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ENZYMOLYSIS TECHNOLOGY OPTIMIZATION FOR PRODUCTION OF ANTIOXIDANT PEPTIDES FROM GOAT MILK CASEIN

– Research paper –

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Abstract: Antioxidant peptides can inhibit lipid peroxidation and scavenging free radicals, maintain the balance of free radicals, and against a variety of diseases. Response surface methodology was used to optimize process conditions for producing antioxidative peptides from goat's milk casein hydrolysate with Alcalase. The results suggested that the optimal process parameters were: temperature at 62.5°C, pH 8.9, E/S ration at 2.5%, substrate concentration at 4.4% and hydrolysis time was 173min). Metal-chelating effect, superoxide anion radical scavenging activity and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity were shown to be 87.21±0.88%, 49.18±1.42% and 69.07±1.26% respectively under the optimal condition. The actual and predicated value were closely which indicated the optimized data fit well to model and the optimized parameters are reliable.

Keywords: *Alcalase, goat's milk casein, response surface methodology, antioxidative peptides*

INTRODUCTION

Oxidation of biomolecules has been identified as a process mediated by free radicals (Hui et al., 2011), which can cause many adverse effects on food and biological systems. Diseases such as arteriosclerosis, cancer and inflammation are associate with metabolism of free radical oxygen in aerobiont (Jomova and Valko, 2011). Free radicals and hydrogen peroxide are highly unstable in vivo and their fast reaction with other substances will lead to the damage of cell or tissue (Halliwell, 2012). Under normal circumstances, the human body can maintain the balance of free radical production and clearance through its own defence system. However, the accumulation of free radicals brings out negative effect when diseases struck (Haliwell, 2012). Therefore, proper intake of antioxidant can prevent the damage caused by free radical and hinder the development of many chronic diseases (Lobo et al., 2010).

As one of the bioactive peptides, antioxidant can achieve the function of resisting disease by scavenging free radicals. Antioxidant peptides can be divided into chemical and natural by source (Ndhlala et al., 2010). Though chemical antioxidant peptides have been widely used in food inhibition of lipid oxidation, security issues limited the further application. The natural antioxidant peptides (Brewer, 2011) become more popular for its remarkable antioxidant activity and high safety. Thus, natural antioxidant peptides become the focus of study. Goat milk (Haenlein, 2004), rich in protein, vitamin, fats and mineral substance, was known as the "king of milk". Goat milk protein consists of whey protein and casein. The content of casein in goat milk were lower than milk, meanwhile, higher content of whey protein was similarly to breast milk. Casein is not easy to digest because of coagulation in stomach. Goat milk protein is more susceptible to digestion because of lower ratio of casein composition. Furthermore, lower content of casein in goat milk make it safer to drink free from anaphylaxis.

Alcalase (Zhang et al., 2014) was screened out as the optimum enzyme for preparation of casein hydrolysates from goat milk in previous work (Shu et al., 2015). Besides, the effect of temperature, time, pH, substrate concentration and enzyme to substrate (E/S) ratio had been

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explored in previous study (Shu et al., 2016). Basing on the results of factorial experiment, temperature of 62.5°C and E/S of 2.5% were determined. Box-Behnken design was used to optimize the condition of enzymatic preparation of antioxidant peptides from goat milk. Concretely, pH, substrate concentration and time were selected as variables; DPPH free radical scavenging activity (Dawidowicz et al., 2012), metal-chelating effect (Gülçin et al., 2007) and superoxide anion radical scavenging activity (Chang et al., 1996) were chosen as response.

MATERIALS AND METHODS

Materials: Goat milk was offered by Hongxing Dairy Co., Ltd. (Shaanxi, China). 1,1- diphenyl – 2-prcrylhudrazyl and Alcalase were bought from Sigma – Aldrich (St. Louis, Mo, USA). Other reagents used in the study were of analytical grade.

Preparation of goat milk casein: Reconstituted milk was obtained by mixing distilled water and goat milk powder at a ratio of 1:8 (W/V). Skim milk was obtained after centrifugation at 5000r/min for 15minutes. Precipitation of casein formed after skim milk heated at 46°C and adjusting pH to 4.6 by 1M HCl. After the second centrifugation at 6500r/min for 15 minutes, the precipitate of casein was dried via vacuum freezing for 24 h.

