Study on the optimization of the enzymatic hydrolysis of antimicrobial protein from Moringa oleifera Leaves by response surface method

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Abstract. Moringa oleifera leaves contains antimicrobial protein, which is absent of optimized extraction and systematic analysis. The aim of this work is to provide a systematic research of its protein isolate hydrolysate on the optimized extraction via the response surface method and the functional properties. The isolated protein was obtained by alkali extraction and acid precipitation, which purity was 81.12%±0.03%. Through the response surface method, the result of the optimization obtained the enzymatic hydrolysis of antimicrobial protein was as follows, the substrate concentration of 5.09%, the Bromelain enzyme dosage of 3883.42 U/g, pH 7.0 and temperature of 55 ℃ for 3.64 h. And the protein hydrolysate was indicated that the purity was 88.59%±0.13%. Moreover, the ash, the crude fat and moisture were declined significantly compared with the protein isolate. This subject could provide basic theoretical basis for the application of M. oleifera leaves protein.

1. Introduction
Originally, Moringa oleifera Lam. derives from India, but is now grown in both tropical and subtropical countries such as South America et al of the world on account of its resilient adaptive features, such as, ability to grow fast, survive in drought condition and its longevity[1]. Its leaves have been reported to have higher proportion of protein (27%~35%) , provitamin A, vitamins B and C, minerals (particularly iron) than those found in other food products such as orange, carrots, milk, bananas, yoghurt and spinach[2-3]. Furthermore, it is a abundant source of essential amino acids such as methionine, cystine, tryptophan and lysine[4]. Moreover, M. oleifera leaves have been identified to have high antimicrobial activity. And, Dahot[5] isolated small protein/peptides from the leaves of M. oleifera possessing antimicrobial characteristic.
The protein of M. oleifera leaves is new but of high quality as well as abundant which shows well-balanced essential amino acids in previous study, possessing enormous potential processing application prospect. At present, most of them are applied as primitive supplement of feed and food due to its rich nutrition and antibacterial activity, so that it only has low added value of applying[6-7]. In addition, studies of M. oleifera leaves protein have focused on its extraction process. There is little information on the functional properties and antibacterial activity of it[8]. On the other hand, plant protein has become the desired substitute for animal protein but with poor machining performance, but enzymolysis can modify the functional properties of the protein meanwhile maintain its inherent nutritional value, and produce hydrolysate with special biological activities[9].

In the above context, there are certified that M. oleifera leaves protein is a valuable source with high nutritional value and well-balanced amino acid composition. However, its application is limited because of its structure[10]. Therefore, the objective of this study is to provide a systematic research of its isolated protein hydrolysate on the optimized extraction via the response surface method and the functional properties in order to provide the theoretical foundation for the research in the future.

2. Materials and methods

2.1 Materials
Moringa oleifera leaves were purchased from the Henan Golden Moringa Biological Technology Co., Ltd. (Henan, China), then crushed for 5 min through a pulverizer, and it was observed that there was no obvious granules, following the sieve was passed through a 60 mesh sieve to obtain a powder of Moringa leaves, which was stored under dry conditions. Bromelain (enzyme activity 6.94×106 U/g), was purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). All other chemicals and reagents used were of analytical grade.

Escherichia coli CICC(B)44103 was purchased from the China Center of Industrial Culture Collection (CICC).

2.2 Preparation of the M. oleifera leaves isolated protein
M. oleifera leaves were grounded using a grinder and defatted with petroleum ether (boiling range: 30-60 °C) at a material/solvent ratio of 1:5 by extracting 24 h. The dispersion was filtered through Buchner funnel at ambient temperature and residue used for next extraction. This process was repeated three times. Finally, the defatted flakes were air-dried in the fume hood until the solvent was removed completely, then ground to powder and store in a refrigerator at 4 ℃.

