Irish Journal of Agricultural and Food Research **52:** 53–65, 2013

Optimisation of hydrolysis conditions for antioxidant hydrolysate production from whey protein isolates using response surface methodology

L. Zhidong[1,3], G. Benheng[2], C. Xuezhong[1], L. Zhenmin[2], D. Yun[4], H. Hongliang[1]† and R. Wen[1]

[1]*East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shanghai, PR China*
[2]*Technical Centre of Bright Dairy and Food Co. Ltd., State Key Laboratory of Dairy Biotechnology, Shanghai, PR China*
[3]*School of Life Sciences and Biotechnology, Shanghai Jiaotong University, Shanghai, PR China*
[4]*School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai, PR China*

The hydrolysates of whey protein isolates (WPI) by papain were found to possess antioxidant activity. Response surface methodology (RSM) was used to improve the antioxidant activity of these hydrolysates. The model was validated and shown to be statistically adequate and accurate in predicting the response. For both 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging activity and reducing power responses, the optimised conditions were achieved at an enzyme to substrate ratio (E/S, w/w) of 2.22%, hydrolysis time of 3.60 h, and hydrolysis temperature of 45.70 °C. Under the optimised conditions, DPPH radical-scavenging activity of the hydrolysates of WPI was 31.48% and the reducing power was 0.612 at 700 nm. The results of confirmation experiments indicated that the model was powerful and suitable for estimation of the experimental value. The hydrolysate of WPI has potential application as an antioxidant in food products.

Keywords: antioxidant activity; papain; response surface methodology; whey protein isolates

†Corresponding author: Huang Hongliang; zdliu1976@163.com

Introduction

Lipid oxidation can cause the deterioration of food quality, shorten the shelf-life of food products and decrease the acceptability of processed foods. Lipid peroxidation can generate free radicals which can also readily react with and oxidise most biomolecules, including carbohydrates, proteins, lipids, DNA and small cellular molecules (Peng, Xiong and Kong 2009). Free radicals are believed to play a significant role in the occurrence of diseases, such as cardiovascular diseases, diabetes mellitus, neurological disorders and Alzheimer's disease (Stadtman 2006). Therefore, it is important to retard lipid oxidation and the formation of free radicals in food and biological systems (Moskovitz, Yim and Choke 2002).

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), n-propyl gallate (PG), and t-butylhydroquinone are commonly used to retard lipid peroxidation and to curtail the formation of free radicals in food and biological systems (Peng *et al.* 2009). However, the application of synthetic antioxidants is restricted due to potential risks related to human health (Hraš *et al*. 2000). Therefore, in order to inhibit lipid peroxidation in food products and enhance the body's antioxidant defense, interest has been developed in identifying natural antioxidants.

There is a growing amount of research on the development of natural antioxidants derived from food. Several studies have demonstrated that some protein enzymatic hydrolysates have antioxidant activity, such as soy protein (Lee *et al*. 2008), casein (Gómez-Ruiz *et al*. 2008), egg-white protein (Dávalos *et al*. 2004), egg-yolk (Sakanaka *et al*. 2004), meat and fish proteins (Je *et al*. 2007; Mendis, Rajapakse and Kim 2005), gelatin obtained from the skin of sole and squid (Gimenez *et al*. 2009), bullfrog skin (Qian, Jung and Kim 2008) and smooth hound muscle (Ali *et al*. 2009).

Whey protein is an abundant and low-cost by-product of the casein or cheese industries, and is widely used as a functional and nutritional ingredient in food products (María *et al*. 2011). Several treatments have been suggested to modify whey proteins to fully exploit their functional attributes. High pressure, heat treatment and enzymatic hydrolysis can effectively alter the structure, thereby changing the functionality of whey protein (Peña-Ramos and Xiong 2001). Enzymatic hydrolysis offers better control of the hydrolysis process and results in a product of reproducible quality. The hydrolysates of protein are distinguished from the original protein mainly due to their smaller molecular size which is more beneficial to human health. Moreover, cleavage is usually accompanied by important structural rearrangements that tend to expose some hydrophobic regions, originally buried within the protein molecule, to the aqueous phase (Korhonen and Pihlanto 2006).

Previous studies have shown that enzyme-hydrolysed whey proteins have antioxidant activity (Hernández-Ledesma *et al*. 2005; María *et al*. 2011; Peña-Ramos and Xiong 2001, 2003). In general, the antioxidant activity of the hydrolysates of protein mainly depends on both the specificity of the enzymes used and the hydrolysis conditions [enzyme to substrate ratio (E/S), time and temperature, pH, etc] (Peña-Ramos and Xiong 2001). Furthermore, the hydrolysates of protein are continuously formed and degraded during hydrolysis and this also has an effect on their antioxidant activity. Therefore, the maximum antioxidant activity of the hydrolysates of protein is a result of a balance between the continuously forming and degrading processes (Cornelly *et al*. 2002). The relationship

between variables and responses is important for predicting the process of hydrolysis by means of a mathematical model. Response surface methodology (RSM) is a collection of mathematical and statistical techniques which is widely applied to build a model of experiments, evaluate the effects of variables and search for the optimum conditions and processes (Amouzgar *et al*. 2010). Predictive equations are used to describe how the variables affect the response, to determine the interrelationship among the variables and to evaluate the combined effect of all the variables in the response (Snehal *et al*. 2008). However, there are little data relating to the application of RSM to the antioxidant hydrolysates derived from whey protein isolates (WPI).

