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Effects on ABTS Radical Inhibition and Functional Groups of Soybean Antioxidant Peptides （SAP） Processed by Microwave Assisted Enzymatic Digestion

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Abstract. Box-Behnken design (BBD) of response surface methodology (RSM) were used to optimize soybean antioxidant peptides (SAP) processed by microwave assisted enzymatic digestion. Antioxidant activity was estimated by ABTS radical inhibition, change of functional group was determined by Mid-infrared spectroscopy (MIR). Compared to enzymatic hydrolysis, ABTS radical inhibition of SAP processed by microwave assisted enzymatic digestion were increased 9.12 % under the optimized conditions of microwave time 37 min, microwave temperature 56 ℃, pH of 8.17, [E/S] of 5:100, [S] of 50 g L^{-1} and microwave power 500 W. SAP processed by microwave assisted enzymatic digestion possess the different functional groups of S-S, C-C, C-S, C-SO_2-C, N=N, ≡C-H. The activity and functional groups of SAP processed by microwave assisted enzymatic digestion technology were difference with enzymatic hydrolysis.

Keywords: Soybean antioxidant peptides (SAP), Microwave assisted enzymatic digestion, ABTS, Mid-infrared spectroscopy (MIR);

1. Introduction

ABTS radical inhibition activity is one of the most commonly used organic radicals for the evaluation of antioxidant activity of complex mixtures and pure compounds [1]. Soybean protein was widely used as functional nutritional ingredients in food products. It was investigated that the soybean antioxidant peptides (SAP) possessed strong antioxidant activity [2]. Enzymatic hydrolysis under controlled conditions is an effective way of improving the functional properties of protein without affecting its nutritive value [3]. Nevertheless, conventional enzymatic hydrolysis methods typically involve several hours or overnight incubations. Microwave irradiation was recognized in the mid-1980s to be an efficient heating source for chemical reactions, where reactions that require several hours under conventional conditions can often be completed in a few minutes with very high yields and reaction selectivity [4]. In the area of peptide biochemistry, the use of microwave-assisted reactions has been very limited. Although the ability of applying microwave irradiation to accelerate enzymatic digestion was well established, digestion products obtained from microwave-assisted and conventional enzymatic hydrolysis have not been investigated regarding differences of the antioxidant activity. Change of functional group was determined by Mid-infrared spectroscopy (MIR) [5].

2. Materials and Methods

2.1. Materials and reagent

Soybean protein powder (protein content of 70 %) was obtained from Ningbo SuoBao Co. (China). Alcalase was purchased from Fanfuer International Chem Co. (Tianjin China). 2, 2-azinobis (3-ethyl-benzothiazo line-6-sulfonic acid) (ABTS) was purchased from Sigma Chemicals Co. (USA). The sodium

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hydroxide, disodium hydrogen phosphate, sodium dihydrogen, phosphate, formaldehyde were sourced from Beijing Chemical Plant (China) and were of analytical grade purity.

2.2. Instruments

Microwave extraction apparatus (MAS-Ⅱ), ultraviolet spectrophotometer (UV-2550), IRrestige－21 Fourier Transform Infrared Spectroscopy (Shimadzu) were provided by the Laboratory of Nutrition and Functional Food of Jilin University.

2.3. The preparation method of SAP

A domestic microwave extraction apparatus was used to conduct the microwave-assisted enzymatic digestion, setting the instrument parameter including extraction time, temperature and microwave power according to the experimental optimization before used. Briefly, soybean protein powder was dissolved in distilled water in a 200 mL microwave extraction bottle followed by a heat treatment at 90℃ in a water bath for 10 min to denature the protein before the suspension mixture was cooled to room temperature. Then, the pH value and temperature of the suspension mixture were adjusted to the required values. Varying amounts of Alcalase enzyme were added to the suspension mixture according to the experimental optimization. Then the suspension mixture was transferred into the microwave extraction apparatus and the agitator of microwave extraction apparatus was initiated. When the reaction was finished, the solution was heated in a water bath at 100 ℃ to inactivate the enzyme and then centrifuged at 8000 × g for 10 min at 4 ℃ The supernatant liquor was freeze-dried, placed in sealed bags, and stored in desiccators until using [6]. The enzymatic hydrolysis time was 4 hours but other experimental condition was same with microwave-assisted enzymatic digestion.

