Analysis of antioxidation activity for the small molecular biomass extracted from the waste of resources

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Abstract. The waste of peanut press cake from oil mill was powdered and used as the feedstock to extract the protein and the extract solution was further hydrolysed by using an enzyme mixture of alcalase and flavourzyme. The peanut protein was become the smaller molecules after enzymatic hydrolysis and the antioxidation activities were analysed by using experimental design. The response surface methodology was applied to analyse the parameters and the interaction between parameters of the enzymatic hydrolysis process for the variation of antioxidation activities of the small molecular protein. On the basis of the experimental results, the optimum responses of antioxidation activities were obtained as the DPPH free radical scavenging ability of 76.85%, reducing power (absorbance value) of 0.1 Abs, and Ferrous ion (Fe²⁺) chelating ability of 17.19% under the operating parameters in solid-liquid ratio of 1:10, enzyme concentration of 10%, and hydrolysis time of 8 hr.

1. Introduction

The small molecular biomass was considered as the important functional material with biological activity. The extraction and analysis of small molecular protein or phytochemicals from plants, e.g. saponin, carotenoids, and polyphenols, was conducted for many researchers. Peanut protein provides many amino acids through the digestion of protein. Protein may become the smaller peptides of amino acids in the enzymatic hydrolysis process. Compare to protein, the small molecular proteins or peptides have better properties on antioxidation activity. The corn protein was hydrolyzed to peptides by using the enzyme mixture of alcalase and flavourzyme [1] and the peanut protein was hydrolyzed to peptides by using an enzyme of esperase. [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 feedstock to make healthy foods or skin care products. As noted the recycling of peanut protein from press cake will increase the additional value of peanut. The natural enzymatic hydrolyzed peanut protein can be substituted the antioxidants from chemical synthesis in skin care products.

The peanut contains plenty protein and rich 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 impressed. [3] The peanut kernel containing 16.1% carbohydrate, sugar, dietary fiber, vitamin B and vitamin E are important for human health. [4]
The protein can be hydrolyzed with the acid solution in the acidic hydrolysis process completely, which is a low cost and high efficiency process. The acidic hydrolysis process is usually operated in the solution with 6M HCl and temperature at 110°C for 24 hours and 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 manufature 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] The major method to produce the hydrolysis products of biologically active protein is to use 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 and application of hydrolyzed products. Since this report is the second part of data discussion of previous study [17], the above literature survey is partly from it. 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. The response surface methodology was applied to analyze the parameters and the interaction between parameters of the enzymatic hydrolysis process for the variation of antioxidation activity of the hydrolyzed protein. The antioxidant activities in different procedures of extraction with microwave digestion hydrolysis and enzymatic hydrolysis were compared.

2. Experiment
The extraction with enzymatic hydrolysis process was conducted with solis-liquid ratio at 1:10, temperature at 55°C, and pH value at 9.0 using 1% enzyme concentration of alcalase, flavourzyme, or the mixture of alcalase and flavourzyme for 3hours. 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. The maximum degree of hydrolysis was observed in the previous study of the operating parameters in temperature of 55°C and pH value of 9.0 for enzymatic hydrolysis process in different protein feedstock. [17]
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 (X₁), enzyme concentration (X₂), hydrolysis time (X₃) and the response were antioxidation activities, including the DPPH free radical scavenging ability (Y₁), the reducing power (Y₂), and the ferrous ion chelating ability (Y₃). The 3 levels (-1, 0, +1) of the factor X₁ were 5, 10, and 15(w/v); the factor X₂ were 5, 7.5, 10%; the factor X₃ were 4, 6, 8hr and the analysis of the variance method was applied to discuss the effects of each variable and the interaction between variables.

3. Results and discussion
3.1. DPPH free radical scavenging ability

There were 15 experimental runs designed by Box-Behnken experimental design methodology for the enzymatic hydrolysis process of this study and the DPPH free radical scavenging ability \( Y_1 \) were obtained and listed in Table 1. The 10th run with maximum DPPH free radical scavenging ability of 76.85% 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.

| Run | Mode | \( X_1 \) | \( X_2 \) | \( X_3 \) | \( Y_1 \) (%) |
|-----|------|----------|----------|----------|-------------|
| 1   | −−0  | 5        | 10       | 6        | 14.18       |
| 2   | +−0  | 15       | 5        | 6        | 6.152       |
| 3   | +0+  | 15       | 7.5      | 8        | 42.34       |
| 4   | −0+  | 5        | 7.5      | 8        | 25.23       |
| 5   | 000  | 10       | 7.5      | 6        | 54.12       |
| 6   | 000  | 10       | 7.5      | 6        | 57.14       |
| 7   | 0−−  | 10       | 5        | 4        | 11.78       |
| 8   | 0−−  | 10       | 10       | 4        | 58.08       |
| 9   | +0−  | 15       | 7.5      | 4        | 24.19       |
| 10  | 0++  | 10       | 10       | 8        | 76.85       |
| 11  | −−0  | 5        | 5        | 6        | 12.2        |
| 12  | −0−  | 5        | 7.5      | 4        | 21.79       |
| 13  | 0−+  | 10       | 5        | 8        | 37.43       |
| 14  | 000  | 10       | 7.5      | 6        | 62.88       |
| 15  | .++0 | 15       | 10       | 6        | 53.91       |