Preparation of antioxidant peptides: 0.1 M NaOH as cosolvent promoted casein dissolution in distilled water. Appropriate amount of Alcalase were added into the casein solution. Then, the solution was adjusted to proper pH. The temperature was also set at the optimum value via constant temperature water bath. Keep pH stable at the optimum value through adding 0.1 M NaOH continuously. Enzymolysis ended by a 15 minutes' killing enzyme in a 95°C thermostatic water bath. pH of hydrolysates was adjusted to 3.4 and supernatant was collected after centrifugation at 6000r/min for 15 minutes, then, regulating the pH to 8.3 and collecting the supernatant after centrifugation at 6000r/min for 15 min.

Data Analysis: Design-Expert software was used to analyse the results of experiment, fit the optimized data with response surface model and verify the reliability of the model.

Quantitative Analysis of antioxidative activity

DPPH free radical scavenging assay: The free radical scavenging activity of hydrolysates on α，α – diphenyl –β– picrylhydrazyl (DPPH) was determined by the method of (Wu et al., 2003) In brief, 2ml of sample was added to 2ml of 0.1mM DPPH - 95% ethanol solution. After shaking and standing for 30 minutes at room temperature, absorbance was measured at 517 nm. The activity was determined by equation 1:

$$DPPH\ free\ radical\ scavenging\ activity\ (\%) = [1 + (D_2/D_0 - D_1/D_0)] \times 100\% \quad (1)$$

In equation (1), D_0 represents absorbance of control group (DPPH-ethanol), D_1 represents absorbance of sample group (sample-DPPH) and D_2 represents absorbance of blank group (sample-ethanol).

Metal chelating assay: Metal chelating effect was evaluated by the method of (Decker and Welch, 1990). Solution containing 0.1 ml of 2 mM $FeCl_2$, 1 ml of hydrolysate sample, 0.2 ml of 5 mM ferrozine and 3.7 ml of distilled water were well mixed in tube. After standing for 20 minutes at room temperature, the absorbance of solution were measured at 562 nm. Chelating rate was calculated by equation 2:

$$Chelating\ effect\ (\%) = (1 - C_1/C_0) \times 100\% \quad (2)$$

In equation 2, C_0 represents the absorbance of control group and C_1 represents the absorbance of sample group.

Superoxide anion radical scavenging assay: The method of (Markland and Markland, 1974) was adopted to measure the effect of hydrolysates on superoxide radical. Mixing 0.2 ml of hydrolysate sample with 5.6 ml of 50 mM Tris- HCl buffer (pH=8.2) in tube. The reaction began with the addition of 0.1 ml of 5m M pyrogallol and the absorbance were measured at 325 nm every 30s last for 5 minutes. Scavenging activity of superoxide anion was evaluated by equation 3:

$$Superoxide\ anion\ radical\ scavenging\ activity\ (\%) = [(S_0/min - S_1/min)/S_0/min] \times 100\% \quad (3)$$

In equation (3), S_0/min represents the absorbance of control group (buffer and distilled water) every minute and S_1/min represents the absorbance of sample group (sample and buffer), every minute.

The Design of Box-Behnken method: Three significant factors including pH, substrate concentration and time were selected for further optimization from the results of factorial experiment. A BBD (Annadurai et al., 1999) factorial 3^3 experimental design was developed with three variables at three levels. The design can be used for establishing a second-order polynomial model. The optimization was accomplished by a small number of experiments (15 runs) due to the superiority of BBD method.

In Table 1, variable A is pH, variable B is substrate concentration and variable C is time.

Table 1. The experimental factor and code levels of production peptide conditions by Alcalase

Level	A	B	C
-1	8.6	4.3	165
0	8.8	4.4	170
1	9.0	4.5	175

RESULTS AND DISCUSSIONS

Design and results of Alcalase enzymatic hydrolysis of casein by Box-Behnken

Box-Behnken design and results were listed in Table 2; variable A is pH, variable B is substrate concentration and variable C is time. Response 1 is DPPH radical scavenging activity, Response 2 is chelating effect and Response 3 is scavenging activity of O_2^-.

