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Optimization of Hydrolyzed Crude Extract from Tartary Buckwheat Protein and Analysis of Its Hypoglycemic Activity in Vitro

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Abstract. Tartary buckwheat was used as substrate for enzymatic hydrolysis to prepare a crude extract of tartary buckwheat protein, hydrolyzed by alcalase to simulate digestion in vivo. On the basis of single factor experiment, buckwheat protein hydrolysis was served as the response value. By Box-Behnken experimental, the optimum conditions were determined as volume of enzyme 40.61 U/g, temperature 46.16°C, time 20.80 min. Under these conditions, the actual degree of hydrolysis of the tartary buckwheat protein could reach to 57.71%, in good agreement with the predicted value (57.69%), and the inhibition of α -amylase activity was 78.69%, respectively.

1. Introduction

Tartary buckwheat belonged to *Polygonaceae* (genus *Fagopyrum*), which was a dicotyledonous cereal small crops^[1]. Tartary buckwheat was precious medicine crop resources^[2]. As one of the main producing areas of tartary buckwheat in the world, China had a planting area of 300,000 hectares and a total output of 800,000 tons^[3]. Moreover, the protein content of tartary buckwheat was rich, accounting for 12% to 19% of the grain^[4]. Coverage had shown that tartary buckwheat protein was one of the main bioactive substances in tartary buckwheat^[5], which had functions of antibacterial, anti-tumor, anti-oxidation, cholesterol lowering and so on^[6], and had broad application prospects in the food and feed fields.

At present, the research on tartary buckwheat protein mainly focused on the extraction of protein technology, the properties of tartary buckwheat protein^[7] and the biological activities of tartary buckwheat protein^[8]. In recent years, more and more studies had been done on tartary buckwheat proteins and peptides. These studies provided a theoretical basis for the preparation of crude extracts of tartary buckwheat and peptide by protease hydrolysis.

2. Material and Reagent

2.1. Materials

The tartary buckwheat were harvested from Xiaodian district, Taiyuan city, Shanxi province in 2018.

2.2. Reagent

Alkaline protease (BR enzyme activity 200 U/mg), Maltose and Bovine serum protein (BR) were purchased from Beijing Chemical Works, China. α -amylase (BR enzyme activity 50 U/mg), α -



glucosidase (BR enzyme activity 58 U/mg) and PNPG were purchased from Sigma-Aldrich, China. All organic solvents used for separation were of analytical grade.

3. Experimental Methods

3.1. Preparation and purity determination of Tartary buckwheat protein

According to the method of X N Guo^[9].

3.2. Preparation of tartary buckwheat proteolytic solution

The tartary buckwheat protein was placed in deionized water, dissolved for 20 min, and then the pH was adjusted to an optimum value with NaOH at a constant temperature water bath. Alkaline protease was added to initiate enzymatic hydrolysis, and the pH and temperature of the solution were kept constant during the hydrolysis. After the hydrolysis was completed, the enzymatic hydrolysate was boiled for 5 min to kill the enzyme, and the suspension was centrifuged at 10,000 g at 4°C for 10 min in a refrigerating centrifuge, the supernatant taken, the pH adjusted to neutral, and tartary buckwheat proteolytic solution was prepared.

3.3. Determination of soluble protein content

Refer to Y Q Zhang g's^[10] method.

3.4. Calculation of protein hydrolysis degree

The Protein hydrolysis degree was calculated by the following equation:

$$M_2(\%) = (M_0 - M_1) / M_0 * 100\%. \quad M_1: \text{the soluble protein mass (mg)} \quad M_0: \text{the total protein mass (mg)}$$

3.5. Determination of amylase inhibition rate and glucosidase inhibition rate

Determination of amylase inhibition rate was determined according to the method of Liu et al.^[11] and S C Jao et al.^[12].