2.4. Optimization of SAP preparation program

RSM was used to evaluate the effects of the variables that might have impacts on the ABTS radical inhibition of SAP, according to the results of the independent variable experiments. Calculations were performed using the Design Expert Software (Trial Version 8.0.0, Stat-Ease Inc., USA). A Box-Behnken design (BBD) with three variables including five replicates at the centre point was used for fitting the second-order response surface. The three independent variables were the microwave time(X_1), microwave temperature(X_2) and pH value(X_3) respectively（presented in Table 1）. The three variables and their ranges were chosen according to the preliminary experiment results. Each of them 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. The order of the experiments was random to minimize the effects of unexplained variability which might be caused by extraneous independent variables. ABTS radical inhibition of soybean peptides was determined using the methodology of Lin et al [7].

Table 1. Independent variables and their levels used for Box-Behnken rotatable design

Independent variable	Level		
	-1	0	1
Microwave time(X_1)	30	35	40
Microwave temperature(X_2)	50	55	60
pH value(X_3)	7.5	8.0	8.5

2.5. Analysis functional groups of SAP by MIR

According to the method described by our previous report [7], [8], MIR was used to analyse the change of functional group between the SAP of processed by microwaved assisted enzymatic digestion and enzymatic hydrolysis. MIR was measured with the spectroscopy at a resolution of 4 cm^{-1}, over the range (4000 - 400 cm^{-1}).

3. Results and Discussion

3.1. Results of statistical analysis and model fitting RSM

RSM was used to optimize the conditions for improving the ABTS radical inhibition of soybean peptides processed by microwave assisted enzymatic digestion. The influence of microwave time, microwave

temperature and pH value on the ABTS radical inhibition is shown in Table 2. A response surface quadratic model was drawn and the statistical analysis for the linear, the quadratic and the interaction of the three variables on the response values is presented in the Table 3. The *P*-value for the model was less than 0.0001, which indicated that the model was significant and could be used to monitor the optimization. Among the three independent variables, all of them exerted extremely significant effects on the ABTS radical inhibition value within a 99 % confidence interval ($P = 0.006 < 0.01$ for X_1, $P = 0.0012 < 0.01$ for X_2, $P = 0.0009 < 0.01$ for X_3), the interaction term $X_1 \times X_2$ ($P < 0.05$) was significant, but $X_1 \times X_3$ and $X_2 \times X_3$ were not significantly influential ($P > 0.05$). Among the three quadratic terms, $X_1 \times X_1$ ($P < 0.01$) and $X_2 \times X_2$ ($P < 0.01$) exerted extremely significant effects, $X_2 \times X_2$ ($P < 0.05$) was significant. Therefore, the change of the response value was very complex, and the effects of each experimental factor on the ABTS radical inhibition was not a simple linear relationship but a second-order relationship. The final mathematical model can be expressed by the following quadratic equations: $Y=71.03 + 1.23X_1 + 1.10X_2 + 1.16X_3 + X_1X_2 + 0.23X_1X_3 + 0.39X_2X_3 - 2.66X_1^2 - 3.58X_2^2 - 1.97X_3^2$, Where Y is the ABTS radical inhibition, and X_1, X_2, X_3 were the variables for the microwave time (min), microwave temperature (℃) and pH value respectively. The statistical analysis for the model (Table 3) showed the "lack of fit" was not significant ($P = 0.0914 > 0.05$). A small value of R-Squared indicates a poor relevance of the dependent variables in the model. When the R-Squared approaches unity, the model fitted well with the experimental data. As shown in Table 3, a good agreement with the experimental results with a coefficient (R-Squared) of 0.9670 was found. "Adjusted R-Squared" was 0.9621, which indicates that the model explains 96.21% of the variation in the data and the experiment error was very small. The value of "Predicted R-Squared" (0.7898) was close to that of "Adjusted R-Squared" (0.9621). "Adequate Precision" measures the signal-to-noise ratio and usually, a ratio greater than 4 is desirable. For this model, the "Adequate Precision" ratio was 17.2360, which is an adequate signal-to-noise ratio. Therefore, this model proved to be powerful for navigating the design space.

