Optimization research on hydrolysis condition of walnut protein

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Abstract. Walnut peptides were obtained through special enzyme or hydrolytic enzyme enzymolysis with walnut residue as raw materials and degree of hydrolysis as index. The results showed that special enzyme was the optimum proteinase. The optimum hydrolytic conditions were as followed: the optimal enzymatic hydrolysis of special enzyme was that substrate concentration was 11%, pH was 10.0, enzyme concentration was 0.5%, enzyme solution temperature was 55 °C, hydrolysis time was 12 h; the optimal enzymatic hydrolysis of hydrolytic enzyme was that substrate concentration was 12%, pH was 9.0, enzyme concentration was 0.5%, enzyme solution temperature was 50 °C and hydrolysis time was 14 h.

Introduction

Walnut (Juglans regial L., Juglandaceae) is one of the important oilseed crops in China. It is rich in essential polyunsaturated fatty acids (especially linoleic acid). The main by-product of the walnut oil extraction process is defatted walnut meal (walnut kernel residue or walnut slag), which has relatively high protein content of 52.5% [1, 2]. Because of different walnut oil extraction methods, the component of defatted walnut meal was complex and function was poor, leading walnut protein is hard to wide application in food processing. So people were always ignore walnut protein resource, most of the walnut slag were used as an animal feed, fish meal, fertilizer or directly discarded, caused the waste of resources [3, 4]. According to related reports, the amino acids of walnut protein are complete compositions of 18 kinds of amino acid, and the contents of 8 kinds of necessary aminophenol are reasonable [5,6]. In recent years, defatted walnut meal have been found to can obviously improve the learning and memory abilities and antioxidant capacity of rats [7]. In addition, the findings of numerous studies have confirmed that walnut polypeptides from walnut protein hydrolysates possess strong antioxidant activity and the ACE inhibitory activity [8-10]. Hence, in order to make effective use of plant proteins in this resource and to expand the fields of walnut protein products in food processing and other applications, this work aimed to enhance the walnut protein hydrolysis process research. It may also provide reference for further developing walnut protein.

Materials and methods

Materials

Walnut kernel residue was purchased from Qingdao city, Shandong province of China. The enzyme preparations used in the experiments were vegetable protein hydrolysis of special enzyme (enzyme activity of 350000 U/g, referred to as special enzyme) and plant protein proteolytic enzyme (enzyme activity of 350000 U/g, referred to as proteolytic enzyme). They were purchased from Nanning dong heng hua dao biological technology Co., Ltd. All other reagents used were of analytical grade.

Preparation of walnut protein

Walnut kernel residue was firstly smashed and then was filtrated at 40 mesh. Then walnut powder was dispersed in NaOH solution (pH 9.0) at ratio 1:9 (w/v) and extracted by ultrasonic at 100 Hz for 1 h. The mixture was centrifuged at 3500 rpm for 10 min and the supernatant was collected. The

supernatant was adjusted to optimal pH (4.5) by addition of 4 N HCl solution to precipitate the proteins, and centrifuged again at 3500 rpm for 10 min and the precipitation was collected. The precipitate was washed several times with distilled water, then dispersed in a small amount of distilled water, and adjusted to pH 7.0 by addition of NaOH solution. Finally, the sediment fraction was freeze-dried using freeze-dryer (Bioblock Scientific Christ ALPHA 1-2) and stored at -4 ℃ for further use.

Acquisition of the standard glycine working curve

Glycine was dried at 105 ℃ until its weight has no change. 25 mg glycine was put into 25 mL volumetric flask with stopper, with adding distilled water to scale, shaking well to obtain 1 $mg \cdot mL^{-1}$ glycine stock solution. 2 mL glycine stock solution was taken out and put into 10 mL volumetric flask with stopper, with adding distilled water to scale, shaking well to obtain 0.2 $mg \cdot mL^{-1}$ glycine reference substance solution. 0.45, 0.50, 0.55, 0.60, 0.65, 0.70 mL glycine reference substance solution was taken out respectively and put into 10 mL test tubes, added distilled water into till 2 mL, added 1mL pH 8.2 phosphate buffer and 1 mL 2% ninhydrin, plugged with plastic wrap, shaken well then placed in a boiling water bath heating 15 min for coloration, then cooled, then added 5 mL distilled water, blended well and stood for 15 min. Then 3.2 mL each of them were filled into 1 cm cuvette to determine their absorbance under 570 nm wavelength, which was kept between 0.1 and 0.8. At last, the standard glycine working curve was acquired.

