establishment of regression model and response surface analysis

Based on the OFAT tests, the effects of hydrolysis variables, including [S] (X_1: 4-6%), [E/S] (X_2: 1-3%) and pH (X_3: 10-11), on the RP values were investigated in an effort to obtain an optimized enzymatic condition and the coefficients of its second-order polynomial equation. The regression and coefficients are summarized in Table 2. The final mathematical model can be expressed by the following quadratic equation, $Y= -43.50691+1.02012X_1-0.028645X_2 + 7.95968X_3 - 0.022582X_1X_2 - 0.066165X_1X_3 + 0.057340X_2X_3 - 0.025306X_1^2 - 0.098388 X_2^2 - 0.36355X_3^2$ for AH, where Y is the absorbance value of the RP activity at 700 nm, X_1, X_2, X_3 are the variables for the concentration of egg white suspension (%), [E/S] (%) and pH, respectively.

The regression model of AH was tested by the analysis of variance (ANOVA), as shown in Table 3. In general, the validity of the model can be judged by lack of fit to check the adequacy of a fitted-response surface model (Stalikas et al., 2009). The P-value of the model was significant ($P < 0.0001$), while the lack of fit value of the mathematical model was 0.086, which meant that the model does not lack fit. The coefficient (R^2) and the ratio of the variations to total variations, was defined to explain the fitness of

