Supplementary data 1: Optimization experiment on proteolysis process of DWMP

In this study, the peptide yield and anti-inflammatory activity of hydrolysates of walnut meal hydrolyzed by six different proteases were investigated. Firstly, the safety of six protease hydrolysates was studied.

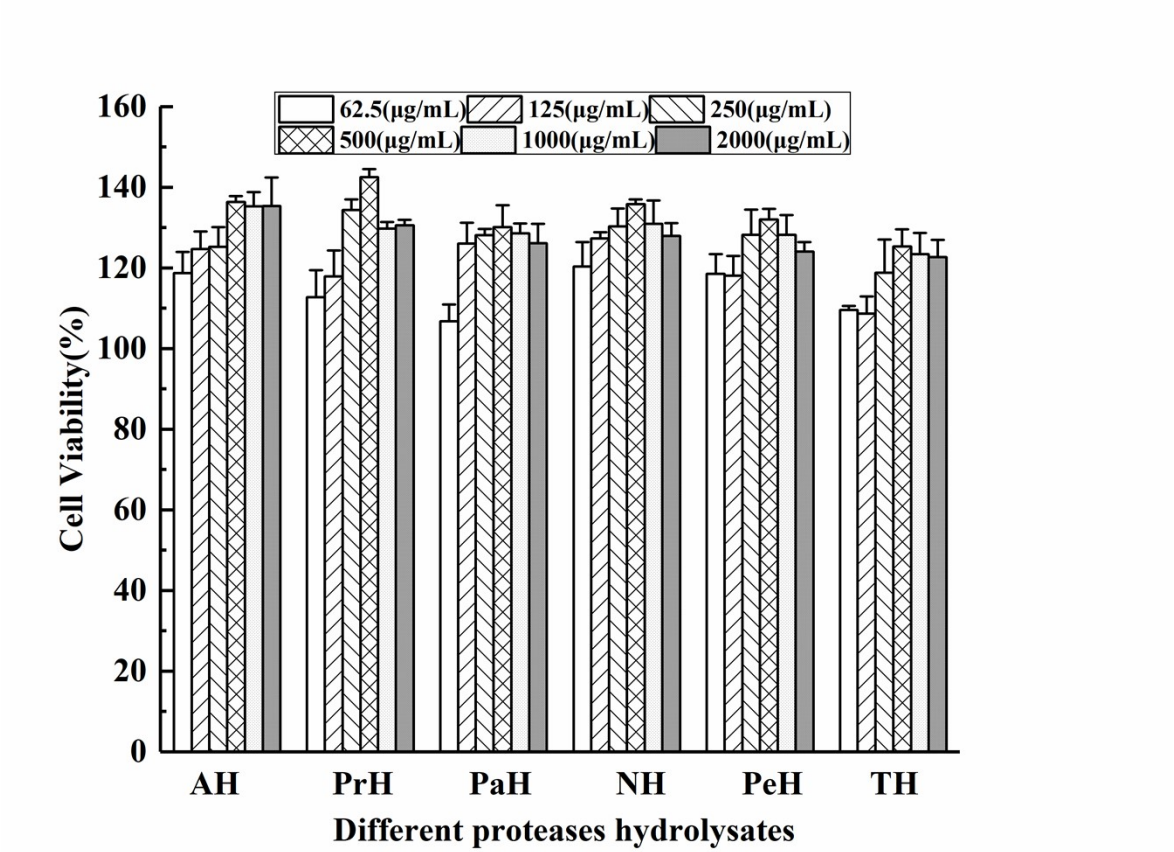

Fig. S1. Effects of different protease hydrolysates on RAW264.7 macrophage activity. AH stands for alcalase hydrolysate, PrH stands for protamex hydrolysate, PaH stands for papain hydrolysate, NH stands for neutral protease hydrolysate, PeH stands for pepsin

hydrolysate, and TH stands for trypsin hydrolysate. Values are means ± SD of three determinations.

As shown in Figure S1, the cell viability of the group without enzymolysis solution was taken as the blank control, and the cell viability was set at 100%. It was found that the activities of RAW264.7 cells were significantly improved by the six protease hydrolysates in the range of 62.5~2000 μg/mL. At low concentration of 62.5~500 μg/mL, they all could promote cell proliferation. As the concentration of hydrolysate continued to increase, the cell viability decreased slightly. It was observed that alcalase hydrolysate (AH), protamex hydrolysate (PrH), pepsin hydrolysate (PeH), trypsin hydrolysate (TH), papain hydrolysate (PaH), neutral protease hydrolysate (NH) showed the highest cell proliferation activity at the concentration was 500 μg/mL, which were 136.31±1.48%, 142.54±1.96%, 130.11±5.45%, 135.80±1.23%, 132.03±2.65% and 125.29±4.28%, respectively. Therefore, the concentration of 500 μg/mL was selected to evaluate the anti-inflammatory activity of walnut meal peptide in the following experiment.