Single factor experiment

For each experiment, 5 g walnut protein was mixed with 50 mL distilled water giving a mixture of walnut protein and distilled water at a 1:10 (w/v) ratio. The mixture was weighted and then heated to 95 °C for 10 min to inactivate endogenous enzymes. After that, it was cooled, compensated weight and placed in a waterbath set at a desired pH and temperature. The mixture was added of different dose of special enzyme (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%). Hydrolysis was stopped by heat treatment at 95°C for 10 min, then hydrolysates were cooled and centrifuged at 3500 rpm for 10 min to separate solid and liquid phase. Measuring and recording the clear liquid product, the supernatant fluid to determine the DH. In the same way, the other factors were determined by single factor tests (substrate concentration: ranged from 10% to 15%. enzyme solution pH: ranged from 6 to 9 or ranged from 8 to 10. enzyme solution temperature: ranged from 35 ℃ to 65 ℃. enzyme solution time: ranged from 2 h to 12 h).

Orthogonal test

According to the results of single factor experiment, we have designed L9 (3^3) orthogonal experiment to investigate the effect of walnut protein substrate concentration, hydrolysis time and hydrolysis pH in special enzyme digestion of walnut protein condition, the experiment design are shown in table 1. Besides, we have chosen walnut protein substrate concentration, enzyme mass fraction and hydrolysis time as three main factors to design L9 (3^3) orthogonal experiment to optimize proteolytic enzyme digestion of walnut protein hydrolysis conditions, design of experiment are shown in table 3.

Results and discussion

Standard working curve for determination of glycine

Fig. 1 shows that there was a good linear relationship between absorbency and glycine content when the glycine content was between 0.09 mg and 0.14 mg with a regression equation y=12.534x-0.9756 and a regression factor R^2=0.9933.

The influence of the enzyme mass fraction on walnut protein hydrolysis

The influence of the enzyme mass fraction on walnut protein hydrolysis was shown in Fig.2. The enzyme mass fraction was ranged from 0.1% to 0.6%. The degree hydrolysis of special enzyme slightly increased with the increase of enzyme mass fraction when enzyme mass fraction was lower than 0.5%. The degree hydrolysis of special enzyme slightly decreased with the increase of enzyme mass fraction when enzyme mass fraction was higher than 0.5%. In other words, the optimal enzyme mass fraction of special enzyme was 0.5% (the degree of hydrolysis was 26.67%). The hydrolysates treated by

proteolytic enzyme for 0.1%～0.6% enzyme mass fraction showed similar DH, which indicated that increasing the enzyme mass did not produce any significant improvement in the DH after 0.4%.

The effect of substrate concentration on walnut protein hydrolysis

The effect of substrate concentration on walnut protein hydrolysis was shown in Fig.3. The substrate concentration was ranged from 10% to15%. The degree hydrolysis of special enzyme and hydrolytic enzyme slightly increased with the increase of substrate concentration when substrate concentration was lower than 12%. The degree hydrolysis of special enzyme and hydrolytic enzyme slightly decreased with the increase of substrate concentration when substrate concentration was higher than 12%. The optimal substrate concentration of special enzyme and proteolytic enzyme were observed 12% (the degree of hydrolysis were 28.82% and 11.75%).

The effect of enzyme solution pH on walnut protein hydrolysis

As shown in Fig.4, the enzyme solution pH of special enzyme was ranged from 6 to 9. Its degree hydrolysis slightly increased with the increase of enzyme solution pH. The degree hydrolysis of hydrolytic enzyme were no significant different compared with other samples.

The effect of enzyme solution temperature on the degree of walnut protein hydrolysis

As shown in Fig.5, the temperature was ranged from 35 to 65℃ .The optimal temperature of special enzyme and proteolytic enzyme were 55 and 50 ℃ (the degree of hydrolysis were 27.95% and 10.04%).