Preliminary research in the laboratory indicated that the antioxidant activity of the hydrolysates of WPI using papain is significantly influenced by the hydrolysis conditions (temperature, time and E/S). Thus, the purpose of this study was to improve the antioxidant activity of the hydrolysates of WPI by optimising the hydrolysis conditions according to the results of the RSM.

Materials and Methods

Materials

Whey protein isolates was obtained from Glanbia Nutritional, Inc. (Monroe, USA). The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was purchased from Sigma Chemicals (St. Louis, MO, USA). Papain was purchased from Guangxi Nanning Javely biological products Co., Ltd. (Nanning, China). Other chemicals used were of analytical grade and obtained from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China).

Determination of the activity of papain

The activity of papain was determined using bovine casein (2% [w/v], pH 7.0) as a substrate. After addition of the enzyme solution, the mixture was incubated at 45 °C for 10 min, 0.4 M trichloroacetic acid (TCA) solution was then added to the mixture, and the content of soluble nitrogen in the supernatant was measured by Lowry-Folin method (Lowry *et al*. 1951). One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under specific conditions.

Hydrolysis of WPI

Whey protein isolates was dissolved in distilled water at a concentration of 5% (w/v) and the conditions for enzymatic hydrolysis of WPI with papain were adjusted with 1 M NaOH to pH 8.0. The hydrolysis time ranged from 2.0 to 6.0 h, the hydrolysis temperature ranged from 35 to 55 °C, and the enzyme-to-substrate ratio ranged from 1% to 3% (w/w). Prior to hydrolysis, the WPI solution was stirred for 15 min at 85 °C. During hydrolysis, the pH was constantly maintained by addition of 1 M NaOH. The hydrolysis reaction was terminated by heating at 95 °C for 15 min. The hydrolysates of WPI were immediately cooled to room temperature, adjusted to pH 7.0, and centrifuged at 10,000×*g* for 30 min at 4 °C. The supernatants were ultrafiltrated through a 5 kDa cut-off membrane (Millipore Corporation, Billerica, MA, USA). The 5 kDa-permeates were freeze-dried and stored at –20 °C until the assessment of the antioxidant activity (María *et al*. 2011).

DPPH radical-scavenging activity assay

The DPPH radical-scavenging activity of the hydrolysates of WPI was measured according to the method of Amarowicz, Naczk and Shahidi (2000). An aliquot of 1.5 mL of 0.25 mM DPPH radical solution in ethanol and 1.5 mL of the sample (2 mg/mL) were mixed. The solution was

mixed rapidly and allowed to reach a steady state in the dark at room temperature for 30 min. A decrease in absorbance at 517 nm showed reduction of the DPPH radical. The DPPH radical scavenging activity was calculated according to the following formula (1):

$$\text{Scavenging rate}=[1-(A_1-A_2)/A_0]\times 100\% \quad (1)$$

where, A_0 was the absorbance of the control (blank, without the sample), A_1 was the absorbance in the presence of the sample and A_2 was the absorbance without DPPH radical.

Reducing power assay

The Fe^{3+} reducing power of the sample solution was determined by the method of Oyaizu (1986) with slight modifications. The sample solution (0.75 mL) at 2 mg/mL was mixed with 0.75 mL of phosphate buffer (0.2 M, pH 6.6) and 0.75 mL of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50 °C in a water bath for 20 min. The reaction was stopped by adding 0.75 mL of TCA solution (10%, w/v) and then centrifuged at 1245×*g* for 10 min. An aliquot of 1.5 mL of the supernatant was mixed with 1.5 mL of distilled water and 0.1 mL of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 min. A higher absorbance at 700 nm indicated greater reducing power.

Experimental design

On the basis of the results of the preliminary experiments, the most influential factors on the antioxidant activity were shown to be E/S (w/w, %) (x_1), hydrolysis temperature (x_2, °C) and time (x_3, h). These were chosen as the independent variables for subsequent experiments. Other related hydrolysis parameters, such as the substrate concentration and pH, which had been ascertained as having less critical effects on the antioxidant activity of the hydrolysates of WPI by the preliminary tests, were not included. The dependent variables y_1 and y_2 were DPPH radical-scavenging activity and reducing power ($A_{700\ nm}$) of the hydrolysates of WPI, respectively.

This experimental design was constructed based on a 3^3 factorial design in one block consisting of 15 sets of experiments according to Box-Behnken design. Table 1 shows the complete design matrix and the results of the experiments employed in this study. As a measurement of precision, three centre experiments were chosen to verify any change in the estimation procedure. Experimental runs were randomised to minimise the effects of unexpected variability in the responses.

Statistical analyses

Design Expert software (Version 8.0.3.1, Stat-Ease, Inc., USA) was used for regression and graphical analysis of the experimental data.