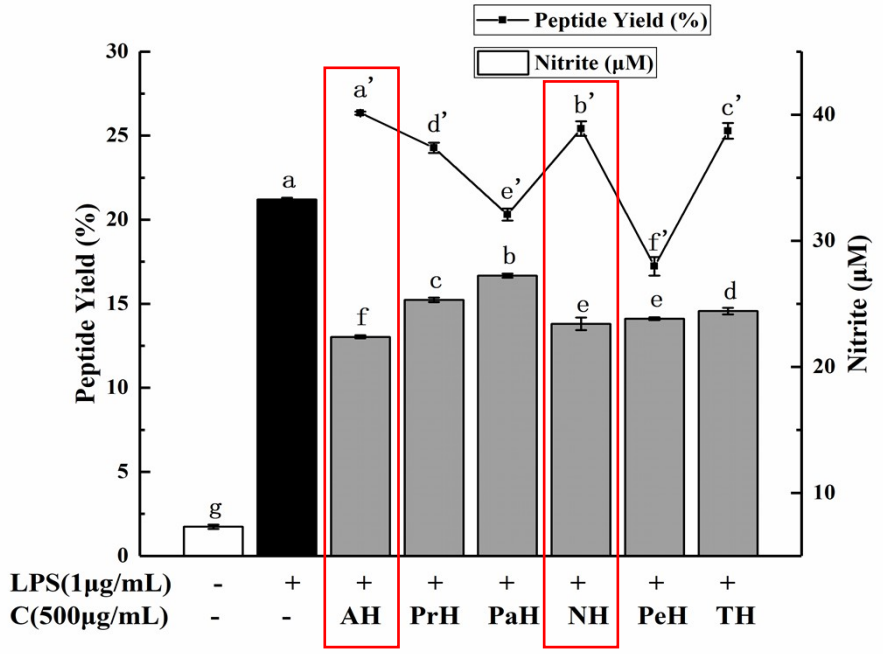

Fig. S2. Peptide yield of different proteases hydrolysates and their effects on NO production in cells. AH stands for alcalase hydrolysate, PrH stands for protamex hydrolysate, PaH stands for papain hydrolysate, NH stands for neutral protease hydrolysate, PeH stands for pepsin hydrolysate, and TH stands for trypsin hydrolysate. Values are means ± SD of three determinations. Different letters (a-g) are means that are significantly different (P<0.05).

Six common commercial proteases were used to hydrolyze DWMP. The peptide yield of hydrolysate of six proteases and the effects on NO production of LPS-induced RAW264.7 macrophages at 500 μg/mL were detected as shown in Fig. S2. Alcalase and neutral protease hydrolyzed walnut meal protein were more thoroughly, and had the highest peptide yield, followed by trypsin and protamex, pepsin and papain hydrolysis effect were the worst. The hydrolysate of walnut meal protein hydrolyzed by alcalase had

the highest inhibition rate of NO production in LPS-induced RAW264.7 cells, followed by eutral protease and protamex, the inhibitory effect of papain was the worst. Therefore, alcalase and neutral protease were selected as the target enzyme to prepare walnut peptides.