The protein isolate of M. oleifera leaves was extracted according to the method of alkaline extraction and acid precipitation described by Paula[9] and modified it appropriately. Briefly, the defatted M. oleifera leaves flour was dispersed in deionized water (1:100, w/v) and pH was adjusted to 9.0 using 0.5 M NaOH. Then extracted for 40 min in water bath at 40 ℃ and it was centrifuged (8000 rpm, 15 min), afterwards the supernatant was obtained. The two supernatants were pooled and pH of supernatants was adjusted to 4~4.5 with 1 M HCl to deposit the protein. The precipitated proteins were recovered by centrifugation for 15 min then resuspended in deionized water, neutralized, dialysis (10 kDa cut off) against water at 4 ℃ for 24 h. After freeze-dried, the protein isolate was stored at -20 ℃ until use.

2.3 Experimental design
Box- Behnken experimental design (BBD) was applied to statistically evaluate main interaction and quadratic effects of the formulation ingredients on response and to optimize the combination of variables in this study[11]. Three extraction variables, concentration of substrate (%) (A), enzymolysis time (h) (B) and enzyme dosage (U/g) (C) and three levels, coded as 1, 0 and -1 for high, intermediate and low level. Coded levels and actual values of the independent variables were showed in Table 1.

The dependent variable was the inhibition zone diameter of E. coli. The triplicates were conducted at all design points in randomized order. The levels of the independent variables utilized in the
experimental design were presented in Table 1. Design Expert software package version 8.0.6 was utilized to analyze the experimental data (mainly analysis of variance, regression analysis, contour plot, and optimization of the data). All the experiments were carried out three times and the inhibition zone diameter of *E. coli* was taken as the response.

Table 1. Box-Behnken design of the levels of factors.

| Independent variables | Coded symbols | Levels |
|-----------------------|---------------|--------|
| concentration of substrate | A             | -1 4 5 6 |
| enzyme dosage         | B             | 3000 4000 5000 |
| enzymolysis time      | C             | 3 4 5 |

2.4 Preparation of and hydrolysate
The protein was hydrolysed using Bromelain as previously described in Ennaas[12] with slight modification. Briefly, lyophilized protein isolate was dissolved in distilled water with the fixed concentration of substrate the fixed dosage of enzyme and hydrolyzed by treatment with Bromelain at 55 °C and pH 7.0. The hydrolysis was allowed to proceed for fixed time which the pH was maintained at 7.0 by addition of 1 M NaOH. At the end of hydrolysis, the enzyme was inactivated by heating at 100 °C for 15 min. The hydrolysate was clarified by centrifugation at 5000 r/min for 15 min at 16 °C to remove insoluble substrate fragments, and the supernatant was lyophilized and kept frozen at -20 °C until further use.

2.5 Chemical analysis
The content of crude protein, crude fat, ash and moisture of protein samples and enzymatic hydrolysis were determined by the standard methods of analysis (AOAC 1990).

2.6 Statistical analysis
All experiments were carried out in triplicate and averaged. The experimental data were mean ± standard deviation and plotted with Origin 8.0 software.

3. Results and discussion

3.1 Optimization of the enzymolysis conditions

3.1.1 Model fitting. According to the principle of box-behnken design, 3 factors and 3 levels were used to optimize the enzymatic hydrolysis conditions for bromelain enzymatic hydrolysis of moringa oleifera isolate. The experimental design and results were shown in table 2.

Table 2. Box-behnken experimental design and results.

| Run | Std | A (%) | B (U/g) | C (h) | Y (actual values) (mm) |
|-----|-----|-------|---------|-------|-----------------------|
| 5   | 1   | 4     | 4       | 3000  | 9.25                  |
| 10  | 2   | 5     | 5       | 3000  | 9.64                  |
| 8   | 3   | 6     | 4       | 5000  | 9.43                  |
| 17  | 4   | 5     | 4       | 4000  | 11.24                 |
| 11  | 5   | 5     | 3       | 5000  | 9.92                  |
| 12  | 6   | 5     | 5       | 5000  | 9.21                  |
| 7   | 7   | 4     | 4       | 5000  | 9.40                  |
| 2   | 8   | 6     | 3       | 4000  | 10.23                 |
| 6   | 9   | 6     | 4       | 3000  | 9.70                  |
| 1   | 10  | 4     | 3       | 4000  | 9.75                  |
| 15  | 11  | 5     | 4       | 4000  | 11.00                 |
| 3   | 12  | 4     | 5       | 4000  | 9.02                  |
According to the table 2, the application of the Design-Expert 8.0.6 indicated that the fitted quadratic model for inhibition zone diameter of E. coli incoded variables was shown in below equation:

\[ Y = 11.15 + 0.12A - 0.44B - 0.14C - 0.12AB - 0.11AC + 0.038BC - 1.01A^2 - 0.63B^2 - 0.70C^2 \]

where \( Y \) represented inhibition zone diameter of E. coli (mm), A, B and C were the actual values of the independent variables.

### Table 3. ANOVA for response surface quadratic model after optimization results.

| Source      | Sum of squares | DF | Mean square | F value  | P value |
|-------------|----------------|----|-------------|----------|---------|
| Model       | 10.8           | 9  | 1.2         | 44.32    | < 0.0001** |
| A           | 0.12           | 1  | 0.12        | 4.43     | 0.0733  |
| B           | 1.52           | 1  | 1.52        | 56.22    | 0.0001** |
| C           | 0.16           | 1  | 0.16        | 5.89     | 0.0456* |
| AB          | 0.053          | 1  | 0.053       | 1.95     | 0.2049  |
| AC          | 0.044          | 1  | 0.044       | 1.63     | 0.2426  |
| BC          | 5.63E-03       | 1  | 5.63E-03    | 0.21     | 0.6624  |
| A^2         | 4.26           | 1  | 4.26        | 157.42   | < 0.0001** |
| B^2         | 1.69           | 1  | 1.69        | 62.44    | < 0.0001** |
| C^2         | 2.06           | 1  | 2.06        | 75.91    | < 0.0001** |
| Residual    | 0.19           | 7  | 0.027       |          |         |
| Lack of Fit | 0.11           | 3  | 0.038       | 2.03     | 0.2525  |
| Pure Error  | 0.075          | 4  | 0.019       |          |         |
| Cor Total   | 10.99          | 16 |             |          |         |

\( R^2=0.9828 \)

\( R^2_{adj}=0.9606 \)

\( CV=1.64 \)

* Significant (p < 0.05).

** Very significant (p < 0.01).

In order to assessing the significance of the coefficient of the models, analysis of variance (ANOVA) was carried out. From Table 3, the coefficient of determination (R2) was 98.28% which indicated contribution of quadratic model was extremely significant (p <0.001), and the adequacy of the applied model. Hence, this equation could be utilized to forecast the actual values of inhibition zone diameter of E. coli.

The linear effect of B, A2, B2, C2 on antibacterial activity of hydrolysates were extremely significant (P<0.05), and those of B, C, A2, B2, C2 were also significant (P<0.01), but A, AB, AC, BC were not significant. All of these results indicated that the relationship between each specific experimental factor and the response surface was not a simple linear but a quadratic parabolic. Moreover, the higher the F value, the greater the influence on the response value. Therefore, the important order of influence of each factor was listed below: B (enzymatic time) > C (enzyme addition) > A (substrate concentration).

3.1.2 Three-dimensional surface response graphs and contour plots analysis. To clearly understand the effect of the independent variables on the response of interest, three-dimensional (3D) surface response and contour plots were illustrated in figure 1.
The effect of substrate concentration and enzymolysis time on the inhibition zone diameter of *E. coli* was shown in Figure 1a. As could be seen from the figure, when the enzyme dosage was fixed at zero level, the diameter of the inhibition zone of *E. coli* increased first and then decreased with the increase of substrate concentration and enzymatic hydrolysis time, and the maximum value was obtained at the center point. The substrate concentration and enzymatic hydrolysis time were both in a parabolic relationship with the diameter of the inhibition zone and the response surface was convex. The closer to the center position, the darker the color was, which indicated that the interaction between the two sides on the inhibition zone was greater. The contour line was close to the circle, which was consistent with the results in Table 1 in which the interaction effect of substrate concentration and enzymatic hydrolysis time on the diameter of the bacteriostasis circle was not significant (P>0.05).

