Analysis of enzymatic hydrolysis process of protein by experimental design method

T-W Chung and M-Y Wang
Chemical Engineering Department / Research Center for Circular Economy, Chung Yuan Christian University, Taoyuan 32023, Taiwan
twcharng@cycu.edu.tw

Abstract. In this study, the defatted press cake of peanut was used as raw material to extract the protein and the extract solution was further hydrolyzed by using an enzyme mixture of alcalase and flavourzyme. Most of the peanut protein were hydrolyzed to be smaller molecules after enzymatic hydrolysis. The response surface methodology was applied to analyze the parameters and the interaction between parameters of the enzymatic hydrolysis process for the protein extraction. On the basis of the experimental results, the maximum responses were obtained as the degree of hydrolysis of 32.2% and percentage of peptides (MW < 5 kDa) of 14.6%.

1. Introduction
Protein becomes the smaller peptides in the enzymatic hydrolysis process. Compare to protein, the small molecular proteins or peptides have better properties on nutrition and antioxidation. The peanut protein was hydrolyzed to peptides by using an enzyme of esperase [1] and the corn protein was hydrolyzed to peptides by using the enzyme mixture of alcalase and flavourzyme. [2] As mentioned above, these small molecular proteins or peptides are easy to be absorbed for human body and with high antioxidant activity, which will be the good raw material to make healthy foods or skin care products. The natural enzymatic hydrolyzed peanut protein can be substituted the antioxidants from chemical synthesis. The recycling of peanut protein from press cake may increase the additional value of peanut.

Other paragraphs are indented (BodytextIndented style). The peanut kernel has good nutrition and contains about 50% oil, which has specific composition of fatty acids. The major unsaturated fatty acid in the peanut oil is linoleic acid, which can reduce the cholesterol in serum of human body. Besides oil, the nutrition can get from peanut protein and it is about 24-36% protein in the peanut kernel. Peanut protein can be digested more than 90% and easy to be absorbed from the human body. It contains 18 amino acids, which is necessary for human body and the value of nutrition is very high. [3] The peanut kernel containing 16.1% carbohydrate (sugar or dietary fiber) and vitamin B and vitamin E are important for human health. [4]

The protein is completely hydrolyzed with the acid solution in the acidic hydrolysis process, which is the low cost and high efficiency process, especially in the solution with 6M HCl and temperature at 110°C for 24 hours. It can reduce the reaction time from 2 to 6 hours and is usually used in protein analysis and production. [5-6] Although the acidic hydrolysis process has the above mentioned advantages, it will destroy the nutrition from amino acids, e.g. tryptophan, methionine, serine, threonine, tyrosine, and produce the toxic by-products. The acidic hydrolysis process will be neutralized for the final product of protein and it will cause the higher contents of salt and glutamic acid. [7] Microwave
digestion hydrolysis technology is usually applied in the protein sample analysis. [8] Martin Weiss and his colleague found that the use of microwave digestion hydrolysis in silk may induce the loss of threonine and serine a little higher than the traditional hydrolysis process and the most of the results are similar in using these two different hydrolysis processes. [9] For the samples needed a long digestion time or not easy digestion, the microwave digestion hydrolysis can get a faster result. However, there is no using microwave digestion hydrolysis for protein degradation directly. It will combined with the acidic hydrolysis or enzymatic hydrolysis to increase the degree of hydrolysis. The enzymatic hydrolysis products can be used as the food additives because it may improve the function of hydrolyzed protein. [10-12] The enzymatic hydrolysis is widely used in special pharmaceutical process to strengthen the function and nutrition features and can be applied to manufacture the health food, low allergy formula, and high energy supplement. After hydrolysis modification, the small molecular peptides are easy to be absorbed for human body. [13-14] It is the major method to produce the hydrolysis products of biologically active protein by using enzymatic hydrolysis method. The hydrolysis process will not damage the amino acid because of the mild operating condition in the enzymatic hydrolysis process. [15] The antioxidants of the hydrolyzed protein from plant or animal will be easy to be absorbed and with very small side effect. [16] The study of enzymatic hydrolysis of natural protein to peptides may increase the value of hydrolyzed products, which are usually used as the feedstock of health foods and skin care products.