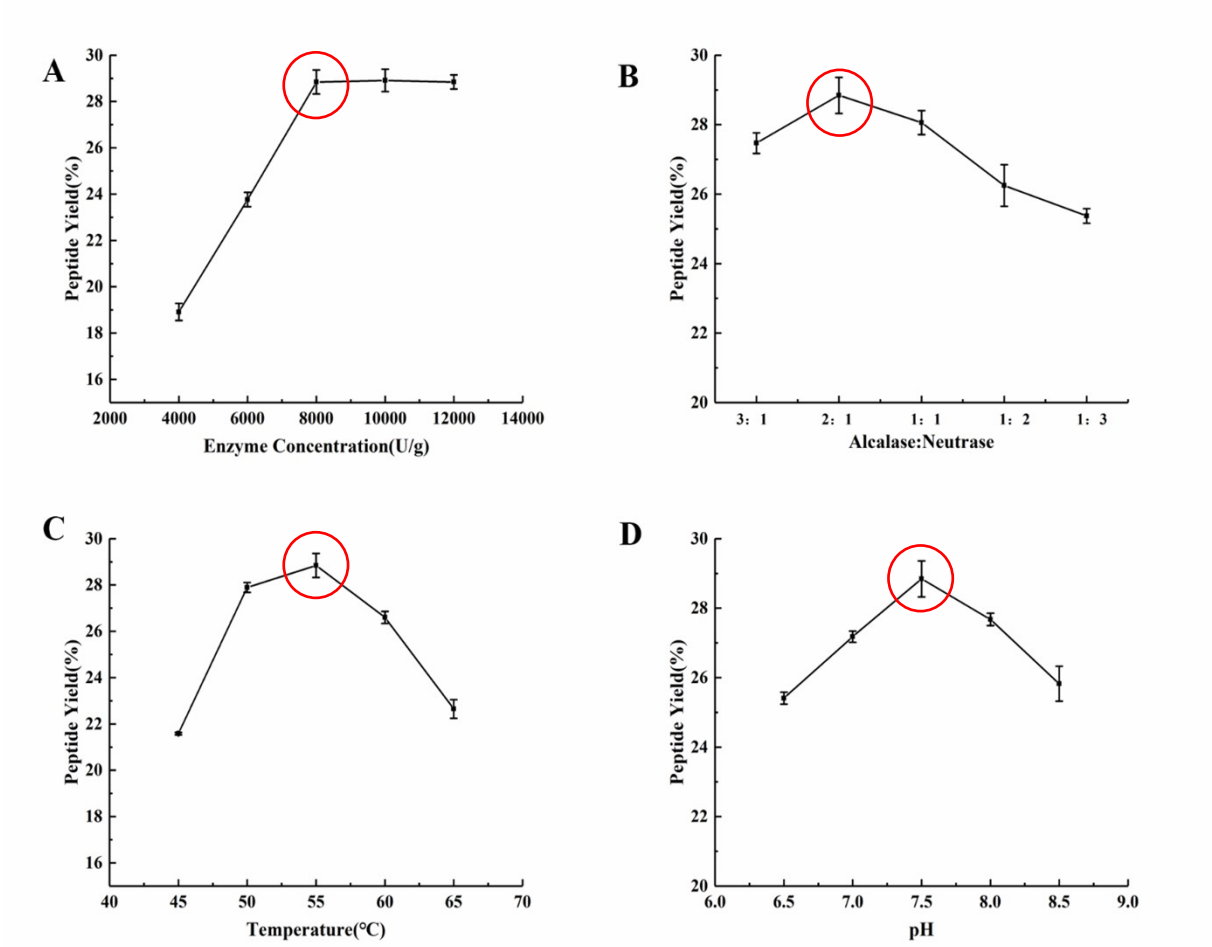

Fig. S3. The effects of different single factors on the peptide yield of DWMPHs. A is enzyme dosage, B is compound enzyme ratio, C is enzymatic hydrolysis temperature, D is pH value. Values are means ± SD of three determinations.

Due to the different hydrolysis conditions of alcalase and neutral protease, single factor experiment combined with response surface methodology was used to optimize the process of their complex enzymatic hydrolysis. As can be seen from Fig. S3, enzyme dosage, compound enzyme ratio, enzymatic hydrolysis temperature, pH value had significant effects on the peptide yield of DWMP. And the peptide yield reached the highest under the following conditions: enzyme dosage 8000U/g, complex enzyme ratio 2∶1, temperature 55 ℃ and PH 7.5. Therefore, the four factors (enzyme dosage, compound enzyme ratio, enzymatic hydrolysis temperature and pH value) were chosen as the response surface factor level.

Table S1. Box-Behnken design and results of hydrolysis of walnut meal protein

Number	A（U/g）	B（A：N）	C（℃）	D	Peptide yield (%)
1	8000	3	50	7.5	26.89
2	8000	2	55	7.5	29.59
3	8000	3	55	7	26.48
4	8000	2	50	8	26.67
5	8000	1	55	7	27.43
6	8000	2	55	7.5	29.64
7	8000	2	60	8	24.34
8	10000	2	55	7	28.64
9	6000	2	55	7	25.06
10	10000	2	50	7.5	27.16
11	6000	3	55	7.5	25.55
12	8000	1	60	7.5	26.56
13	6000	2	55	8	25.04
14	10000	1	55	7.5	27.02
15	8000	2	55	7.5	28.93
16	8000	2	50	7	27.58
17	6000	2	50	7.5	25.15
18	6000	2	60	7.5	25.39
19	8000	2	60	7	25.24
20	8000	1	55	8	25.18
21	6000	1	55	7.5	26.55
22	8000	3	60	7.5	26.1
23	10000	3	55	7.5	27.88
24	10000	2	55	8	25.43
25	8000	3	55	8	27.19
26	8000	1	50	7.5	27.09
27	8000	2	55	7.5	28.67
28	10000	2	60	7.5	26.84
29	8000	2	55	7.5	29.96