The effect of enzyme solution time on the degree of walnut protein hydrolysis

As shown in Fig.6, the enzyme solution time was ranged from 2 to 12 h. The degree hydrolysis of special enzyme showed a trend of fluctuations with the increase of enzyme solution time, when the hydrolysis time was 10 h, its degree of hydrolysis was 37.79%. The degree hydrolysis of hydrolytic enzyme were no significant different compared with other samples, when the hydrolysis time was 12 h, its degree of hydrolysis was 8.73%. So 10 h and 12 h were chosen as the optimal enzyme solution time of special enzyme and proteolytic enzyme.

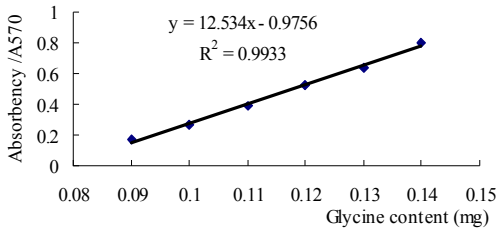

Fig. 1 Standard glycine working curve

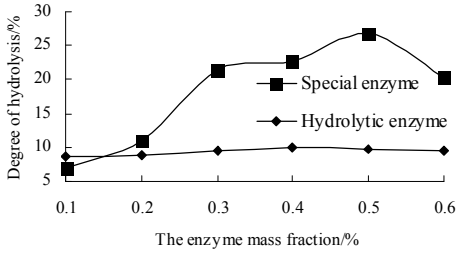

Fig.2 The influence of the enzyme mass fraction on walnut protein hydrolysis

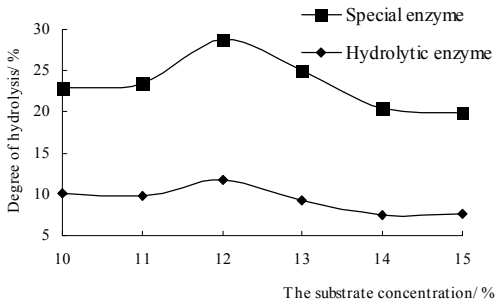

Fig.3 The effect of substrate concentration on walnut protein hydrolysis degree

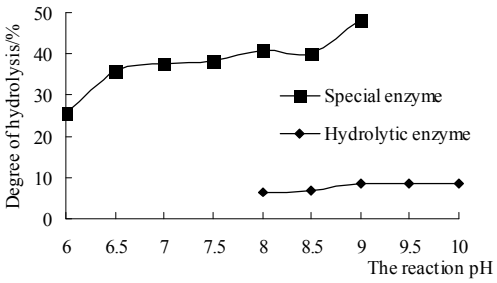

Fig.4 The effect of enzyme solution pH on walnut protein hydrolysis

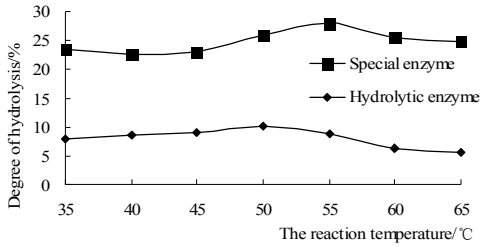

Fig.5 The effect of enzyme solution temperature on the degree of walnut protein hydrolysis

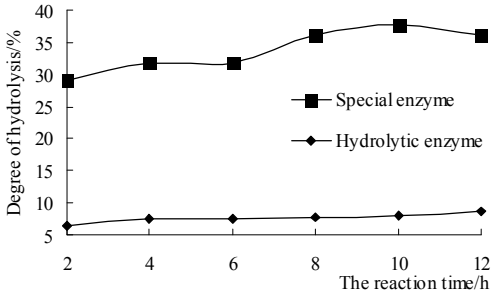

Fig.6 The effect of enzyme solution time on the degree of walnut protein hydrolysis

Special enzyme orthogonal test

An orthogonal test L9 (3^3) design was used to investigate the optimal formula of special enzyme hydrolyse walnut protein. As seen from table 1, the experiment was carried out with 3 factors and 3 levels.