The DPPH free radical scavenging ability \( (Y_1) \) in the 15 runs and their operating factors were regressed to obtain a second-order polynomial equation as follow.

\[
Y_1 = 58.05 + 6.65 \left( \frac{(X_2-10)}{5} \right) + 16.93 \left( \frac{(X_2-7.5)}{2.5} \right) + 8.25 \left( \frac{(X_3-6)}{2} \right) + 11.44 \left( \frac{(X_1-10)}{5} \right) \left( \frac{(X_2-7.5)}{5} \right) + 3.68 \left( \frac{(X_1-10)}{5} \right) \left( \frac{(X_3-6)}{2} \right) - 1.72 \left( \frac{(X_2-7.5)}{2.5} \right) \left( \frac{(X_3-6)}{2.5} \right) - 27.04 \left( \frac{(X_1-10)}{5} \right) \left( \frac{(X_3-6)}{5} \right) - 9.39 \left( \frac{(X_2-7.5)}{2.5} \right) \left( \frac{(X_2-7.5)}{2.5} \right) - 2.62 \left( \frac{(X_1-10)}{2} \right) \left( \frac{(X_3-6)}{2} \right) \left( \frac{(X_2-7.5)}{2} \right) \left( \frac{(X_3-6)}{2} \right)
\]  

The experimental results of the DPPH free radical scavenging ability and the predictions from Equation 1 were plotted in Figure 1. 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 0.96.

The effects of the factors on the response \( Y_1 \) can be identified in Table 2. 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 2, the P-values of enzyme concentration \( (X_2) \), and the hydrolysis time \( (X_3) \) are smaller than 0.05. It represents the effect of these two factors are significant and the order of the effect of these three factors on the response are \( X_2 > X_3 > X_1 \). It also can be observed from Table 2, the interaction between factors are significant in \( X_1X_2 \).
Table 2. Effect of variables on the DPPH free radical scavenging ability.

| Term   | Estimate | Standard Error | t ratio | P-value |
|--------|----------|----------------|---------|---------|
| Intercept | 58.05 | 4.41 | 13.16 | <.0001* |
| $X_1$   | 6.65    | 2.70 | 2.46 | 0.0571 |
| $X_2$   | 16.93   | 2.70 | 6.27 | 0.0015* |
| $X_3$   | 8.25    | 2.70 | 3.06 | 0.0283* |
| $X_1X_2$ | 11.44  | 3.82 | 3.00 | 0.0302* |
| $X_1X_3$ | 3.68   | 3.82 | 0.96 | 0.3800 |
| $X_2X_3$ | -1.72  | 3.82 | -0.45 | 0.6712 |
| $X_1X_3$ | -27.04 | 3.98 | -6.80 | 0.0010* |
| $X_2X_3$ | -9.39  | 3.98 | -2.36 | 0.0645 |
| $X_1X_3$ | -2.62  | 3.98 | -0.66 | 0.5396 |

*Significant

The effect of each variable on the response of the DPPH free radical scavenging ability was shown in Figure 2. The maximum DPPH free radical scavenging ability was obtained at $X_1$ of 1:10, however, the DPPH free radical scavenging ability was increased with the increasing of $X_2$ and $X_3$.

3.2. Reducing power

There were 15 experimental runs designed by Box-Behnken experimental design methodology for the enzymatic hydrolysis process of this study and the absorbance value for reducing power ($Y_2$) were obtained and listed in Table 3. The 8th run with maximum reducing power of 0.097 Abs was obtained in the operating factors of solid-liquid ratio ($X_1$) at 1:10, enzyme concentration ($X_2$) at 7.5%, and hydrolysis time ($X_3$) at 6 hours.
Table 3. The reducing power in the 15 experimental runs of RSM.