3.6. Single-Factor Experiment

According to the method in section 2.2 and 2.3, respectively investigated the substrate concentration (2.5%, 3.0%, 3.5%, 3.0%, 4.5%, 5.0%), volume of enzyme (20, 40, 60, 80, 100, 120 U/g), enzymolysis time (10, 20, 30, 40, 50, 60 min), electrolysis temperature (15, 25, 35, 45, 55, 65 °C) of buckwheat protein hydrolysis, and the optimal parameters were determined.

3.7. Box-behnken experimental design

According to the single-factor test results above, volume of enzyme, time and temperature were selected as the main factors affecting the enzymatic hydrolysis of tartary buckwheat protein. With tartary buckwheat protein hydrolysis degree serving as the index, the process was optimized by response surface analysis. Experimental factors and horizontal coding are shown in table 1.

Table 1. Box-Behnken test factors and levels of encoding

| Factors | Levels | | |
|--------------------------------|--------|----|----|
| | -1 | 0 | 1 |
| A Volume of enzyme (U/g) | 20 | 40 | 60 |
| B Enzymolysis temperature (°C) | 35 | 45 | 55 |
| C Enzymolysis time (min) | 10 | 20 | 30 |

4. Results and Discussion

4.1. Single-Factor Experiment results

4.1.1. Effect of substrate concentration on the hydrolysis degree of tartary buckwheat protein

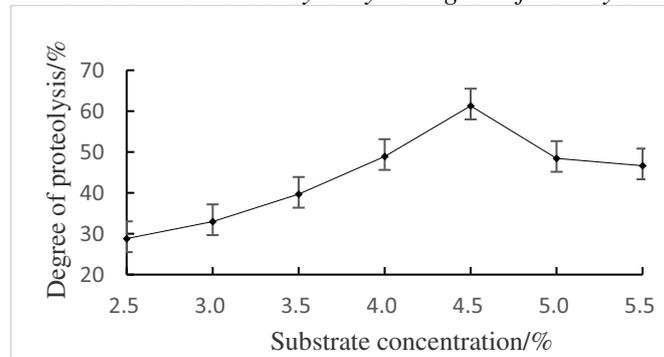


Fig. 1. Effect of substrate concentration on the hydrolysis degree of tartary buckwheat protein
According to Fig.1, with the extension of substrate concentration, the hydrolysis degree of tartary buckwheat protein increased. When the substrate concentration exceeded 4.5%, the degree of proteolysis began to decrease and remained constant. The effect of enzymatic hydrolysis was affected by the substrate concentration. When the substrate concentration was too high, it would in turn inhibit the enzymatic hydrolysis reaction. Considering comprehensively, 4.5% substrate concentration was selected.

4.1.2. Effect of volume of enzyme on the hydrolysis degree of tartary buckwheat protein

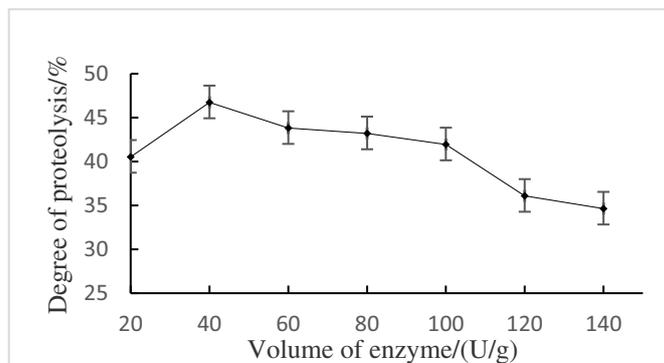


Fig. 2. Effect of volume of enzyme on the hydrolysis degree of tartary buckwheat protein
From Fig. 2, with the rise of volume of enzyme, an increase can be seen the hydrolysis degree of tartary buckwheat protein. But, it lowered gradually when volume of enzyme was increased from 40 U/g to 140 U/g. The effect of enzymatic hydrolysis was affected by the volume of enzyme. Too much enzyme would in reverse inhibit the reaction speed. Account the saving of the cost of enzymatic hydrolysis, it was more appropriate to select the volume of 40 U/g.