Table 1. Box-Behnken design matrix and the responses of the two dependent variables

Run	x_1 E/S (w/w,%)	x_2 (temp, °C)	x_3 (time, h)	y_1 (%)	y_2
1	1	40	4.0	18.75	0.438
2	1	50	4.0	23.28	0.528
3	3	40	4.0	24.04	0.533
4	3	50	4.0	26.31	0.592
5	2	40	3.5	19.31	0.462
6	2	40	4.5	26.23	0.573
7	2	50	3.5	25.43	0.549
8	2	50	4.5	26.85	0.585
9	1	45	3.5	19.02	0.456
10	3	45	3.5	29.32	0.597
11	1	45	4.5	27.34	0.591
12	3	45	4.5	26.71	0.582
13	2	45	4.0	31.86	0.603
14	2	45	4.0	30.96	0.617
15	2	45	4.0	31.62	0.615

y_1 is DPPH radical-scavenging activity, y_2 is reducing power.

E/S=enzyme to substrate ratio.

The responses (y) of the hydrolysis process were used to develop a quadratic polynomial equation that correlates antioxidant activity of the hydrolysates of WPI as a function of the independent variables and their interactions, and were fitted to the following equation:

$$y=b_0+\sum_{i=1}^{3} b_i x_i+\sum_{i=1}^{3} b_{ii} x_i^2+\sum_{i<j=2}^{3} b_{ij} x_i x_j+\varepsilon \qquad (2)$$

where y is the response value, b_0, b_i, b_{ii} and b_{ij} are constants and regression coefficients of the model (b_0 for constant, b_i for the first order model coefficient, b_{ii} for the quadratic coefficient for the variable i, and b_{ij} for the linear model coefficient for the interaction between variables i and j, respectively), x_i and x_j are the independent variables, respectively, while ε is the error. Response surface methodology (RSM) was chosen as the method for the hydrolysis process to determine the optimum operating conditions (Amouzgar *et al*. 2010).

The quality of the fit of the model was evaluated by the correlation coefficient (R^2), while the F-test for analysis of variance (ANOVA) was used to evaluate the statistical significance of the model equation. The regression equation was expressed as response surface in order to visualise the relationship between the response and experimental levels of the selected variables and to deduce the optimum conditions based on the criterion of desirability. The confirmation experiments were conducted in triplicate to verify the model.

Results and Discussion

Statistical analysis and the regression model

Papain is an enzyme with broad specificity and hydrolyses sites with hydrophobic amino acids, such as Lys and Arg, at their C-terminal. Papain (2×10^4 U/g) was used to obtain antioxidant hydrolysates from WPI. Non-hydrolysed WPI showed a slight antioxidant capacity (DPPH radical-scavenging activity was 10.29±0.26% and the reducing power was 0.227±0.018 OD, respectively) but hydrolysis with papain induced a notable increase in the antioxidant activity of WPI. This result implies that the low molecular weight of the hydrolysates or peptides obtained from WPI were mainly responsible for the antioxidant activity (Moosman and Behl 2002).

Effect of variables on DPPH radical-scavenging activity

Radical quenching is a primary mechanism by which antioxidants inhibit oxidative processes (Peng *et al.* 2009). The DPPH radical is a relatively stable organic radical and can become a more stable diamagnetic molecule by accepting an electron (Bondet, Brand-Williams and Berset 1997). It has been widely used as a substrate to evaluate the efficacy of antioxidants. The increasing DPPH radical-scavenging activity of the hydrolysed WPI was probably due to the structural changes of the protein, leading to a greater radical quenching ability. Park *et al*. (2001) characterised the antioxidative peptides derived from the hydrolysates of lecithin-free egg yolk and concluded that the efficacy of proteins or peptides depended on their molecular weight; a 5 kDa permeate was found to have the strongest antioxidant activity.

The effects of variables on the linear, quadratic, and interaction terms of the model were evaluated for adequacy, fitness and significance by ANOVA (Table 2). The Model F-value of 94.65 implies that the model is significant. There is only 0.01% chance that a "Model F-Value" this large could occur due to noise in the experiments. Values

Table 2. Analysis of variance (ANOVA) for response surface quadratic model analysis of DPPH radical-scavenging activity

Source	df	Mean square	F-value	Prob>F	
Model	9	28.91	94.65	<0.0001	Significant
x_1	1	40.46	132.44	<0.0001	
x_2	1	22.92	75.02	0.0003	
x_3	1	24.68	80.78	0.0003	
$x_1 x_2$	1	1.28	4.18	0.0963	
$x_1 x_3$	1	29.87	97.78	0.0002	
$x_2 x_3$	1	7.56	24.76	0.0042	
x_1^2	1	48.42	158.51	<0.0001	
x_2^2	1	83.79	274.31	<0.0001	
x_3^2	1	18.88	61.81	0.0005	
Residual	5	0.31			
Lack of fit	3	0.36	1.68	0.3947	Not significant
Pure error	2	0.22			

of "Prob > F" less than 0.05 indicates that the model is significant. This result showed that three linear coefficients (x_1, x_2, x_3), three quadratic coefficients (x_1^2, x_2^2, x_3^2), and two cross-product coefficients (x_1x_3, x_2x_3) were significant for DPPH radical-scavenging activity. The R^2 value of the model was 0.9942, which further indicates that the model was suitable for adequately representing the relationships among the variables. The "Pred R-Squared" (Predicted correlation coefficient) value of 0.9295 is in reasonable agreement with the "Adj R-Squared" (Adjusted determination coefficient) value of 0.9837. The "Lack of Fit F-value" of 1.68 implies that the Lack of Fit is not significant relative to the pure error. There is a 39.47% chance that a "Lack of Fit F-value" this large could occur due to noise in the experiments. Non-significant lack of fit is good. The value of R^2 indicates that there is a good agreement between the experimental values and predicted values obtained from the model. In general, the P-value determines the significance of each coefficient in the model. However, in order to minimise error, all of the coefficients were considered in the design.