According to the experimental results (Table 2), using Design-Expert software to build response surface model and analyse the data, the regression equations of this experiment are 4, 5 and 6. In the equations 4-6, R1 means the predicted value of DPPH free radical scavenging activity, R2 means the predicted value of chelating effect and R3 means the predicated value of scavenging activity of O_2^-. A, B and C represent pH, substrate concentration and time, respectively.

$$R_1=70.70 + 0.57A - 0.91B + 0.62C + 0.44AB - 1.52AC + 0.14BC - 4.44A^2 - 7.52B^2 - 1.05C^2 \quad (4)$$
$$R_2=84.66 - 1.13A + 0.42B + 1.56C + 0.12AB + 0.45AC + 0.81BC - 5.7A^2 - 0.62B^2 + 3.91C^2 \quad (5)$$
$$R_3=50.18 - 0.52A - 1.21B + 0.014C - 2.74AB - 0.41AC + 1.09BC - 4.54A^2 - 5.75B^2 - 5.66C^2 \quad (6)$$

Table 2. The B-B design and results for the responses of production peptide conditions by Alcalase

Run	A	B	C	R1(%)	R2(%)	R3(%)
1	9	4.5	170	59.52	77.89	35.21
2	9	4.3	170	60.62	76.04	45.71
3	8.8	4.5	165	60.98	85.49	38.39
4	8.8	4.3	175	63.01	88.79	36.99
5	9	4.4	165	65.49	80.04	38.16
6	8.6	4.5	170	55.99	80.41	39.58
7	8.6	4.3	170	58.85	79.04	39.1
8	8.8	4.4	170	71.59	85.53	49.04
9	8.8	4.4	170	70.31	83.23	50.53
10	8.6	4.4	175	67.99	84.82	42.64
11	8.8	4.3	165	62.93	87.04	40.41
12	8.6	4.4	165	62.82	82.72	40.52
13	8.8	4.4	170	70.21	85.23	50.98
14	8.8	4.5	175	61.61	90.49	39.33
15	9	4.4	175	64.56	83.94	38.63

As shown in Table 3, F value of 21.59 and P< 0.01 indicated a high significance of the model. Lack of fit (P=0.2308>0.05) also reflected the effectiveness of model. The results showed that the model of regression equation suit well to R1 (DPPH free radical scavenging activity). Coefficient determination (R^2= 0.9749) indicated high consistence

between R1 and the value predicted by model. Adjusted determination coefficient (R_{adj}^2 = 0.8883) showed that over 88.83% of the response value were affected by the change of variable. Thus, the model is reliable to analyse and predicate the trend of R1 when variables changed. The effects of the independent variable on the dependent variable were determined by F-test of the variance analysis of the regression equation. Thus, the order of the factors affecting the DPPH free radical scavenging activity was obtained as follow: B>A>C. Substrate concentration indicated a highest effect on R1, pH and time were secondly. Low significance of variable A and C reflected a non-linear correlation between variables and R1. F value of variable B also showed its certain contribution to model. Higher F value of A2 and B2 contribute more to model. The p value of AC (p=0.0538<0.1) indicated the interaction between them has a significant effect on R1.

Table 3. The ANOVA of Box-Behnken of DPPH free radical scavenging activity

Source	SS	DF	MS	F	Pr > F	Significance
Model	286.76	9	31.86	21.59	0.0017	***
A (pH)	2.58	1	2.58	1.75	0.2436	
B (substrate concentration)	6.68	1	6.68	4.53	0.0867	*
C (time)	3.068	1	3.06	2.08	0.2092	
AB	0.778	1	0.77	0.52	0.5013	
AC	9.308	1	9.30	6.30	0.0538	*
BC	0.076	1	0.075	0.051	0.8299	
A^2	72.72	1	72.728	49.28	0.0009	***
B^2	208.82	1	208.828	141.50	< 0.0001	***
C^2	4.07	1	4.078	2.76	0.1575	
Residual	7.38	5	1.48			
Lack of Fit	6.198	3	2.068	3.49	0.2308	
Pure Error	1.188	2	0.598			
Cor Total	294.148	14				

* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$

Variance analysis of DPPH free radical scavenging rate regression equation

In order to evaluate the significance of regression equation and explore the significant factors, variance analysis was performed using Design-Expert. The results were shown in Tables 3-5.

The interaction of various factors on the optimum hydrolysis condition were analysed by response surface methodology. Response surface graphs based on the polynomial quadratic regression model were constructed to make a clear understanding of interactive effect between variables on response one. Mutual interaction between A×C which can be found from the elliptic contour plots in Figure 2 had a significant effect on R1. Though elliptic contour plots were also shown in figures 1-3, the mutual interaction of A × B and B × C were not significant. Maximum cannot be found when the variables close to the centre as the 3D surface plots shown in figures 1-3.