Table S2. Regression coefficients and analysis of variance for quadratic model

Source	Sure of squares	Df	Mean square	F-value	*P*-value	Significance
Model	60.88	14	4.35	12.31	<0.0001	**
A	8.72	1	8.72	24.69	0.0002	**
B	5.63E-03	1	5.63E-03	0.016	0.9013	
C	3.07	1	3.07	8.69	0.0106	*
D	3.61	1	3.61	10.22	0.0065	**
AB	0.86	1	0.86	2.45	0.1399	
AC	0.078	1	0.078	0.22	0.6448	
AD	2.54	1	2.54	7.2	0.0178	*
BC	0.017	1	0.017	0.048	0.83	
BD	2.19	1	2.19	6.2	0.0259	*
CD	2.50E-05	1	2.50E-05	7.08E-05	0.9934	
A^2	15.94	1	15.94	45.14	<0.0001	**
B^2	7.04	1	7.04	19.92	0.0005	**
C^2	17.77	1	17.77	50.32	<0.0001	**
D^2	19.79	1	19.79	56.02	<0.0001	**
Residual error	4.94	14	0.35			
Lack of fit	3.79	10	0.38	1.32	0.4254	
Pure error	1.15	4	0.29			
Sum	65.82	28				
R^2=0.9249		Adj R^2=0.8498		Pre R^2=0.6408		

* means significant difference，P<0.05；** means extremely significant difference，P<0.001

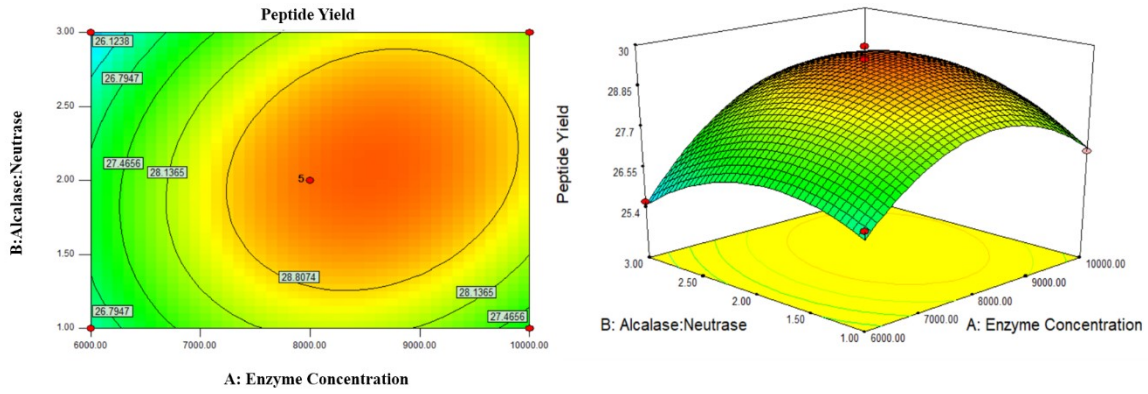

Fig. S4. Responsive surface plots and corresponding contour plots of the peptide yield of DWMPHs. Interaction of enzyme concentration and the ratio of enzymes.