Figure 1b showed that there was no significant interaction between substrate concentration and
enzyme dosage on the diameter of the inhibition zone of *E. coli* (P>0.05). When the enzymatic hydrolysis time was fixed, the antibacterial ability of the enzymatic hydrolysate increased first and then decreased with the increase of substrate concentration and enzyme dosage. Compared with the changes of the contour lines, it was found that the contour maps of the enzyme dosage and substrate concentration were close to a circle, that was to say, the interaction effect of the two on the diameter of the inhibition zone were weaker.

According to the contour line of figure 1.c and the 3D corresponding surface map, when the substrate concentration was fixed, the transform of inhibition zone diameter of *E. coli* with changes of enzymolysis time and enzyme dosage which was similar to that of the other two changes hydrolysis, and also showed a parabolic change. However, compared with the contour plots, the contours of enzymatic hydrolysis time and enzyme dosage were more densely distributed and closer to the elliptical shape, indicating that the interaction between the two had a stronger effect on the antibacterial activity of the hydrolysate. Although the results of cross-variation analysis of enzymatic hydrolysis time and enzyme dosage were not significant (P>0.05), but according to the trend of contour change, it could be concluded that the two factors still had a certain interaction, so that the contour line had obvious trend to elliptical.

According to figure 1, all the response surface images were convex, and the opening were downward. It could be seen that the inhibition zone diameter of *E. coli* and the antibacterial activity of the hydrolysate changed with the change of the independent variable, and the magnitude of the change was different. The interaction between the two factors was basically consistent with the effects of each single factor, and each interaction factor could find a center maximum value. It indicated that the substrate concentration, enzymatic hydrolysis time and enzyme dosage had a certain interaction effect on the antibacterial activity of the hydrolysate.

### 3.1 Verification of predictive models

The optimum conditions for enzymolysis were determined by a typical analysis of an experimental model, and obtained the following results: the concentration of substrate was 5.09%, the dosage of enzyme was 3883.42 U/g and hydrolyzed by Bromelain at 55 °C and pH 7.0 for 3.64 h. At the optimum conditions, the diameter of the inhibition zone of *E. coli* was 11.2412 mm. A verification test was performed to confirming the reliability of the model, and the result of the validation test showed that the inhibition zone diameter of *E. coli* was 11.2396 mm which was closed to the predicted value with deviation below 0.0016 mm. As a result, it could be seen that the model was valid to optimize the process of inhibition zone diameter of *E. coli* as well as the antibacterial activity of hydrolysate.

### 3.2 Chemical compositions

**Table 4. Nutrition composition analysis of protein isolate and hydrolysate of *Moringa* leaves.**

| Chemical component | Moisture (%) | Crude fat (%) | Protein content (%) | Ash (%) |
|--------------------|--------------|---------------|---------------------|--------|
| protein isolate    | 8.49±0.11    | 0.21±0.11     | 81.12±0.03          | 0.90±0.04 |
| hydrolysate        | 7.01±0.27    | 0.19±0.02     | 88.59±0.13          | 0.18±0.12 |

The chemical compositions of protein isolate and hydrolysate were shown in Table 4. It was found that ash and fat of the protein content decreased significantly, but the purity increased to 88.59%±0.13% after enzymolysis. It also indicates that impurities of *M. oleifera* leaves protein can be removed after the enzymatic treatment.

### 4. Conclusion

In this study, according to the inhibition zone diameter of E. coli as the dependent variable, the Box-Behnken experimental design (BBD) was applied to statistically evaluate main interaction and quadratic effects of the antimicrobial protein on response and to optimize the combination of variables. The optimization of enzymatic hydrolysis of antimicrobial protein was as follow, hydrolyzed at pH 7.0.
and 55 °C for 3.64 h, herein the substrate concentration of 5.09% and the enzyme dosage of 3883.42 U/g. Moreover, the obtained hydrolysate had been indicated that possessed antimicrobial activity and excellent functional properties. In a word, new orientation of isolated protein was explored, and the prospects of hydrolysate and protein isolate were broaden.

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