In this study, the press cake from the residue of peanut oil press was chosen as the feedstock in the protein extraction and enzymatic hydrolysis experiments. The protein enzymatic hydrolysis experiments were conducted in one factor at a time method first and select three significant parameters for the experimental design with response surface methodology.

2. Experiment
The enzymatic hydrolysis process was conducted in the extraction and hydrolysis process with solis-liquid ratio at 1:10, temperature at 55℃, and pH value at 9.0 using 1% enzyme concentration of alcalase, flavourzyme, or the mixture of alcalase and flavourzyme for 3 hours. As shown in Figure 1, the degree of hydrolysis of using enzyme mixture of alcalase and flavourzyme was better than those of using single enzyme. Therefore, the enzyme mixture was selected in this study for the further experiments.

Since the degree of hydrolysis was varied with the temperature and pH value, the 1% enzyme mixture in 1:1 volume ratio of alcalase and flavourzyme, solid-liquid ratio at 1:10 were selected in the enzymatic hydrolysis reaction for 3 hours and the result was shown in Figure 2.

The optimum operating parameters of temperature and pH value for enzymatic hydrolysis process was varied in different protein feedstock. The enzymatic hydrolysis of peanut protein had better parameters in temperature of 55℃ and pH value of 9.0. Since the characteristic of hydrolysate was affected in the condition with higher pH values, the pH values were considered in less than 9.0 and the hydrolysis parameters were selected in the temperature of 55℃ and pH value of 9.0 for the experiments in this study.

![Figure 1. Effect of different enzyme on the degree of hydrolysis of peanut protein.](image1)

![Figure 2. Effect of different temperature and pH value on the degree of hydrolysis of peanut protein.](image2)
The response surface methodology (RSM) was conducted in this study for the experimental design in 3 factors and 3 levels. The 3 factors for the enzymatic hydrolysis process were selected as solid-liquid ratio ($X_1$), enzyme concentration ($X_2$), hydrolysis time ($X_3$) and the response were the degree of hydrolysis ($Y_1$) and the percentage of small molecular weight (less than 5kDa) protein ($Y_2$). The 3 levels (-1, 0, +1) of the 3 factors were listed in Table 1 and the analysis of the variance method was applied to discuss the effects of each variable and the interaction between variables.

| Table 1. The 3 levels of the 3 factors in this study.               |
|-------------------------------------------------------------------|
| Level | $X_1$ (w/v) | $X_2$ (%) | $X_3$ (hr) |
|-------|-------------|-----------|------------|
| -1    | 5           | 5         | 4          |
| 0     | 10          | 7.5       | 6          |
| +1    | 15          | 10        | 8          |

3. Results and Discussion

3.1. Degree of hydrolysis
There were 15 experimental runs designed by Box-Behnken experimental design methodology for the enzymatic hydrolysis process of this study and the degree of hydrolysis ($Y_1$) were obtained and listed in Table 2. The 10th run with maximum degree of hydrolysis of 32.2% was obtained in the operating factors of solid-liquid ratio ($X_1$) at 1:10, enzyme concentration ($X_2$) at 10%, and hydrolysis time ($X_3$) at 8 hours.

| Table 2. The degree of hydrolysis in the 15 experimental runs of RSM. |
|---------------------------------------------------------------------|
| Run | Mode | $X_1$ | $X_2$ | $X_3$ | $Y_1$ (%) |
|-----|------|------|------|------|-----------|
| 1   | ++0  | 5    | 10   | 6    | 23.58     |
| 2   | +0-  | 15   | 5    | 6    | 24.17     |
| 3   | +0+  | 15   | 7.5  | 8    | 29.2      |
| 4   | -0+  | 5    | 7.5  | 8    | 20.8      |
| 5   | 000  | 10   | 7.5  | 6    | 30.72     |
| 6   | 000  | 10   | 7.5  | 6    | 29.35     |
| 7   | 0--  | 10   | 5    | 4    | 25.02     |
| 8   | 0+-  | 10   | 10   | 4    | 30.5      |
| 9   | +0-- | 15   | 7.5  | 4    | 24.18     |
| 10  | 0++  | 10   | 10   | 8    | 32.2      |
| 11  | --0  | 5    | 5    | 6    | 19.42     |
| 12  | -0-- | 5    | 7.5  | 4    | 18.99     |
| 13  | 0-+  | 10   | 5    | 8    | 26.25     |
| 14  | 000  | 10   | 7.5  | 6    | 28.91     |
| 15  | .+0  | 15   | 10   | 6    | 27.63     |