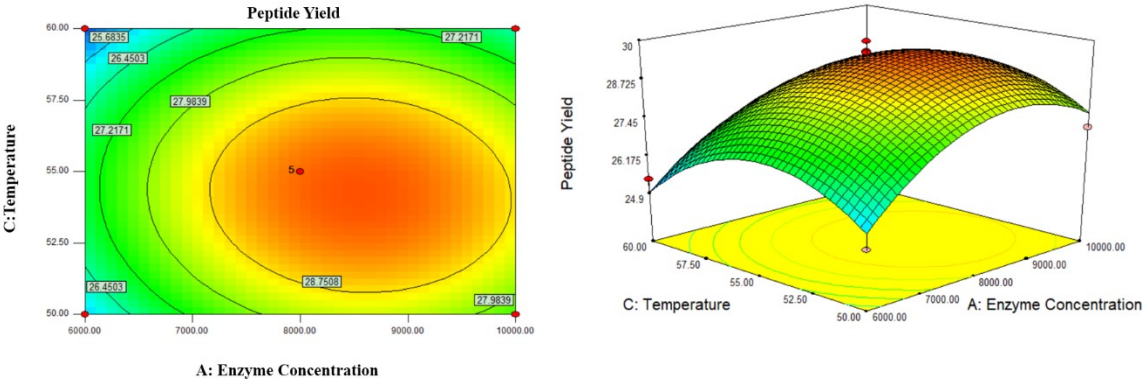

Fig. S5. Responsive surface plots and corresponding contour plots of the peptide yield of DWMPHs. Interaction of enzyme concentration and temperature.

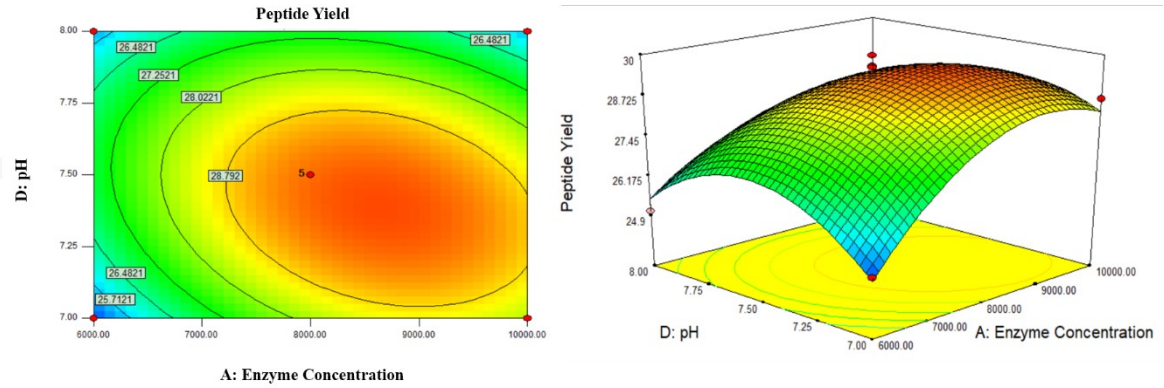

Fig. S6. Responsive surface plots and corresponding contour plots of the peptide yield of DWMPHs. Interaction of enzyme concentration and pH.

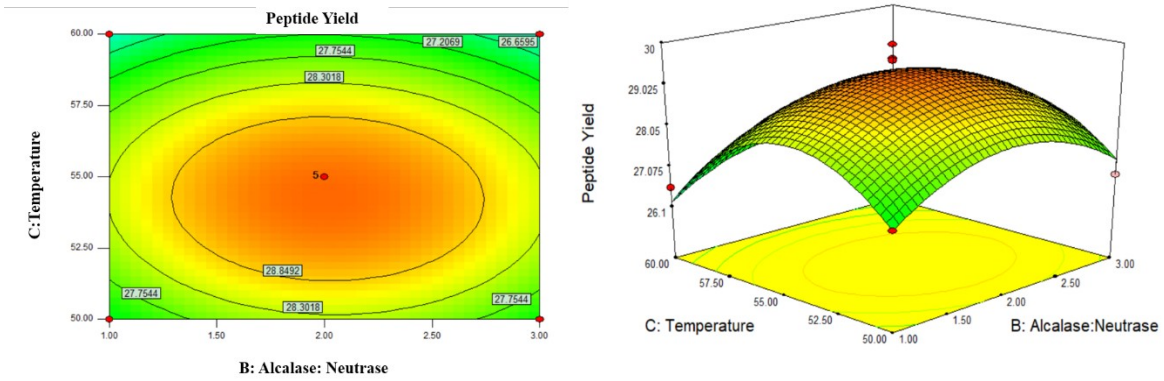

Fig. S7. Responsive surface plots and corresponding contour plots of the peptide yield of DWMPHs. Interaction of the ratio of enzymes and temperature.