4.1.3. Effect of enzymolysis temperature on the hydrolysis degree of tartary buckwheat protein

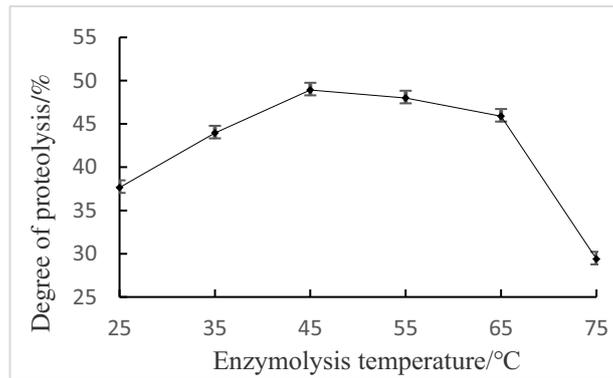


Fig. 3. Effect of Enzymolysis temperature on the hydrolysis degree of tartary buckwheat protein
 It could be seen from Fig.3, with the temperature increased to 65°C, the degree of proteolysis decreased significantly. Enzymatic reaction was sensitive to temperature, and it was easy to hydrolyze at 45°C. Too low temperature would inhibit protease activity and excessive temperature would inactivate the enzyme, so the enzyme solution temperature 45°C was elected .

4.1.4. Effect of enzymolysis time on the hydrolysis degree of tartary buckwheat protein

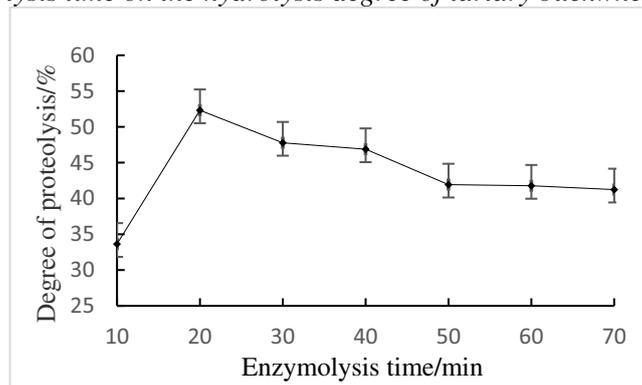


Fig. 4. Effect of Enzymolysis time on the hydrolysis degree of tartary buckwheat protein
 As was shown in Fig.4, with the increase of enzymatic hydrolysis time, the hydrolysis degree of tartary buckwheat protein showed a trend of slow decrease after increasing, and the degree of protein hydrolysis tended to be balanced in the enzymatic hydrolysis time of 30~70 min. After comprehensive consideration, 30 min was selected as the enzymatic hydrolysis time.

4.2. Box-behnken experimental results

Box-behnken experimental design was carried out with volume of enzyme(A), enzymolysis time (B), and enzymolysis temperature (C) as independent variables and degree of proteolysis as response values. 1, 0, and -1 were used to represent the 3 levels of independent variables, respectively. The experimental design level and response values were shown in table 2, anova in table 3.

Table 2. Box-behnken design with response value

| Ordinal | Volume enzyme (U/g) | of Enzymolysis time (min) | Enzymolysis temperature (°C) | Degree of proteolysis (%) |
|---------|---------------------|---------------------------|------------------------------|---------------------------|
| 1 | -1(20) | 1(30) | -1(35) | 38.73 |
| 2 | 0(40) | -1(10) | -1 | 36.33 |
| 3 | 0 | 1 | -1 | 35.48 |
| 4 | 1(60) | 0(20) | -1 | 39.48 |
| 5 | -1 | -1 | 0(45) | 47.45 |

| | | | | |
|----|----|----|-------|-------|
| 6 | -1 | 1 | 0 | 50.49 |
| 7 | 0 | 0 | 0 | 57.63 |
| 8 | 0 | 0 | 0 | 57.55 |
| 9 | 0 | 0 | 0 | 57.32 |
| 10 | 0 | 0 | 0 | 57.39 |
| 11 | 0 | 0 | 0 | 57.08 |
| 12 | 1 | 1 | 0 | 49.66 |
| 13 | 1 | -1 | 0 | 49.47 |
| 14 | -1 | 0 | 1(55) | 44.90 |
| 15 | 1 | -1 | 1 | 39.83 |
| 16 | 1 | 1 | 1 | 43.69 |
| 17 | 1 | 0 | 1 | 44.84 |