Variance analysis of chelating effect regression equation

As shown in table 4, F value of 25.92 and P< 0.01 indicated a high significance of the model. Lack of fit (P=0.7876>0.05) also reflected the effectiveness of model. The results show that the model of regression equation suit well to R2 (chelating effect). Coefficient determination (R^2= 0.9790) indicated high consistence between R2 and the value predicted by model. Adjusted determination coefficient (R_{adj}^2= 0.9412) showed that over 94.12% of the response value were affected by the change of variable. Thus, the model is reliable to analyse and predicate the trend of R2 when variables changed.

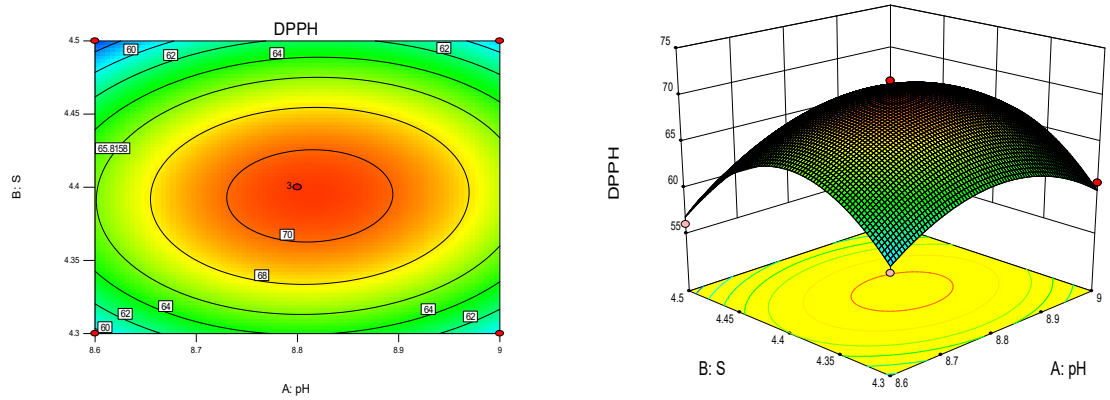
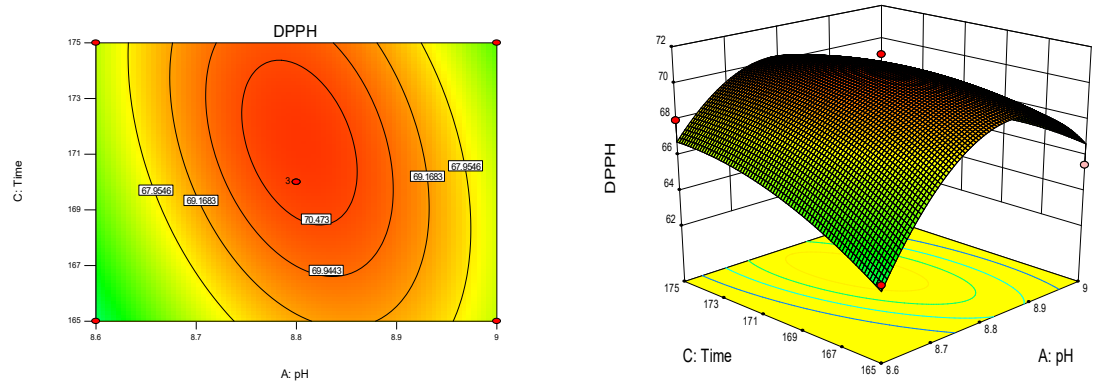


Figure 1. Effect of mutual interaction between pH and substrate concentration on DPPH free radical scavenging activity