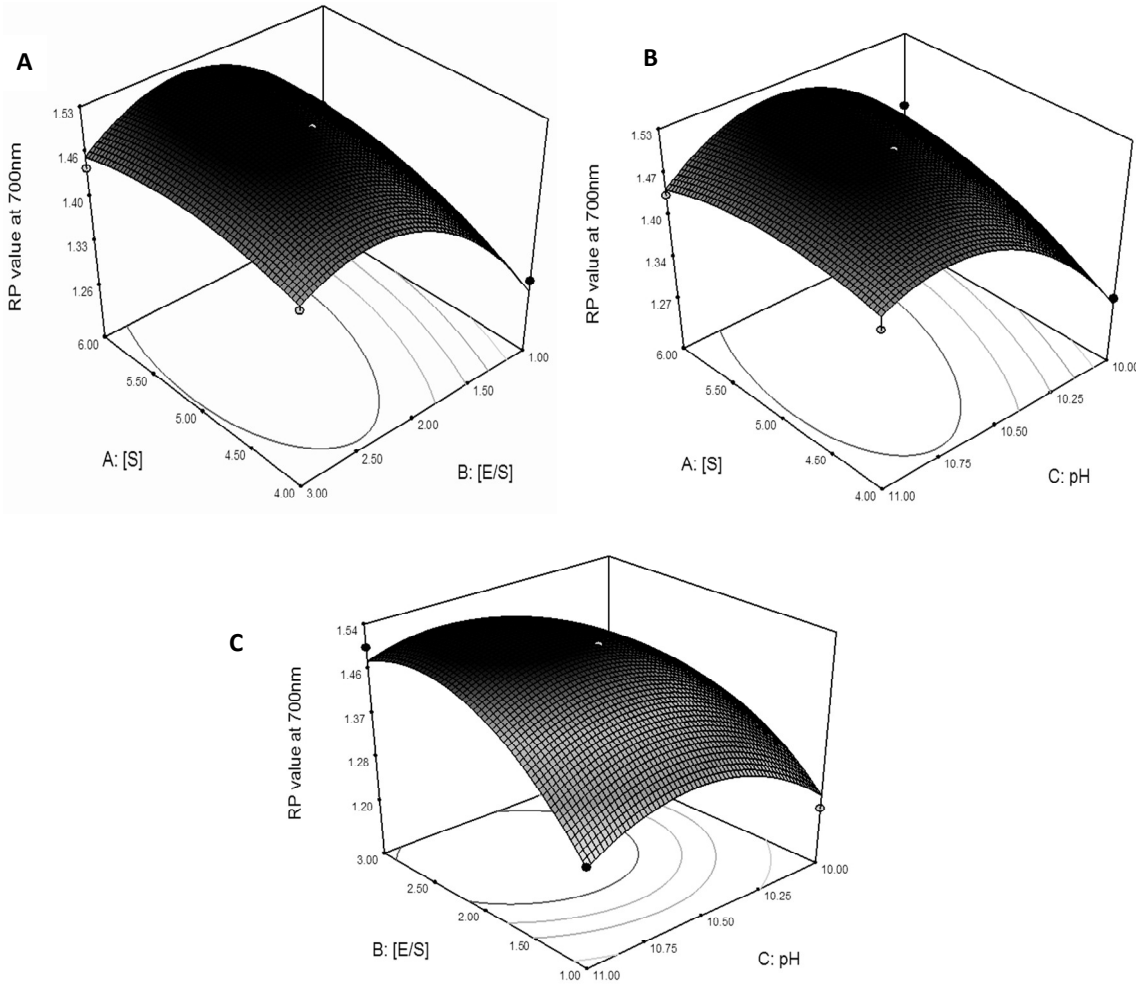


Figure 3. The response surface plots of RP values of the hydrolysates from EWP catalyzed by alcalase (A to C). The RP value is shown as a function of the interactions between either [S] or [E/S] (A) or [S] and pH (B), or pH and [E/S] (C).

the regression models (Nath and Chattopadhyay, 2007). A small value of R^2 indicates a poor relevance of the dependent variables in the model. When the R^2 approaches unity, the model fitted well with the experimental data. By analysis of variance (ANOVA), a good agreement with the experimental results with a coefficient (R^2) of 0.9790 was found. The statistical analysis showed that the AH regression model could significantly (*P = 0.0011*) match the real RP values. Coincidently, the predicted results for AH were further experimentally validated and found to be very close to the observed experimental responses. Therefore, the model of AH can be used to analyze and predict the effect of the independent variables on the antioxidant activity.

The results of response surface analysis generated by combining two of three independent variables versus the corresponding RP values are profiled in Figure 3. The contour plots at the bottom of the response surface presenting ellipse means the interaction effect was significant. The Equation of AH exhibited a great accuracy on predicting the RP activity andinteraction effects between two combined independent variables. Therefore, the optimal condition for preparing antioxidant peptides from the alcalase treatment could be obtained by RSM as 5.16% of EWP, 2.37% of [E/S] and 10.66 pH.

Effect of hydrolysate MW on antioxidant activity

In this study, the egg white hydrolysates, were prepared by the alcalase under its optimal condition, were sequentially separated by the ultrafiltration membranes in the cut-off MW at 30, 10 and 1 kDa. The collected fractions within different molecular weights were freezed-dried and compared for their RP activities because many studies have reported that peptides with different MW