The equation of the predicted model (based on the actual value) by which DPPH radical-scavenging activity was estimated is given in Eq. (3):

$$y_1 = 31.48+2.25\,x_1+1.69\,x_2+1.76\,x_3 -0.56\,x_1x_2-2.73\,x_1x_3-1.38\,x_2x_3 -3.62\,x_1^2-4.76\,x_2^2-2.26\,x_3^2 \quad (3)$$

where y is the response value, DPPH radical-scavenging activity (%), x_1, x_2, and x_3 are the values of the independent factors, E/S (w/w, %), hydrolysis temperature (°C), and hydrolysis time (h), respectively. The model was regressed by only considering the significant terms. The model proved suitable for the adequate representation of the real relationship among the selected factors.

Effect of variables on reducing power

The reducing power assay showed that the hydrolysates of WPI were able to reduce a Fe^{3+} complex to a Fe^{2+} complex. The reducing power assay is the most effective means to evaluate the ability of antioxidant to donate electrons (Peng *et al*. 2009). The strong reducing power of the WPI hydrolysates may be due to the increasing availability of hydrogen ions produced by peptide hydrolysis.

Table 3. Analysis of variance (ANOVA) for response surface quadratic model analysis of reducing power

Source	df	Mean square	F-value	Prob>F	
Model	8	6.083E-003	42.96	<0.0001	Significant
x_1	1	0.011	74.75	0.0001	
x_2	1	7.688E-003	54.29	0.0003	
x_3	1	8.911E-003	62.93	0.0002	
$x_1 x_3$	1	5.625E-003	39.72	0.0007	
$x_2 x_3$	1	1.406E-003	9.93	0.0198	
x_1^2	1	5.146E-003	36.34	0.0009	
x_2^2	1	9.825E-003	69.38	0.0002	
x_3^2	1	1.174E-003	8.29	0.0281	
Residual	6	1.416E-004			
Lack of fit	4	1.838E-004	3.20	0.2517	Not significant
Pure error	2	5.733E-005			

The effect of variables on the regression model was evaluated for adequacy, fitness and significance by ANOVA (Table 3). The Model F-value of 42.96 implies the model is significant. There is only 0.01% chance that a "Model F-Value" this large could occur due to noise in the experiments. Values of "Probe > F" less than 0.05 indicates that the model is significant. The result shows that three linear coefficients (x_1, x_2, x_3), three quadratic coefficients (x_1^2, x_2^2, x_3^2), and two cross-product coefficients (x_1x_3, x_2x_3) were significant model terms for reducing power. The R^2 value of the model was 0.9828, which indicated that the model was suitable for adequately representing the real relationships among the selected reaction variables. The "Lack of Fit F-value" of 3.20 implies that the Lack of Fit is not significantly related to the pure error. There is a 25.17% chance that a "Lack of Fit F-value" this large could occur due to noise factors (uncontrollable factors) such as human errors or experimental errors. Non-significant lack of fit is good. The "Pred R-Squared" of 0.8644 is in reasonable agreement with the "Adj R-Squared" of 0.9600. This model is adequate enough to describe the data of reducing power of the hydrolysates of WPI.

The equation of the predicted model (based on the actual value) by which reducing power was estimated is given in Eq. (4):

$$y_2 = 0.612+0.036\,x_1+0.031\,x_2+0.033\,x_3 -0.037\,x_1x_3-0.018\,x_2x_3-0.037\,x_1^2 -0.052\,x_2^2-0.018\,x_3^2 \quad (4)$$

where y is the response value reducing power (OD), x_1, x_2, and x_3 are the values of the independent factors, E/S (w/w, %), hydrolysis temperature (°C), and hydrolysis time (h), respectively. A positive sign in front of the terms indicates a synergistic effect while a negative sign indicates an antagonistic effect. The final model only considered the significant terms. The model can be used for the prediction of the variables within the given ranges. The importance of the effect of the variables on the antioxidant activity of the hydrolysates of WPI could be ranked in the following order: E/S (x_1)>hydrolysis time (x_3)>hydrolysis temperature (x_2).

The maximum predicted values and the dependent variables agreed well with the optimum values and the dependent variables obtained by the equations of

the predicted models. According to the regression equations, comprehensively assessing these main factors, the maximum antioxidant activity of the response could be obtained an E/S (w/w) value of 2.22%, a hydrolysis time of 3.60 h and a hydrolysis temperature of 45.7 °C, respectively. The maximum predicted values (y_1 and y_2) were DPPH radical-scavenging activity of 31.48% and reducing power of 0.612 ($A_{700\ nm}$), respectively.