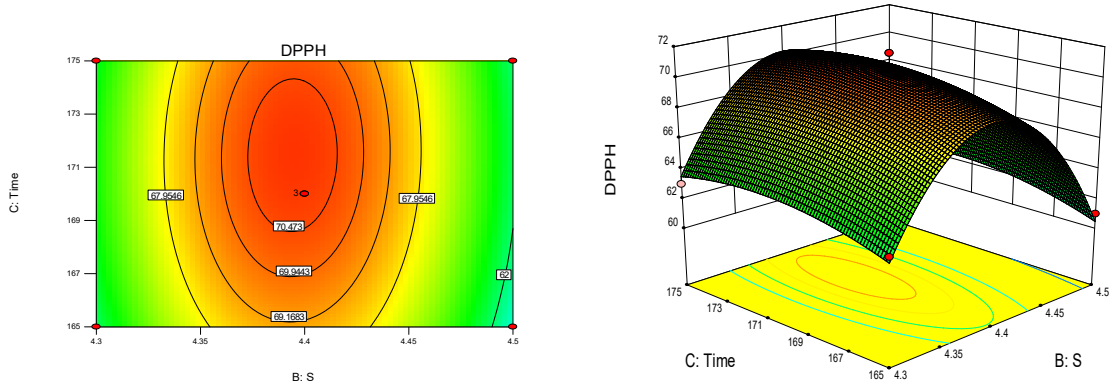


Figure 3. Effect of mutual interaction between substrate concentration and time on DPPH free radical scavenging activity

The effects of the independent variable on the dependent variable were determined by F-test of the variance analysis of the regression equation (Table 4). Thus, the order of the factors affecting the chelating effect was obtained as follow: C>A>B. Time indicated a highest effect on R2, pH and substrate concentration were secondly. High F value of variable A and C reflected significant effect on R2. Lower F value of variable B also showed its certain contribution to model. Higher F value of A^2 and C^2 contribute more to model. The lower p value of AB, AC and BC ($p=0.0538<0.1$) indicated the interaction between them had few significant effect on R2.

Table 4. The ANOVA of metal-chelating effect

Source	SS	DF	MS	F	Pr > F	Significance
Model	226.5307	9	25.17	25.92	0.0011	***
A (pH)	10.31	1	10.31	10.61	0.0225	**
B (substrate concentration)	1.42	1	1.42	1.46	0.2807	
C (time)	20.32	1	20.32	20.93	0.0060	***
AB	0.058	1	0.058	0.059	0.8173	
AC	0.81	1	0.81	0.83	0.4030	
BC	2.64	1	2.64	2.72	0.1601	
A^2	119.77	1	119.77	123.34	0.0001	***
B^2	1.43	1	1.433	1.48	0.2787	
C^2	56.51	1	56.51	58.19	0.0006	***
Residual	4.86	5	0.97			
Lack of Fit	1.73	3	0.58	0.37	0.7876	
Pure Error	3.13	2	1.56			
Cor Total	231.386	14				

* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$

The interaction of various factors and the optimum hydrolysis condition were analysed by 3D surface plots and contour plots. As shown in figure 4, oval contour plots indicated the significant mutual interaction between A × B. 3D surface plots is convex showed increase trend of R2 when A and B increased. The maximum value cannot be found on the plots and the predicated maximum value may appear beyond the scope of experiment. Irregular contour in figures 5, 6 indicated the mutual interaction of A × C and B × C were not significant. 3D surface plots is saddle shaped and saddle point was selected as stable point.

Variance analysis of superoxide anion scavenging activity regression equation

The results of variance analysis were shown in table 5. F value of 6.45 and P< 0.05 reflected a high significance of the model. Lack of fit (P=0.1088>0.05) also indicated the effectiveness of model. The results showed that the model of regression equation suit well to R3 (superoxide anion radical scavenging activity). Coefficient determination (R^2= 0.9207) indicated high consistence between R3 and the value predicted by model. Adjusted determination coefficient (R_{adj}^2= 0.7781) showed that over 77.81% of the response value were affected by the change of variable. In summary, the order of the factors affecting the chelating effect was obtained as follow: B>A>C. Substrate concentration indicated a highest effect on R3, pH and time were secondly. Higher F value of AB showed that mutual interaction between A×B had significant effect on R3. Higher F value of A^2 and C^2 contribute more to the model.