The degree of hydrolysis ($Y_1$) in the 15 runs and their operating factors were regressed to obtain a second-order polynomial equation as follow.

\[
Y_1 = 29.66 + 2.8 \frac{(X_1-10)}{5} + 2.38 \frac{(X_2-7.5)}{2.5} + 1.22 \frac{(X_3-6)}{2} - 0.17 \frac{(X_1-10)}{5} \frac{(X_2-7.5)}{2.5} - 0.80 \frac{(X_1-10)}{5} \frac{(X_3-6)}{2} + 0.12 \frac{(X_2-7.5)}{2.5} - 5.58 \frac{(X_1-10)}{5} - 0.38 \frac{(X_2-7.5)}{2.5} - 0.79 \frac{(X_3-6)}{2}\]

(1)
The experimental results of the degree of hydrolysis and the predictions from Equation 1 were plotted in Figure 3. It can be found that most of the predictions are coincident with the experimental data and the $R^2$ of the above regression equation is 0.97.

The effects of the factors on the response $Y_1$ can be identified in Table 3. The student’s $t$ test was applied to identify the significance of the effect of each factor or the interaction between factors. In usual, the $P$-value will be reduced with the increase of $t$ ratio. The smaller of the $P$-value is, the effect of the factor on the response will be more significant. From the results in Table III, the $P$-values of solid-liquid ratio ($X_1$), enzyme concentration ($X_2$), and the hydrolysis time ($X_3$) are smaller than 0.05. It represents the effect of these three factors are significant and the order of the effect of these three factors on the response are $X_1$>$X_2$>$X_3$. It also can be observed from Table 3, the interaction between factors are not significant.

| Term       | Estimate | Standard Error | $t$ ratio | $P$-value |
|------------|----------|----------------|-----------|-----------|
| Intercept  | 29.66    | 0.75           | 39.67     | <.0001*   |
| $X_1$      | 2.8      | 0.46           | 6.11      | 0.0017*   |
| $X_2$      | 2.38     | 0.461          | 5.20      | 0.0035*   |
| $X_3$      | 1.22     | 0.46           | 2.66      | 0.0448*   |
| $X_1X_2$   | -0.17    | 0.65           | -0.27     | 0.7985    |
| $X_1X_3$   | 0.80     | 0.65           | 1.24      | 0.2709    |
| $X_2X_3$   | 0.12     | 0.65           | 0.18      | 0.8618    |
| $X_1X_1$   | -5.582   | 0.67           | -8.28     | 0.0004*   |
| $X_2X_2$   | -0.38    | 0.67           | -0.56     | 0.5971    |
| $X_3X_3$   | -0.79    | 0.67           | -1.17     | 0.2956    |

*Significant

The effect of each variable on the response of the degree of hydrolysis was shown in Figure 4. The maximum degree of hydrolysis was obtained at $X_1$ at 1:11, however, the degree of hydrolysis was increased with the increasing of $X_2$ and $X_3$.

3.2. Percentage of peptides ($MW < 5$ kDa)
There were 15 experimental runs designed by Box-Behnken experimental design methodology for the enzymatic hydrolysis process of this study and the percentage of peptides ($Y_2$) were obtained and
listed in Table 4. The 8th run with maximum percentage of peptides of 14.6% was obtained in the operating factors of solid-liquid ratio (X_1) at 1:10, enzyme concentration (X_2) at 10%, and hydrolysis time (X_3) at 4 hours.

Table 4. The percentage of peptides in the 15 experimental runs of RSM.

| Run | Mode | X_1 | X_2 | X_3 | Y_2 (%) |
|-----|------|-----|-----|-----|---------|
| 1   | -+0  | 5   | 10  | 6   | 3.90    |
| 2   | ++0  | 15  | 5   | 6   | 7.91    |
| 3   | +0+  | 15  | 7.5 | 8   | 10.70   |
| 4   | -0+  | 5   | 7.5 | 8   | 5.21    |
| 5   | 000  | 10  | 7.5 | 6   | 5.78    |
| 6   | 000  | 10  | 7.5 | 6   | 3.52    |
| 7   | 0--  | 10  | 5   | 4   | 7.62    |
| 8   | 0+-  | 10  | 10  | 4   | 14.60   |
| 9   | +0-  | 15  | 7.5 | 4   | 14.30   |
| 10  | 0++  | 10  | 10  | 8   | 12.50   |
| 11  | 0−0  | 5   | 5   | 6   | 2.92    |
| 12  | 0−−  | 5   | 7.5 | 4   | 5.36    |
| 13  | 0−+  | 10  | 5   | 8   | 7.69    |
| 14  | 000  | 10  | 7.5 | 6   | 6.27    |
| 15  | ++0  | 15  | 10  | 6   | 12.60   |