Table 3. Analysis of variance for the Box-Behnken design

| Source | Squares | df | Square | F-value | P-value |
|------------------------|---------|---------------------------------------|--------|----------|----------|
| Model | 1004.57 | 9 | 111.62 | 3465.33 | 0.0001** |
| A | 0.44 | 1 | 0.44 | 13.72 | 0.0076* |
| B | 4.87 | 1 | 4.87 | 151.11 | 0.0001** |
| C | 67.51 | 1 | 67.51 | 2096 | 0.0001** |
| AB | 2.03 | 1 | 2.03 | 63.04 | 0.0001** |
| AC | 0.16 | 1 | 0.16 | 5.09 | 0.0586 |
| BC | 5.55 | 1 | 5.55 | 172.18 | 0.0001** |
| A ² | 26.02 | 1 | 26.02 | 807.72 | 0.0001** |
| B ² | 133.97 | 1 | 133.97 | 4159.29 | 0.0001** |
| C ² | 702.93 | 1 | 702.93 | 21823.33 | 0.0001** |
| Residual | 0.23 | 7 | 0.032 | | |
| Lack of Fit | 0.041 | 3 | 0.014 | 0.3 | 0.8253 |
| Pure Error | 0.18 | 4 | 0.046 | | |
| Cor Total | 1004.79 | 16 | | | |
| R ² =0.9998 | | R ² _{Adj} =0.9995 | | | |

Note: * significant difference ($p < 0.05$); ** the difference was extremely significant ($p < 0.01$).

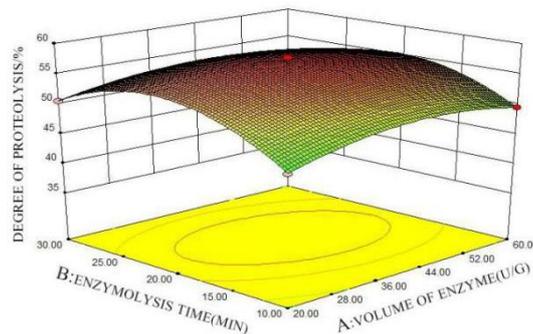
It could be seen from table 3 that the lack of fit was not significant ($p=0.8253>0.05$), indicating that the regression equation did not lose its fitting, indicating that the selected model was suitable and could be used to fit the test. The equation model was extremely significant ($p=0.0001<0.01$), indicating that the multiple regression equation could better fit the experimental results. The model determination coefficient R^2 was 0.9998 and R^2_{Adj} was 0.9995, indicating that the correlation and interpretation of the model were good. In this model, the significance test of regression equation shown that the first-order term A reached the significant level. The influence of primary terms B and C, square terms A^2 , B^2 , C^2 and interaction terms AB and BC reached extremely significant level. The order of the effects of the three factors on the proteolysis degree of enzymatic hydrolysis of tartary buckwheat was $C>B>A$. The quadratic term was fitted by the software.

The regression equation is:

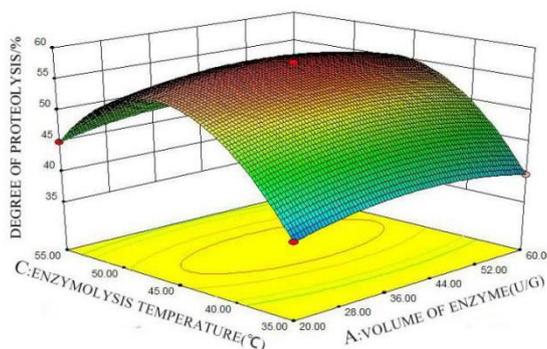
$$Y=57.39+0.24A+0.78B+2.91C-0.71AB-0.20AC+1.18BC-2.49A^2-5.64B^2-12.92C^2 .$$