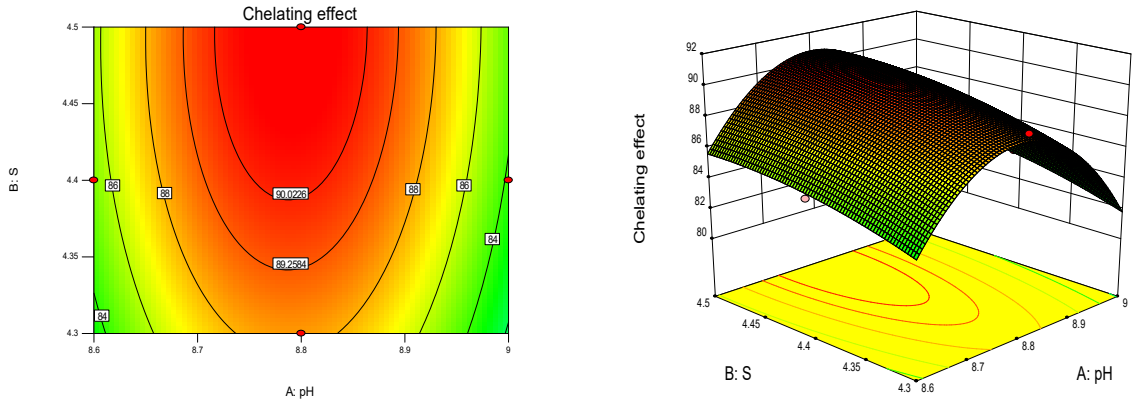


Figure 4. Effect of mutual interaction between pH and substrate concentration on Chelating effect

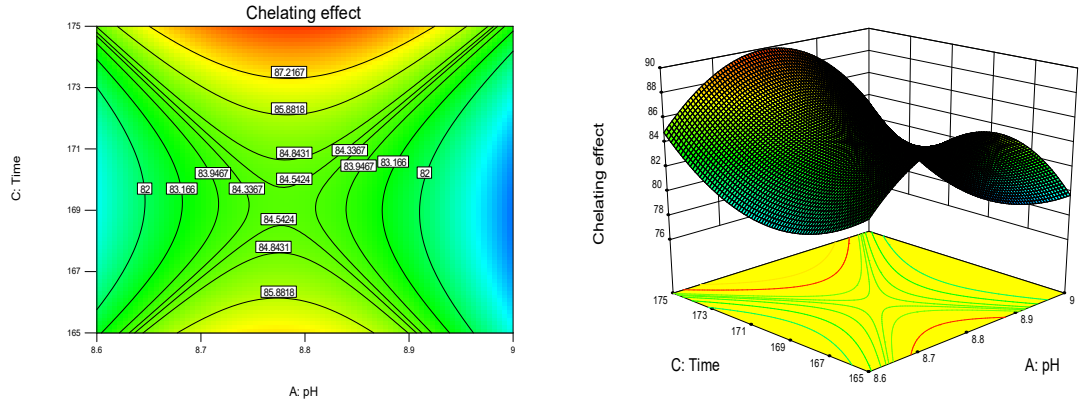


Figure 5. Effect of mutual interaction between pH and time on chelating effect

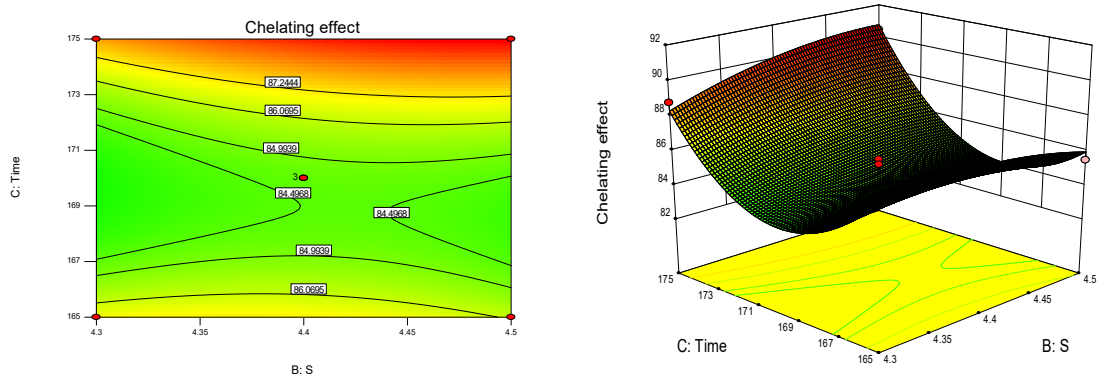


Figure 6. Effect of mutual interaction between substrate concentration and time on chelating effect