Table 2. Box-Behnken design matrix and response values for ABTS radical inhibition

Experimental number	X_1	X_2	X_3	Actual ABTS radical inhibition (%)	Predicted ABTS radical inhibition (%)
1	0	1	-1	64.64 ±0.37	65.03
2	0	0	0	70.82 ±0.12	71.03
3	-1	0	-1	64.78 ±0.36	64.24
4	-1	0	1	66.34 ±0.07	66.09
5	1	-1	0	64.36 ±0.16	64.20
6	-1	1	0	63.78 ±0.09	63.94
7	-1	-1	0	62.54 ±0.26	63.18
8	0	0	0	70.64 ±0.25	71.03
9	0	0	0	71.58 ±0.32	71.03
10	1	1	0	68.48 ±0.28	67.84
11	0	0	0	71.24 ±0.07	71.03
12	0	1	1	68.04 ±0.24	68.14
13	0	-1	1	65.54 ±0.39	65.15
14	0	0	0	70.87 ±0.18	71.03
15	1	0	1	68.48 ±0.56	69.02
16	0	-1	-1	63.71 ±0.22	63.62
117	1	0	-1	65.98 ±0.18	66.23

3.2. Results of response surface

The responses were obtained using Expert 7.0 software. Effects of microwave time, microwave temperature and pH value on ABTS radical inhibition were illustrated in Fig. 1. The effects of microwave time and microwave temperature were illustrated in Fig. 1A where the pH value remained constant at 8.0. The behavior of Fig.1A indicated that the ABTS radical inhibition increased at first and then decreased with the increasing of microwave time, (X_1) and temperature (X_2). The maximum ABTS radical inhibition was observed at around 36min and 56 ℃. This result also showed that the ABTS radical inhibition had a

curvilinear relationship with the microwave temperature and the quadratic term was significant. Effects of microwave time (X_1) and pH value (X_3) were shown in Fig. 2B, where the microwave temperature was constant at 55 ℃. A maximum ABTS radical inhibition was observed at around 35 ~ 37.5 min and about pH 8.0 ~ 8.25. Fig.2C showed that the pH value (X_3) and microwave temperature (X_2) demonstrated quadratic effects on the ABTS radical inhibition when the microwave time was fixed at 35 min. The ABTS radical inhibition increased at first and then decreased with the increasing of microwave temperature and pH value. The results confirmed that the predicted maximum ABTS inhibition (Y) of 71.50 % was obtained with the following conditions: microwave time 36.36 min, microwave temperature 56 ℃, pH value 8.17, [E/S] of 5:100, [S] of 50 g L^{-1}, microwave power of 500 W. While the actual maximum ABTS inhibition (Y) of 72.06 ±0.32% was obtained with the following conditions: microwave time 37 min, microwave temperature 56 ℃ and pH value 8.17, [E/S] of 5:100, [S] of 50 g L^{-1}, microwave power 500 W. The error of between practical value and actual value was less than 1%, so the model was valid.

Table 3. Regression coefficients estimate and significance test for the quadratic polynomial model

Model term	Coefficient estimate	Standard error Sum of square		Mean square	F-value	Probability	significance
Model	146.46	9.00	16.27	46.13	<0.0001	Significant	**
X_1	12.15	1.00	12.15	34.45	0.0006		**
X_2	9.66	1.00	9.66	27.38	0.0012		*
X_3	10.79	1.00	10.79	30.58	0.0009		*
$X_1 \times X_2$	2.07	1.00	2.07	5.88	0.0458		*
$X_1 \times X_3$	0.22	1.00	0.22	0.63	0.4547		
$X_2 \times X_3$	0.62	1.00	0.62	1.75	0.2278		
X_1^2	29.88	1.00	29.88	84.70	< 0.0001		
X_2^2	53.85	1.00	53.85	152.66	< 0.0001		**
X_3^2	16.36	1.00	16.36	46.38	0.0003		**
Residual	2.47	7.00	0.35				
Lack of fit	1.90	3.00	0.63	4.46	0.0914		
Pure Error	0.57	4.00	0.14				
Cor Total	148.93	16.00					

R= 0.9834, R-Squared=0.9670, Adjusted R^2= 0.9621,; predicated R^2= 0.7898, Adeq Precision=17.2360. ** The extremely significant difference was determined with *P<0.01*. * The significant difference was determined with *P<0.05*.