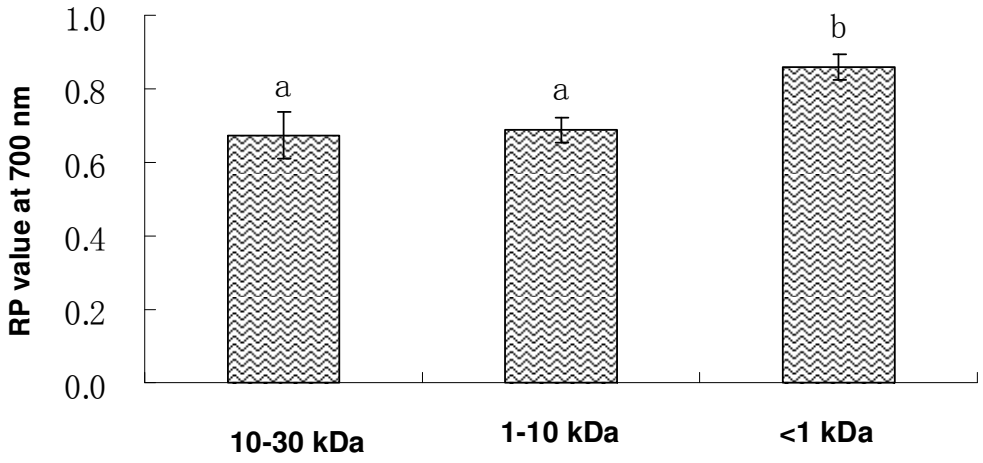


Figure 4. The RP activities of different fractions prepared from AH. Different lowercase letters denote that the variances of
RP ability are significant (*P < 0.05*).

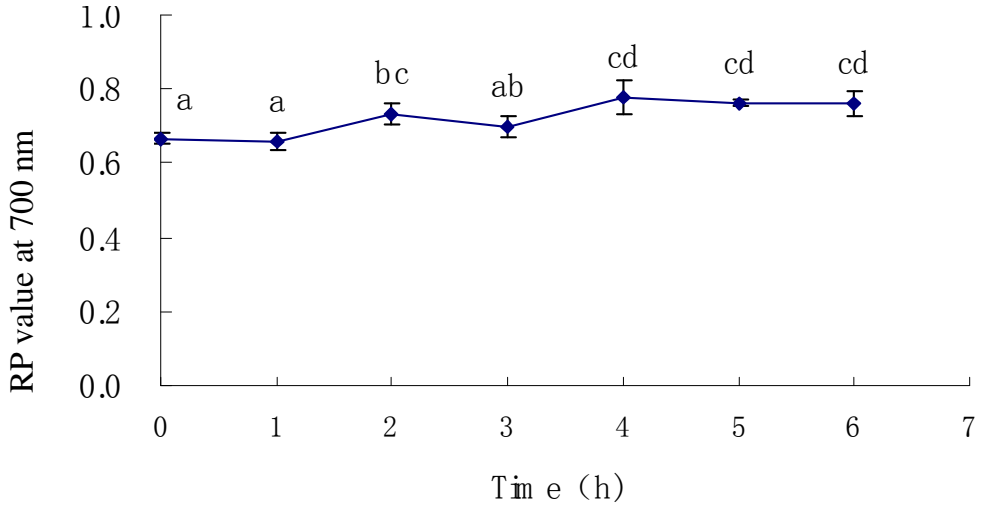


Figure 5. The trend of the RP activity of <1 kDa fraction prepared by AH within 6 h after PEF treatment. Different lowercase letters denote statistical difference at *P < 0.05*. The applied PEF treatment conditions consisted of the electric field strength (10 kV/cm), the pulse frequency (3000 Hz) and the pulse number (300).

the results in this study and the published reports, the fraction of <1 kDa prepared by UF for AH was chosen to be treated by PEF to investigate its effect on the antioxidant activity.

Effects of PEF on antioxidant peptide

The PEF with fixed parameters (electric field strength at 10 kV/cm; pulsed number at 300; the frequency at 3000 Hz) was applied to the <1 kDa fraction prepared from AH and the RP ability was detected per hour after the treatment within 6 h. The relationship of treating time versus RP is shown in Figure 5. The results showed that the RP ability of the sample increased significantly (*P < 0.05*) at different stages. The RP underwent a relative stability from 0 to 1 h and increased significantly from 1 to 2 h, then achieved the highest value of 0.778 after 4 h, then slightly decreased (*P > 0.05*). Compared with the untreated sample (time = 0 h), the PEF treatment significantly increased the antioxidant activity of AH (*P < 0.05*) at the first 4 h. Barsotti et al. (2001) reported that a series of 20 exponential decay pulses of 31.5 kV/cm at 1 Hz applied to ovalbumin in a buffer solution induced at least a partial unfolding of the protein structure or enhanced the ionization of SH groups into a more reactive S^- form.