Table. 5 The ANOVA of superoxide anion radical scavenging activity

Source	SS	DF	MS	F	Pr > F	Significance
Model	324.16	9	36.02	6.45	0.0269	**
A (pH)	2.136	1	2.13	0.38	0.5636	
B (substrate concentration)	11.766	1	11.76	2.107	0.2063	
C (time)	0.00156	1	0.0015	0.00027	0.9875	
AB	30.146	1	30.14	5.40	0.0677	*
AC	0.686	1	0.68	0.12	0.7412	
BC	4.756	1	4.75	0.85	0.3985	
A^2	76.03	1	76.03	13.62	0.0141	**
B^2	121.88	1	121.88	21.83	0.0055	***
C^2	118.20	1	118.20	21.18	0.0058	***
Residual	27.91	5	5.58			
Lack of Fit	25.84	3	8.61	8.36	0.1088	
Pure Error	2.06	2	1.031			
Cor Total	352.07	14				

* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$

The contour plots and 3D-surface plot were shown in figures 7-9. The contour plots of A × C is oval shaped which revealed the significant mutual interaction between them. Circular shape of B × C and A × C in figures 8 and 9 suggested their mutual interaction were not significant. All the 3D surface plots are convex shaped and maximum value could be found near the centre.

pH, substrate concentration and time were selected as main factors for antioxidative activity of casein hydrolysates. 8.0 of pH was chose as the optimum value in previous factorial experiment. The active site (He et al., 2011) of enzyme consists of binding site and catalytic site. Only enzyme maintains a certain spatial conformation can reach the optimum catalytic result. The active site group are sensitive to the change of pH and its dissociation state will affected by pH. Conformation of enzyme molecule will be affected by the change of active site. In a word, the effect of pH (Kumar et al., 2016) on antioxidative activity of casein hydrolysates is many-sided. Dissociation of necessary group on active site and ionization state of proton donor and proton acceptor (Isogawa et al., 2009) are all affected by the change of pH. Besides, dissociation degree of substrate and coenzyme are also affected by pH which has effects on the binding of enzyme to substrate. Thus, 8.9 of pH were selected as the optimum result for antioxidative activity of casein hydrolysates to make a further optimization.

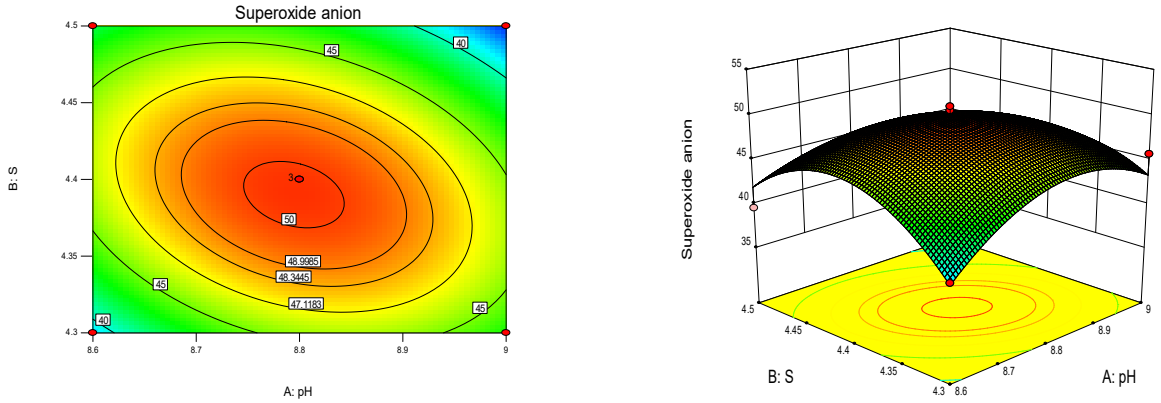


Figure 7. Effect of mutual interaction between pH and substrate concentration on superoxide anion radical scavenging activity