| Run | Mode | X₁ | X₂ | X₃ | Y₂ (Abs) |
|-----|------|----|----|----|---------|
| 1   | ++0  | 5  | 10 | 6  | 0.048   |
| 2   | +−0  | 15 | 5  | 6  | 0.054   |
| 3   | +0+  | 15 | 7.5| 8  | 0.062   |
| 4   | −0+  | 5  | 7.5| 8  | 0.038   |
| 5   | 000  | 10 | 7.5| 6  | 0.097   |
| 6   | 000  | 10 | 7.5| 6  | 0.090   |
| 7   | 0−−  | 10 | 5  | 4  | 0.050   |
| 8   | 0++  | 10 | 10 | 4  | 0.086   |
| 9   | +0−  | 15 | 7.5| 4  | 0.047   |
| 10  | 0++  | 10 | 10 | 8  | 0.001   |
| 11  | −0−  | 5  | 5  | 6  | 0.005   |
| 12  | −0−  | 5  | 7.5| 4  | 0.027   |
| 13  | 0−+  | 10 | 5  | 8  | 0.053   |
| 14  | 000  | 10 | 7.5| 6  | 0.090   |
| 15  | .++0 | 15 | 10 | 6  | 0.084   |

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

$$Y_2 = 0.092 + 0.0160 \frac{(X_1-10)}{5} + 0.02 \frac{(X_2-7.5)}{2.5} + 0.005 \frac{(X_3-6)}{2}$$

$$- 0.003 \frac{(X_1-10)}{5} \frac{(X_2-7.5)}{2.5} + 0.001 \frac{(X_1-10)}{5} \frac{(X_3-6)}{2}$$

$$+ 0.003 \frac{(X_2-7.5)}{2.5} \frac{(X_3-6)}{2} - 0.037 \frac{(X_1-10)}{5} \frac{(X_1-10)}{5}$$

$$- 0.008 \frac{(X_2-7.5)}{2.5} \frac{(X_2-7.5)}{2.5} - 0.012 \frac{(X_3-6)}{2} \frac{(X_3-6)}{2}$$

(2)

The experimental results of the percentage of peptides and the predictions from Equation 2 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 0.98.

The effects of the factors on the response $Y_2$ can be identified in Table 4. 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 4, 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 these three factors on the response are $X_2>X_1>X_3$. It also can be observed from Table 4, the interaction between factors are not significant.
### Table 4. Effect of variables on the reducing power.

| Term   | Estimate | Standard Error | t ratio | P-value |
|--------|----------|----------------|---------|---------|
| Intercept | 0.092    | 0.004          | 21.91   | <.0001* |
| $X_1$  | 0.016    | 0.003          | 6.21    | 0.0016* |
| $X_2$  | 0.02     | 0.003          | 7.58    | 0.0006* |
| $X_3$  | 0.005    | 0.003          | 2.08    | 0.0920  |
| $X_1X_2$ | -0.003  | 0.004          | -0.84   | 0.4372  |
| $X_1X_3$ | 0.001    | 0.004          | 0.23    | 0.8286  |
| $X_2X_3$ | 0.003    | 0.004          | 0.71    | 0.5111  |
| $X_1X_1$ | -0.037   | 0.004          | -9.63   | 0.0002* |
| $X_2X_2$ | -0.008   | 0.004          | -2.12   | 0.0877  |
| $X_3X_3$ | -0.012   | 0.004          | -3.24   | 0.0231* |

*Significant

The effect of each variable on the response of the reducing power was shown in Figure 4. The maximum reducing power was obtained at $X_1$ of 1:10 and at $X_3$ of 6 hours, however, reducing power was increased with the increasing of $X_2$.

**Figure 3.** Comparison of the predicted and experimental data of the reducing power.

**Figure 4.** Effect of each variable on the response $Y_2$.

### 3.3. Ferrous ion chelating ability

There were 15 experimental runs designed by Box-Behnken experimental design methodology for the enzymatic hydrolysis process of this study and the ferrous ion chelating ability ($Y_3$) were obtained and listed in Table 5. The 10th run with maximum ferrous ion chelating ability of 26.66% was obtained in the operating factors of solid-liquid ratio ($X_1$) at 1:15, enzyme concentration ($X_2$) at 10%, and hydrolysis time ($X_3$) at 6 hours.
Table 5. The ferrous ion chelating ability in the 15 experimental runs of RSM.

| Run | Mode  | X1 | X2 | X3 | Y1 (%) |
|-----|-------|----|----|----|-------|
| 1   | −+0   | 5  | 10 | 6  | 11.63 |
| 2   | +−0   | 15 | 5  | 6  | 8.46  |
| 3   | +0+   | 15 | 7.5| 8  | 23.51 |
| 4   | −0+   | 5  | 7.5| 8  | 11.15 |
| 5   | 000   | 10 | 7.5| 6  | 24.61 |
| 6   | 000   | 10 | 7.5| 6  | 20.84 |
| 7   | 0−−   | 10 | 5  | 4  | 10.31 |
| 8   | 0−+   | 10 | 10 | 4  | 14.58 |
| 9   | +0−   | 15 | 7.5| 4  | 15.20 |
| 10  | 0++   | 10 | 10 | 8  | 17.19 |
| 11  | −−0   | 5  | 5  | 6  | 9.80  |
| 12  | −0−   | 5  | 7.5| 4  | 11.26 |
| 13  | 0−+   | 10 | 5  | 8  | 4.52  |
| 14  | 000   | 10 | 7.5| 6  | 21.63 |
| 15  | ++0   | 15 | 10 | 6  | 26.66 |