RSM analysis

Three-dimensional response surfaces and contour plots of the models are generally used for graphical representation of the regression equation to determine the optimum values of the variables within the given ranges. Response surfaces with a contour (at the base) plot showed the interaction of two independent variables when another variable was fixed at zero. The maximum predicted value was indicated by the surface confined in the smallest ellipse in the contour diagram. The elliptical contours are obtained when there is a perfect interaction between the independent variables.

Figures 1–3 present the response surface curves established for DPPH radical-scavenging activity in this study. The maximum DPPH radical-scavenging activity was obtained at the point of intersection of the major and minor axes of the ellipse. Figure 1 shows the effect of the interaction between E/S and the hydrolysis temperature on DPPH radical-scavenging activity of the hydrolysates of WPI at any constant hydrolysis time. It was noted that DPPH radical-scavenging activity increased with increasing E/S and hydrolysis temperature at first, and then decreased with increasing E/S and hydrolysis temperature within a given range. The main reason for this phenomenon may be the inaction of papain with increasing temperature, and the equilibrium between the hydrolysates and protein continuously

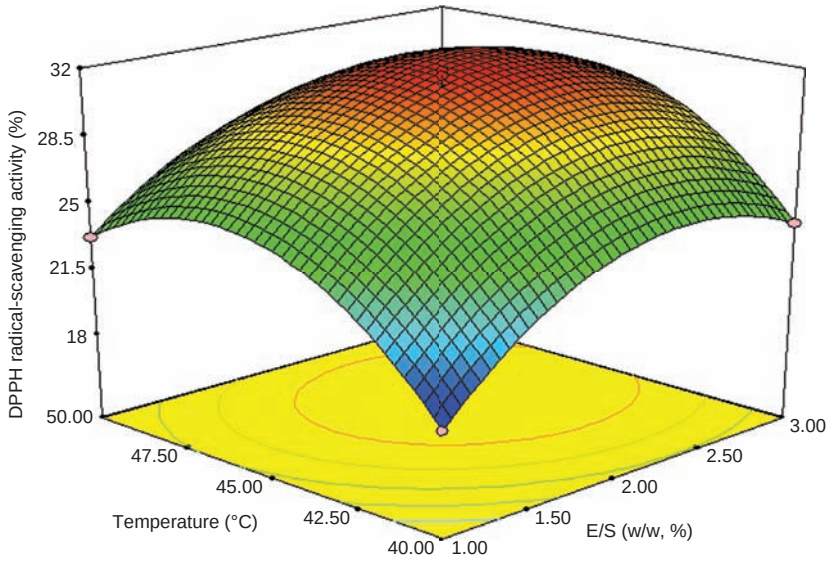

Figure 1. Effects of E/S and hydrolysis temperature on DPPH radical-scavenging activity in three-dimensional response surface.

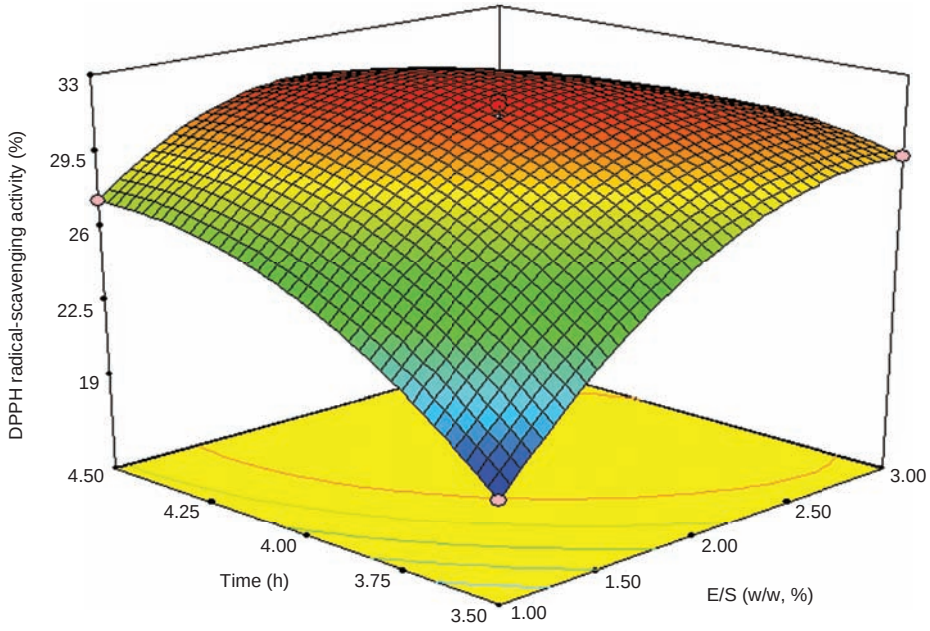

Figure 2. Effects of E/S and hydrolysis time on DPPH radical-scavenging activity in three-dimensional response surface.