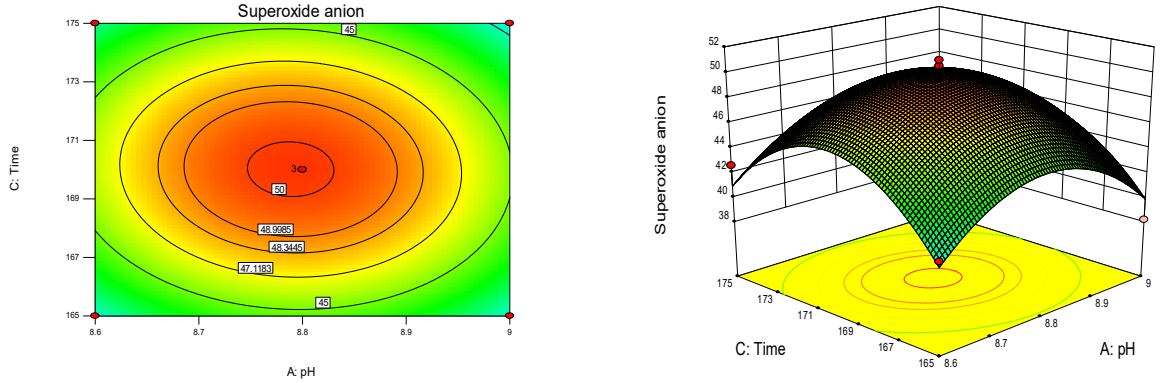


Figure 8. Effect of mutual interaction between pH and time on superoxide anion radical scavenging activity

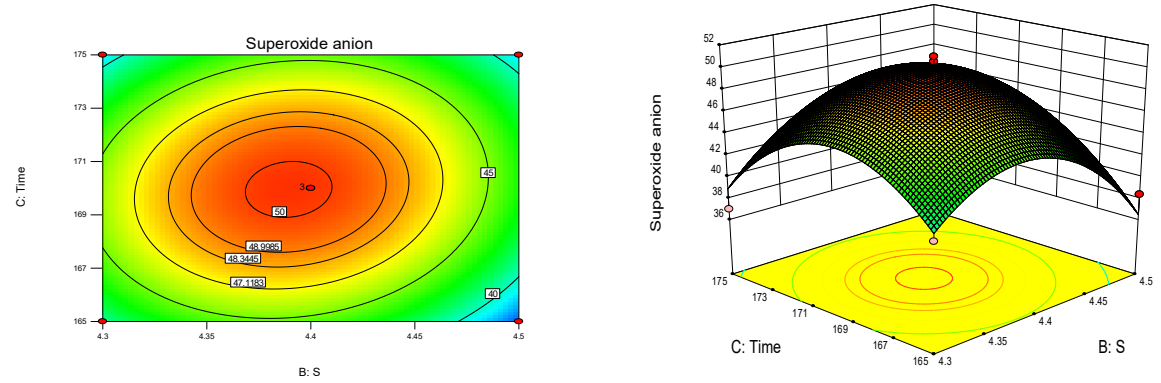


Figure 9. Effect of mutual interaction between substrate concentration and time on superoxide anion radical scavenging activity

Time have a significant influence on antioxidative activity of casein hydrolysates. Most of time, because more antioxidant peptides accumulated during casein hydrolysis, the antioxidative activity increased with time went by. After the critical time, the efficiency of hydrolysis decrease which led to a decline trend of antioxidative activity. Therefore, 150 minutes was selected as the optimum value in previous factorial experiment and a better result of 173 minutes was obtained by further optimization in this study. Furthermore, substrate concentration was also found as a main factor on antioxidative activity of casein hydrolysates. Proper concentration of substrate will promote antioxidative activity of casein hydrolysates efficiently. 4% of substrate concentration was selected as the optimum value in previous factorial experiment and a better result of 4.4% was obtained by further optimization in this study. In this study, mutual interaction between pH and time has a positive effect on DPPH free radical scavenging activity (Peng et al., 2010). For metal-chelating effect (Kim et al., 2007), mutual interaction between pH and substrate concentration play a key role. Besides, the mutual interaction between pH and substrate also indicated a positive effect on superoxide anion radical scavenging activity (Mao et al., 2011).

CONCLUSIONS

Main effects of pH, time and substrate on antioxidative activity of casein hydrolysates were optimized by response surface methodology. The mathematical model is established, and variance analysis indicated remarkable significance of model. The optimum conditions were as follows: 62.5°C of temperature, 8.9 of pH, 4.4% of substrate concentration, 2.5% of E/S and the hydrolysis time was 173 minutes. Under this condition, the DPPH free radical scavenging ability was 69.07 ± 1.26%, activity of metal-chelating was 87.21 ± 0.88%, and superoxide anion radical scavenging activity was 49.18 ± 1.42%. There nearly no difference between the results and predicated value. Thus, the optimized data fit well to model and the optimized parameters are reliable.

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