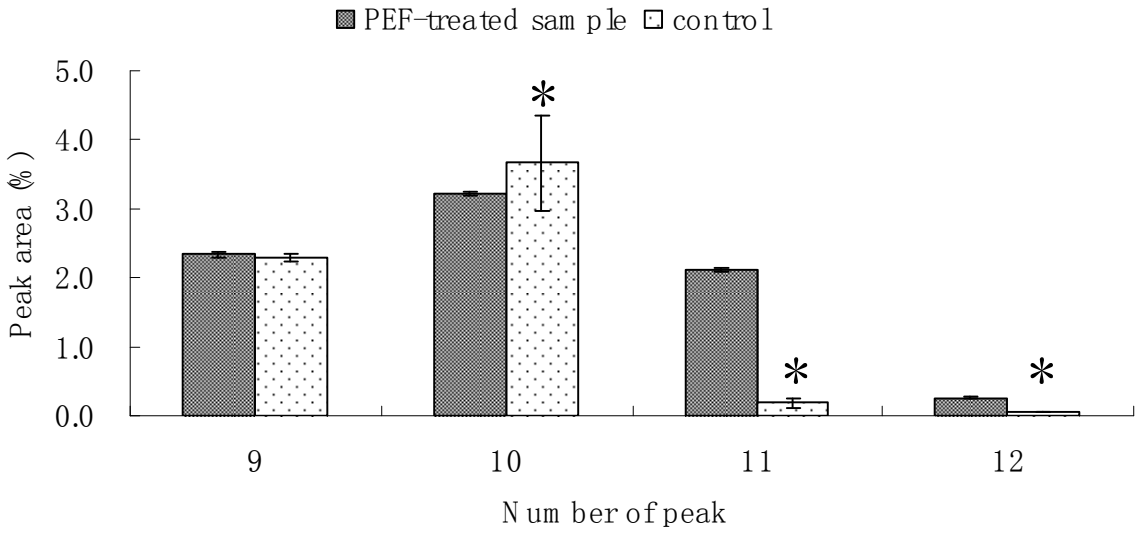


Figure 6. Comparison of peak area of PEF-treated sample and control. * Variance of treated and non-treated samples is significant ($P < 0.05$).

The number of pulses and the energy applied per pulse significantly influenced SH group reactivity. However, these modifications appeared to be transient when the pulse applied to the ovalbumin solution was kept at 4°C for more than 30 min, the extent of the observed changes decreased and were completely reversed after 24 h. The results indicate that the PEF had exerted some effects on the antioxidant peptides, but the effects might be reversible. In order to explore the specific mechanism of PEF on peptide, the PEF-treated sample and the control were further analyzed by HPLC at exact 4 h when the PEF exerted the best influence.

Effect of PEF treatment on the peptide

The results of HPLC analyses are shown in Figure 6. It could be concluded that some separated antioxidant peptides underwent significant changes in their amounts after the PEF treatment. The peak 10 in the control was significantly larger than that of the PEF-treated sample ($P < 0.05$), while the peaks 11 and 12 of PEF-treated samples presented lager peak areas than the control ($P < 0.05$). It clearly demonstrated that PEF caused the larger peptides decomposition into smaller peptides. The smaller antioxidant peptides might have exerted better biological effects (Hernández-Ledesma et al., 2005). It was hypothesized that the PEF could induce the polarization of peptide molecules and destroy the non-covalent bonds such as hydrophobic interactions, electrostatic interactions, hydrogen bonds and dissociated more subunits leading to primary and higher structure of protein being disorganized (Perez and Pilosof, 2004). As a result, it is hypothesized that the PEF treatment might have facilitated the production of more smaller peptides and exposure of His, Pro, Cys, Tyr, Try, Phe and Met residues to increase the antioxidant activity of peptides. Nevertheless, this unique observation needs further investigation. Characterization of the antioxidant peptides and the effect of PEF on these peptides were investigated by HPLC-MS in our laboratories.

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