formed and degraded during hydrolysis. Figure 2 shows the effect of the interaction between the hydrolysis time and E/S on DPPH radical-scavenging activity of the hydrolysates of WPI. DPPH radical-scavenging activity increased with increasing hydrolysis time, and then decreased with increasing hydrolysis time, due to the inactivation of papain. Figure 3 shows the response surface plot of DPPH radical-scavenging activity as a function of hydrolysis temperature and hydrolysis time. At low hydrolysis temperature, DPPH radical-scavenging activity was mainly affected by hydrolysis time. However, at higher reaction temperature, DPPH radical-scavenging activity reached a maximum value, and then decreased due to the inaction of papain. Therefore, the increasing DPPH radical-scavenging activity of the hydrolytes of WPI was probably due to the structural changes of the protein and the exposure of the specific groups of the hydrolysates. Wu, Chen and Shiau (2003) reported similar results in mackerel protein hydrolysates, where the DPPH radical-scavenging activity gradually increased with increasing hydrolysis time.

The response surfaces and contours (at the base) for reducing power on E/S, hydrolysis temperature and hydrolysis time are shown in Figures 4 and 5, respectively. Figure 4 shows the response surface curve of reducing power as a function of E/S and hydrolysis time. The predicted reducing power increased until it reached a maximum value with increasing hydrolysis time within a given range, and then decreased on further increasing hydrolysis time. The reducing power increased with increasing E/S at lower E/S. A further increase in the E/S resulted in a reversal of this trend. Figure 5 shows that hydrolysis temperature and hydrolysis time within a

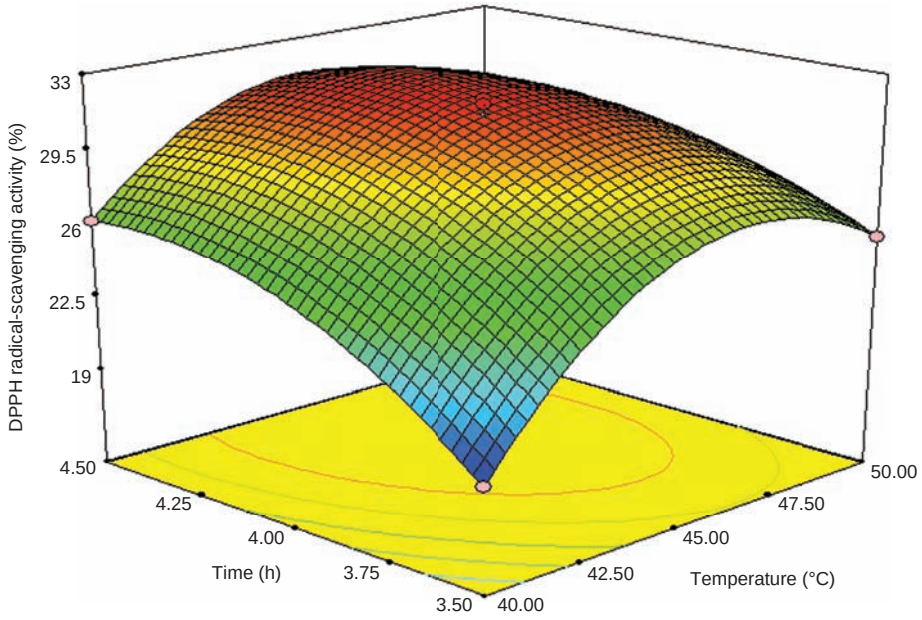

Figure 3. Effects of hydrolysis temperature and hydrolysis time on DPPH radical-scavenging activity in three-dimensional response surface.

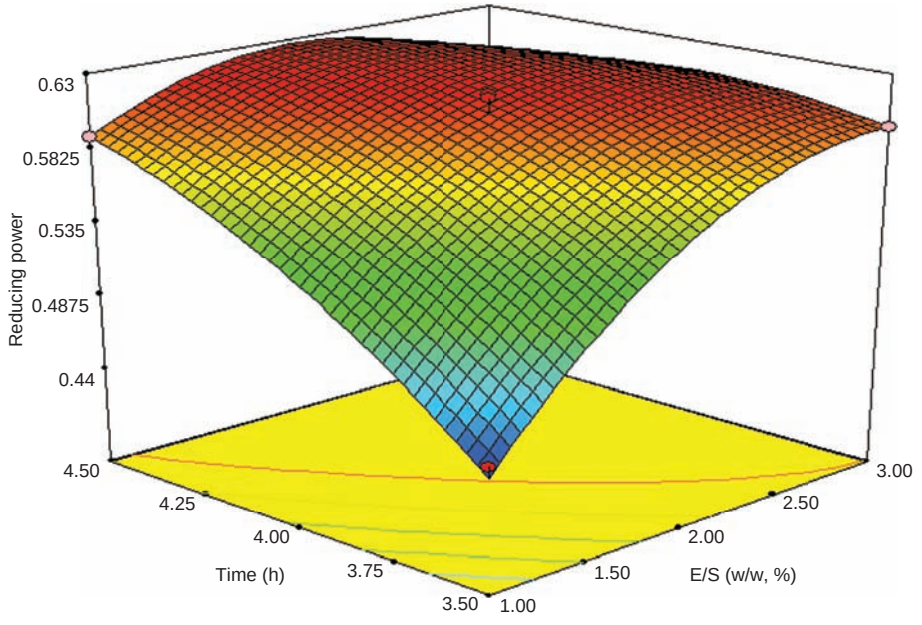

Figure 4. Effects of E/S and hydrolysis time on reducing power in three-dimensional response surface.