4.3. Response surface analysis

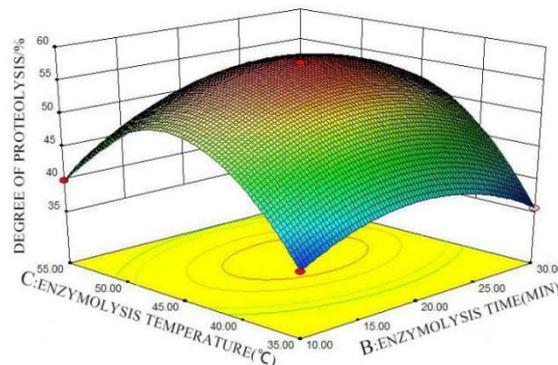
In order to study the interaction strength between experimental factors and determine the optimal level range of each factor, multiple regression fitting was conducted on table 2 data using design-expert 8.0.6, and the response surface of the regression equation obtained was shown in figure 5. As could be seen from the following three contour maps, the contour lines of enzyme uptake and enzymatic hydrolysis time, enzymatic hydrolysis time and enzymatic hydrolysis temperature tended to be oval, indicating significant interaction. The contour lines of enzyme uptake and enzymatic hydrolysis temperature tended to be round, and the slope of the slope was large, indicating that the interaction was not significant.



a. Volume of enzyme and enzymolysis time



b. Volume of enzyme and enzymolysis temperature



c. Enzymolysis time and enzymolysis temperature

Fig. 5. Response surface plots showing the interactive effects of various factors on the proteolysis degree

It could be seen from Fig. 5a, 5b, when the volume of enzyme was between 20~60 U/g, the proteolysis degree rose firstly and then decreased with the increase of the volume of enzyme, and there was the maximum point of proteolysis degree. Therefore, the control of the volume of enzyme in the appropriate range was conducive to tartary buckwheat protein hydrolyzes completely.

The Fig. 5b and 5c shown that, the degree of proteolysis kept an upward trend with temperature elevated, because the temperature would influence the activity of the enzyme. Low temperature or high temperature would slow down the reaction, so the protein hydrolysis occurred at 45°C, the maximum points.

According to Fig.5c and 5a, the time surface changed a lot, and the degree of proteolysis amplified with the prolonging of time, and then decreased slightly to the state of equilibrium. That was because excessive fermentation time would reduce the degree of proteolysis. Therefore, the time needed to be controlled within a suitable range. The time had a great influence on the degree of proteolysis.

4.4. Parameter optimization and validation

By SPSS 17.0, optimized conditions were as follows: volume of enzyme 40.62 U/g, time 20.79 min, temperature 46.15°C, the degree of proteolysis of buckwheat protein of predictive value was 57.59%. Under the condition of the three parallel verification experiments, the actual average value obtained from the measurements was 57.75%, the relative error was 0.3%. It had practical application value. Under these conditions, the inhibition rate of α -amylase was 78.68%, and the inhibition rate of α -glucosidase was 89.68%.

5. Conclusion

The hydrolysis degree of tartary buckwheat by alkaline protease including substrate concentration, volume of enzyme, time and temperature were screened and Box-Behnken design was carried out. On the basis of single factor experiment, buckwheat protein hydrolysis was served as the response value. By Box-Behnken experiment, the optimum conditions were determined as volume of enzyme 40.61 U/g, temperature 46.16°C, time 20.80 min. Under these conditions, the theoretical value of proteolysis degree content was 57.59 mg/g, the actual measured value was 57.45 mg/g, and the inhibition rate of α -amylase activity was 78.68%, and the inhibition rate of α -glucosidase activity was 89.68%. It provided a new direction for the further processing and high-value utilization of tartary buckwheat, and provided the corresponding technological parameters and theoretical basis for the large-scale industrial production of tartary buckwheat protein and polypeptides.

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