Table 1 The factors and the levels of Special enzyme orthogonal test

	factor		
level	A (substrate concentration) /%	B (hydrolysis pH) /%	C (hydrolysis time)/h
1	11	8	8
2	12	9	10
3	13	10	12

Table2 The results and analysis of special enzyme orthogonal test

No.	A	B	C	DH /%
1	1	1	1	45.76
2	1	2	2	46.63
3	1	3	3	44.33
4	2	1	2	31.19
5	2	2	3	41.58
6	2	3	1	40.24
7	3	1	3	35.97
8	3	2	1	35.10
9	3	3	2	39.48
k1	45.573	37.64	40.367	
k2	37.67	41.103	39.1	
k3	36.85	41.35	40.627	
R	8.723	3.710	1.527	

In view of orthogonal analysis, we adopt statistical software to calculate the values of k and R. According to the R value, we can judge walnut protein concentration (A), hydrolysis pH (B)and hydrolysis time(C) three factors influence the degree of hydrolysis of walnut protein were listed in decreasing order as follows: A> B > C. Besides, k_{A1}, k_{A2} and k_{A3} were different, it means that changes in the levels of A factor can influence the test results. At the same time, according to the k value, we can judge A1, A2 and A3 three levels influence on test index were listed in decreasing order as follows: A1 > A2 > A3. So we concluded that A1 was A factor of optimal level. Similarly, B3 and C3 were the optimal levels of B and C factors. Therefore, the optimum process conditions of enzymatic hydrolysis of walnut protein enzyme were $A_1B_3C_3$, namely, the substrate concentration was 11%, the hydrolysis pH was 10, and the hydrolysis time was 12 h

Hydrolytic enzyme orthogonal test

An orthogonal test L9 (3^3) design was used to investigate the optimal formula of hydrolytic enzyme hydrolyse walnut protein. As seen from table 3, the experiment was carried out with 3 factors and 3 levels.

Table 3 The factors and the levels of Hydrolytic enzyme orthogonal test

level	factor		
	A (substrate concentration) /%	B (enzyme mass fraction) /%	C (hydrolysis time)/h
1	11	0.3	10
2	12	0.4	12
3	13	0.5	14

Table 4 The results and analysis of hydrolytic enzyme orthogonal test

No.	A	B	C	DH /%
1	1	1	1	12.18
2	1	2	2	9.19
3	1	3	3	10.06
4	2	1	2	8.24
5	2	2	3	8.26
6	2	3	1	13.85
7	3	1	3	9.84
8	3	2	1	10.06
9	3	3	2	10.16
K1	10.477	10.087	12.03	
K2	10.117	9.17	9.197	
K3	10.02	11.357	9.387	
R	0.457	2.187	2.833	

In view of orthogonal analysis, we adopt statistical software to calculate the values of k and R. According to the R value, we can judge walnut protein concentration (A), enzyme mass fraction (B)and hydrolysis time(C) three factors influence the degree of hydrolysis of walnut protein were listed in decreasing order as follows: C>B>A. Besides, k_{A1}, k_{A2} and k_{A3} were different, it means that changes in the levels of A factor can influence the test results. At the same time, according to the k value, we can judge A1, A2 and A3 three levels influence on test index were listed in decreasing order as follows: A1 > A2 > A3. So we concluded that A1 was A factor of optimal level. Similarly, B3 and C1 were the optimal levels of B and C factors. Therefore, the optimum process conditions of enzymatic hydrolysis of walnut protein enzyme were $A_1B_3C_1$, namely, the substrate concentration was 11%, the enzyme mass fraction was 0.5%, and the hydrolysis time was 14 h.

Conclusion

With walnut protein as raw materials and degree of hydrolysis as indicators, special enzyme and hydrolytic enzyme were optimized by single factor and orthogonal experiments, the results were as follows: The optimal enzymatic hydrolysis of special enzyme was that substrate concentration was 11%, pH was 10.0, enzyme concentration was 0.5%, enzyme solution temperature was 55 °C, hydrolysis time was 12 h; the optimal enzymatic hydrolysis of hydrolytic enzyme was that substrate concentration was 12%, pH was 9.0, enzyme concentration was 0.5%, enzyme solution temperature was 50 °C and hydrolysis time was 14 h.

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