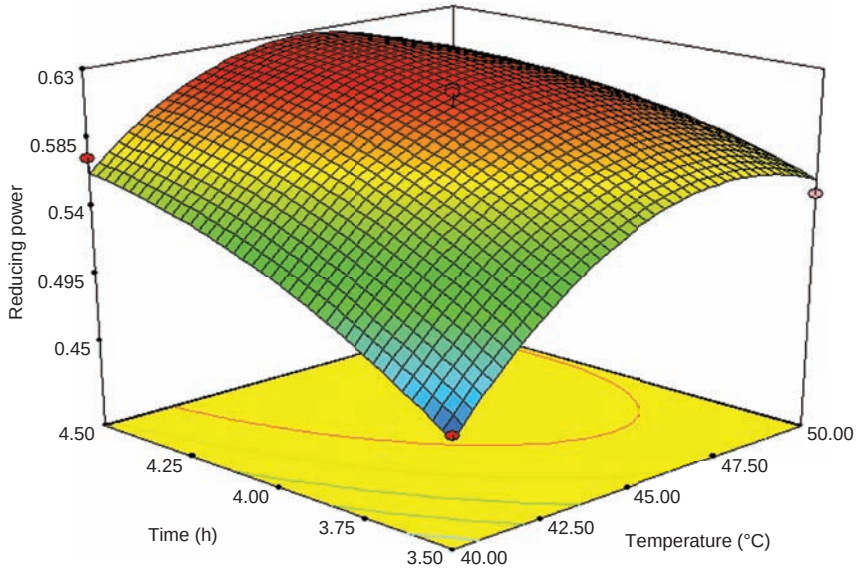

Figure 5. Effects of hydrolysis time and hydrolysis temperature on reducing power in three-dimensional response surface.

given range improved the reducing power of the hydrolysates of WPI, but at high temperature, the reducing power was decreased. Surprisingly, the interaction between E/S and the hydrolysis temperature was not significant, indicating that the interactive effects of E/S and the hydrolysis temperature on the reducing power did not play a key role. Cumby *et al*. (2008) also reported a similar result for canola protein hydrolysate, its reducing power increased with increasing hydrolysis time. Therefore, the hydrolysis reaction is likely to have changed the structure of WPI and exposed amino acid residues which are electron donors and could react with free radicals, converting them to more stable products and terminating the free radical chain reaction.

Verification of model

In order to verify the optimised results, the suitability and validity of the model for predicting the optimum response values were tested for DPPH radical-scavenging activity and reducing power of absorbance at 700 nm, respectively. Under these optimised conditions, the confirmation experiments were conducted in triplicate. The experimental values of DPPH radical-scavenging activity and reducing power were 31.36% and 0.607, respectively (Table 4). The experimental values were in agreement with the predicted

Table 4. Predicted and experimental values of the responses at optimum conditions[a]

Response variables	Predicted value	Experimental value[a]
DPPH radical-scavenging activity (%)	31.48±0.00	31.36±0.55
Reducing power	0.612±0.000	0.607±0.010

[a]Mean value of three experiments.

values of the model within a 95% confidence interval, indicating that the model was powerful and suitable for estimation of the experimental value.

Conclusions

The values of the variables, E/S (2.22%, w/w), hydrolysis time (3.60 h), and hydrolysis temperature (45.70 °C) were found to be optimum for improvement of the antioxidant activity of the hydrolysates of WPI for both DPPH radical-scavenging activity (31.36±0.55%) and reducing power (0.607±0.010 OD), respectively. These experimental values were well within the predicted values of the model. The results indicated that RSM is an effective tool for optimisation of hydrolysis parameters in order to obtain the optimum antioxidant activity the hydrolysates of WPI.

Acknowledgments

This work was supported by the fund for National Science Technology Minister of China (No. 2012AA092304, No. 2012AA092303).

References

Ali, B., Mohamed, H., Rafik, B., Imen, L., Yosra, T. and Moncef, N. 2009. Antioxidant and free radical scavenging activities of smooth hound (*Mustelus mustelus*) muscle protein hydrolysates obtained by gastrointestinal proteases. *Food Chemistry* **114:** 1198–1205.

Amarowicz, R., Naczk, M. and Shahidi, F. 2000. Antioxidant activity of various fractions of non-tannin phenolics of canola hulls. *Journal of Agricultural and Food Chemistry* **48:** 2755–2759.

Amouzgar, P., Abdul Khalil, H.P.S., Salamatinia, B., Abdullah, A.Z. and Issam, A.M. 2010. Optimization of bioresource material from oil palm trunk core drying using microwave radiation; a response surface methodology application. *Bioresource Technology* **101:** 8396–8401.

Bondet, V., Brand-Williams, W. and Berset, C. 1997. Kinetics and mechanism of antioxidant activity using the DPPH free radical methods. *LWT-Food Science and Technology* **30:** 609–615.