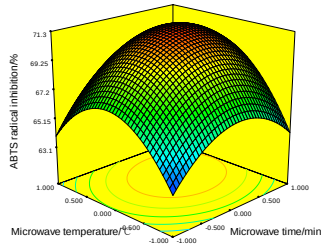

Fig.1A

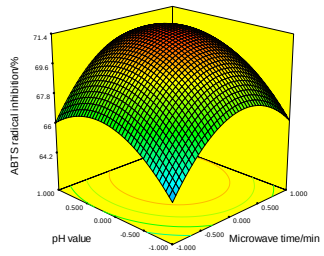

Fig.2B

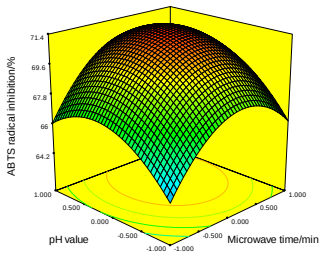

Fig.3C

Fig. 1: Response surface of ABTS radical inhibition of the soybean peptide.

3.3. Analysed the change of functional group by MIR

MIR spectra of SAP processed by microwave assisted enzymatic digestion in Fig. 2A and enzymatic hydrolysis was show in Fig. 2B. The Fig. 2A was observed in 3288.63, 3066.82, 2960.73, 2935.66, 2877.79, 1662.64, 1654.92, 1616.35, 1558.48, 1541.54, 1398.39, 1317.38, 1246.02, 1074.35, 1049.28, 700.16, 624.94, 601.79, 592.15, 574.79, 555.5 and 540.07 cm^{-1}. While，the Fig. 2B was observed at 3292.49, 3066.82, 2960.73, 2935.66, 1662.64, 1654.92, 1541.92, 1541.12, 1446.61, 1398.39, 1242.16, 1074.35, 1049.28 cm^{-1}. Therefore the =C-H, O-H, N-H, -C-H, C=C, C=N, N=N, C=C, C-NO_2, N=N, -CH_3, -CH_2, C-SO_2-C, C-O-C, C-SO-C, C=S, C-S, C-C and S-S, were observed in soybean peptides processed by microwave assisted enzymatic digestion. The =C-H, O-H, N-H, -C-H, C=N, C=C, C-NO_2, N=N, -CH_3, -CH_2, C-O-C and C=S were observed in soybean peptides processed by enzymatic hydrolysis. The fraction of peptide bonds in α-

helical, β -sheet, and aperiodic conformations can be accurately estimated by analysis of the amide band (1600–1700 cm^{-1}) in the mid-IR region.

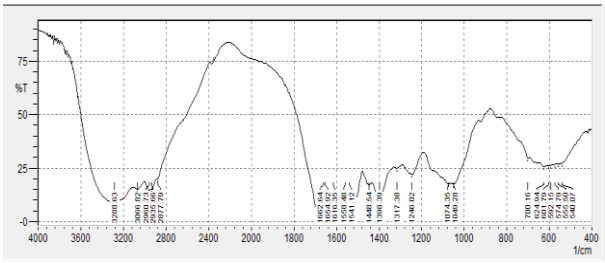

Fig.2A

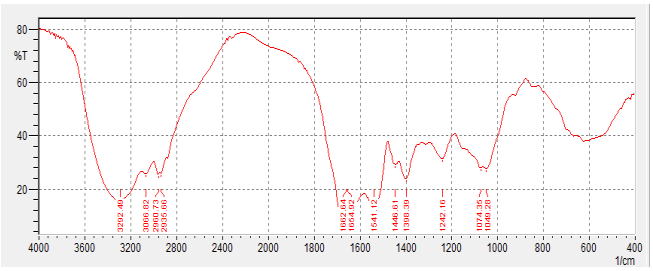

Fig.2B

Fig. 2: The MIR spectra of peptide fractions

4. Conclusions

The effect of microwave assisted enzymatic digestion on improving ABTS radical inhibition of the SAP was observed. The ABTS radical inhibition of SAP can be improved by microwave assisted enzymatic digestion processing. Based on the experiments results, it can be seen that microwave time, microwave temperature, and pH value had significant effects on the response values. Optimized conditions showed an increase in ABTS radical inhibition of 9.12 %, compared to the soybean peptides obtained from enzymatic hydrolysis (time was 4 hours but same other experimental condition), which is increased significantly. It can be concluded that the activity and functional groups of SAP processed by microwave assisted enzymatic digestion technology were difference with enzymatic hydrolysis.

5. Acknowledgements

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