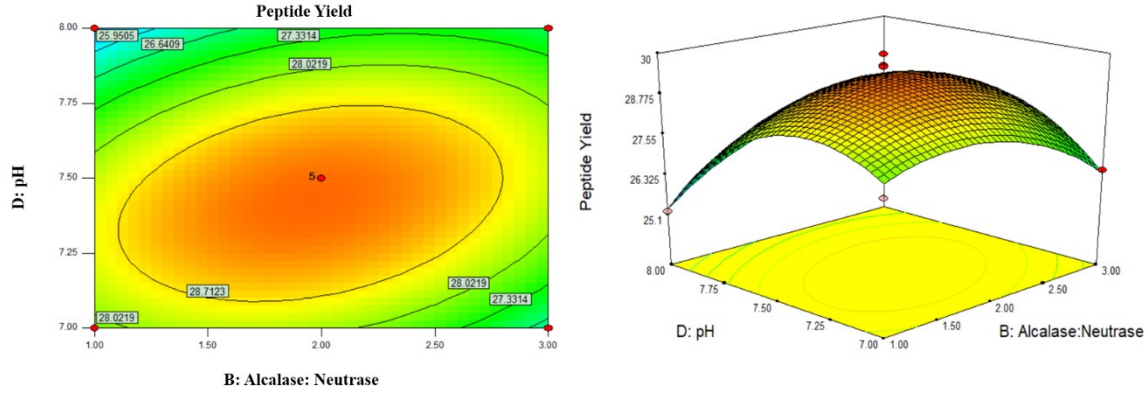

Fig. S8. Responsive surface plots and corresponding contour plots of the peptide yield of DWMPHs. Interaction of the ratio of enzymes and pH.

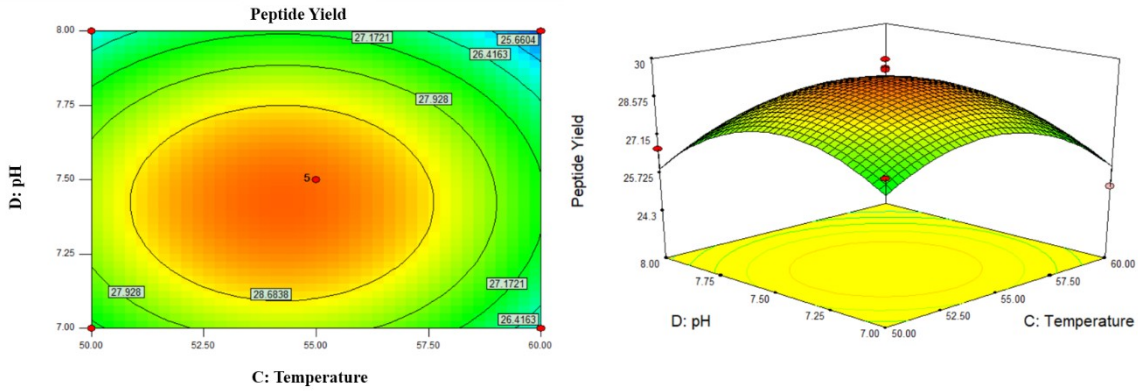

Fig. S9. Responsive surface plots and corresponding contour plots of the peptide yield of DWMPHs. Interaction of temperature and pH.

Response surface methodology was used to analyze the optimal parameters for the enzymatic hydrolysis process. Enzyme dosage (A: 6000-10000 U/g), compound enzyme ratio (B: 1-3), a range of temperature (C: 50-60 ℃), and pH value (D: 7.5-8) were determined by single-factor experiments. The design scheme and results have been detailed in Table S1 and S2. The polynomial regression equation was used to fit the relationship of each factor with the corresponding response value. The peptide yield of DWMP was taken as the response value. After regression analysis, the influence of these factors on the response value could be expressed by the following function:

$$Y(\%)=29.36+0.85A+0.022B-0.51C-0.55D+0.46AB-0.14AC-0.80AD-0.065BC+0.74BD+0.0025CD-1.57A^2-1.04B^2-1.66C^2-1.75D^2$$

According to response surface test, the optimal conditions of proteolysis of DWMP were as follows: enzyme dosage 8678.17 U/g, compound enzyme ratio 2.01∶1, the temperature 54.16 ℃, pH 7.38, with the peptide yield of 30.11 ± 0.82%.

Due to the limitations of practical operation, the optimal enzymatic hydrolysis process conditions were adjusted to be enzyme dosage 8500 U/g, compound enzyme ratio 2∶1, the temperature 55 ℃, pH 7.4. Under these conditions, the actual peptide yield of walnut meal protein was 30.08 ± 0.67%, which was basically consistent with the theoretical value.