Cornelly, V., Harry, G., Dries, B.B.A. and Alphons, G.V. 2002. Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. *International Dairy Journal* **12:** 813–820.

Cumby, N., Zhong, Y., Naczk, M. and Shahidi, F. 2008. Antioxidant activity and water-holding capacity of canola protein hydrolysates. *Food Chemistry* **109:** 144–148.

Dávalos, A., Miguel, M., Bartolomé, B. and López-Fandiño, R. 2004. Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection* **67:** 1939–1944.

Gimenez, B., Alemán, A., Montero, P. and Gómez-Guillén, M.C. 2009. Antioxidant and functional properties of gelatin hydrolysates obtained from skin of sole and squid. *Food Chemistry* **114:** 976–983.

Gómez-Ruiz, J.A., López-Expósito, I., Pihlanto, A., Ramos, M. and Recio, I. 2008. Antioxidant activity of ovine casein hydrolysates: identification of active peptides by HPLC-MS/MS. *European Food Research and Technology* **227:** 1061–1067.

Hraš, A.R., Hadolin, M., Knez, Z. and Bauman, D. 2000. Comparison of antioxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. *Food Chemistry* **71:** 229–233.

Hernández-Ledesma, B., Dávalos, B., Bartolomé, B. and Amigo, L. 2005. Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry* **53:** 588–593.

Je, J.Y., Qian, Z.J., Byun, H.G. and Kim, S.K. 2007. Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. *Process Biochemistry* **42:** 840–846.

Korhonen, H. and Pihlanto, A. 2006. Bioactive peptides: production and functionality. *International Dairy Journal* **16:** 945–960.

Lee, J.S., Yoo, M.A., Koo, S.H., Baek, H.H. and Lee, H.G. 2008. Antioxidant and ACE inhibitory activities of soybean hydrolysates: effect of enzyme and degree of hydrolysis. *Food Science and Biotechnology* **17:** 873–877.

Lowry, O.H., Rosenbrough, N.J., Fair, A.L. and Randall, R.J. 1951. Protein measurement with the Folin-phenol reagents. *Journal of Biological Chemistry* **193:** 265–275.

María, M.C., Blanca, H.L., Lourdes, A., Pedro, J.M. and Isidra, R. 2011. Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: optimization by response surface methodology. *LWT- Food Science and Technology* **44:** 9–15.

Mendis, E., Rajapakse, N. and Kim, S.K. 2005. Antioxidant properties of a radical scavenging peptide purified from enzymatically prepared fish skin gelatin hydrolysate. *Journal of Agricultural and Food Chemistry* **53:** 581–587.

Moosman, B. and Behl, C. 2002. Secretory peptide hormones are biochemical antioxidants: structure-activity relationship. *Molecular Pharmacology* **61:** 260–268.

Moskovitz, J., Yim, K.A. and Choke, P.B. 2002. Free radicals and disease. *Archives of Biochemistry and Biophysics* **397:** 354–359.

Muthu, M., Lejla, P. and Vijayaraghavan, K. 2009. Optimization of growth media for obtaining high-cell density cultures of halophilic archaea (family *Halobacteriaceae*) by response surface methodology. *Bioresource Technology* **100:** 3107–3112.

Oyaizu, M. 1986. Studies on products of browning reactions: antioxidant activities of products of browning reaction prepared from glucose amine. *Japanese Journal of Nutrition* **44:** 307–315.

Park, P.J., Jung, W.K., Nam, K.S., Shahidi, F. and Kim, S.K. 2001. Purification and characterization of antioxidative peptides from lecithin-free egg yolk protein. *Journal of the American Oil Chemists' Society* **78:** 651–656.

Peña-Ramos, E.A. and Xiong, Y.L. 2001. Antioxidative activity of whey protein hydrolyzates in a liposomal system. *Journal of Dairy Science* **84:** 2577–2583.

Peña-Ramos, E.A. and Xiong, Y.L. 2003. Whey and soy protein hydrolysates inhibit lipid oxidation in cooked pork patties. *Meat Science* **64:** 259–263.

Peng, X.Y., Xiong, Y.L. and Kong, B.H. 2009. Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance. *Food Chemistry* **113:** 196–201.

Qian, Z.J., Jung, W.K. and Kim, S.K. 2008. Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, *Rana catesbeiana Shaw*. *Bioresource Technology* **99:** 1690–1698.

Sakanaka, S., Tachibana, Y., Ishihara, N. and Juneja, L.R. 2004. Antioxidant activity of egg-yolk protein hydrolysates in a linoleic acid oxidation system. *Food Chemistry* **86:** 99–103.

Snehal, R.M., Bhalchandra, K.V., Renuka, M.J., Kiran, M.D. and Sanjay, N.N. 2008. Use of response surface optimization for the production of biosurfactant from *Rhodococcus* spp. MTCC 2574. *Bioresource Technology* **99:** 7875–7880.

Stadtman, E.R. 2006. Protein oxidation and aging. *Free Radical Research* **40:** 1250–1258.

Wu, H.C., Chen, H.M. and Shiau, C.Y. 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International* **36:** 949–957.

Received 13 June 2012