The percentage of peptides (Y_2) in the 15 runs and their operating factors were regressed to obtain a second-order polynomial equation as follow.

\[
Y_2 = 5.86 + 3.52 \frac{(X_1-10)}{5} + 2.18 \frac{(X_2-7.5)}{2.5} - 0.71 \frac{(X_3-6)}{2} + 0.92 \frac{(X_1-10)}{5} \frac{(X_2-7.5)}{2.5} - 0.86 \frac{(X_1-10)}{5} \frac{(X_3-6)}{2} \]

\[
- 0.53 \frac{(X_2-7.5)}{2.5} \frac{(X_4-6)}{2} - 0.37 \frac{(X_1-10)}{5} \frac{(X_1-10)}{5} + 1.33 \frac{(X_2-7.5)}{2.5} \frac{(X_2-7.5)}{2.5} + 3.41 \frac{(X_3-6)}{2} \frac{(X_3-6)}{2}
\]

(2)

The experimental results of the percentage of peptides and the predictions from Equation 1 were plotted in Figure 5. It can be found that most of the predictions are coincident with the experimental data and the R^2 of the above regression equation is 0.97.

The effects of the factors on the response Y_1 can be identified in Table 5. The student’s t test was applied to identify the significance of the effect of each factor or the interaction between factors. In usual, the P-value will be reduced with the increase of t ratio. The smaller of the P-value is, the effect of the factor on the response will be more significant. From the results in Table 5, the P-values of solid-liquid ratio (X_1) and enzyme concentration (X_2) are smaller than 0.05. It represents the effect of the two factors are significant and the order of the effect of the two factors on the response are X_1>X_2. It also can be observed from Table 5, the interaction between factors are not significant.

Table 5. Effect of variables on the percentage of peptides.

| Term   | Estimate | Standard Error | t ratio | P-value |
|--------|----------|----------------|---------|---------|
| Intercept | 5.86     | 0.61           | 9.68    | 0.0002* |
| X_1    | 3.52     | 0.37           | 9.49    | 0.0002* |
| X_2    | 2.18     | 0.37           | 5.87    | 0.0020* |
| X_3    | -0.71    | 0.37           | -1.92   | 0.1123  |
| X_1X_2 | 0.92     | 0.52           | 1.76    | 0.1390  |
| X_1X_3 | -0.86    | 0.52           | -1.64   | 0.1621  |
X₂X₁ -0.53 0.52 -1.01 0.3605
X₁X₁ -0.37 0.55 -0.67 0.5330
X₂X₂ 1.33 0.55 2.44 0.0586
X₃X₃ 3.41 0.55 6.25 0.0015*

*Significant

The effect of each variable on the response of the percentage of peptides was shown in Figure 6. The minimum percentage of peptides was obtained at X₃ at 6 hours, however, percentage of peptides was increased with the increasing of X₁ and X₂.

Figure 5. Comparison of the predicted and experimental data of the percentage of peptides.

Figure 6. Effect of each variable on the response Y₂.

4. Conclusion

In this study, the response surface methodology was conducted for the enzymatic hydrolysis of peanut protein to discuss the operating parameters and the optimum operating conditions. On the basis of the experimental data, the best degree of hydrolysis was obtained in 32.2% (X₁ = 1:10, X₂ = 10%, X₃ = 8 hr). The effects of solid-liquid ratio (X₁), enzyme concentration (X₂) and hydrolysis time (X₃) on the response of degree of hydrolysis (Y₁) were significant. The best percentage of peptides was obtained in 14.6% (X₁ = 1:10, X₂ = 10%, X₃ = 4 hr). The effects of solid-liquid ratio (X₁) and enzyme concentration (X₂) on the response of percentage of peptides (Y₂) were significant.

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