The ferrous ion chelating ability \((Y_3)\) in the 15 runs and their operating factors were regressed to obtain a second-order polynomial equation as follow.

\[
Y_3 = 22.36 + 3.75 \left( \frac{X_1-10}{5} \right) + 4.62 \left( \frac{X_2-7.5}{2.5} \right) + 0.63 \left( \frac{X_3-6}{2} \right) \\
+ 4.09 \left( \frac{X_1-10}{5} \right) \left( \frac{X_2-7.5}{2.5} \right) + 2.11 \left( \frac{X_1-10}{5} \right) \left( \frac{X_3-6}{2} \right) \\
+ 2.10 \left( \frac{X_2-7.5}{2.5} \right) \left( \frac{X_3-6}{2} \right) - 2.30 \left( \frac{X_1-10}{5} \right) \left( \frac{X_1-10}{5} \right) \\
- 5.93 \left( \frac{X_2-7.5}{2.5} \right)^2 - 4.78 \left( \frac{X_3-6}{2} \right)^2 - 2.30 \left( \frac{X_1-10}{5} \right) \left( \frac{X_2-7.5}{2.5} \right) \\
+ 2.10 \left( \frac{X_1-10}{5} \right) \left( \frac{X_3-6}{2} \right) - 2.30 \left( \frac{X_1-10}{5} \right) \left( \frac{X_1-10}{5} \right) \\
- 5.93 \left( \frac{X_2-7.5}{2.5} \right)^2 - 4.78 \left( \frac{X_3-6}{2} \right)^2 (3)
\]

The experimental results of the ferrous ion chelating ability and the predictions from Equation 3 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 0.96.

The effects of the factors on the response \(Y_3\) can be identified in Table 6. 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 6, the P-values of enzyme concentration (\(X_1\)), and the hydrolysis time (\(X_2\)) are smaller than 0.05. It represents the effect of these two factors are significant and the order of the effect of these three factors on the response are \(X_2>X_1>X_3\). It also can be observed from Table 2, the interaction between factors are not significant.


Table 6. Effect of variables on the ferrous ion chelating ability.

| Term     | Estimate | Standard Error | t ratio | P-value |
|----------|----------|----------------|---------|---------|
| Intercept| 22.36    | 1.32           | 16.95   | <.0001* |
| $X_1$    | 3.75     | 0.81           | 4.64    | 0.0056* |
| $X_2$    | 4.62     | 0.81           | 5.72    | 0.0023* |
| $X_3$    | 0.63     | 0.81           | 0.78    | 0.4718  |
| $X_1X_2$ | 4.09     | 1.14           | 3.58    | 0.0158* |
| $X_1X_3$ | 2.11     | 1.14           | 1.84    | 0.1245  |
| $X_2X_3$ | 2.10     | 1.14           | 1.84    | 0.1255  |
| $X_1X_1$ | -2.30    | 1.19           | -1.93   | 0.1113  |
| $X_2X_2$ | -5.93    | 1.19           | -4.98   | 0.0042* |
| $X_3X_3$ | -4.78    | 1.19           | -4.02   | 0.0101* |

*Significant

The effect of each variable on the response of the ferrous ion chelating ability was shown in Figure 6. The maximum ferrous ion chelating ability was obtained at $X_2$ of 7.5% and $X_3$ of 6 hours, however, the ferrous ion chelating ability was increased with the increasing of $X_1$.

4. Conclusion

In this study, the analysis of variances (ANOVA) method was conducted for the experimental design of enzymatic hydrolysis of peanut protein to obtain the optimum operating conditions. The best DPPH free radical scavenging ability was obtained in 74.66 % ($X_1 = 1 : 12.2$, $X_2 = 10.1\%$, $X_3 = 9.1$ hr). The effects of enzyme concentration ($X_2$) and hydrolysis time ($X_3$) on the response of degree of hydrolysis ($Y_1$) were significant. The best reducing power was obtained in 0.11 Abs ($X_1 = 1 : 10.8$, $X_2 = 10.6\%$, $X_3 = 6.7$ hr). The effects of solid-liquid ratio ($X_1$) and enzyme concentration ($X_2$) on the response of percentage of peptides ($Y_2$) were significant. The best ferrous ion chelating ability was obtained in 30.64 % ($X_1 = 1 : 22.6$, $X_2 = 11.1\%$, $X_3 = 7.9$ hr). The effects of solid-liquid ratio ($X_1$) and enzyme concentration ($X_2$) on the response of percentage of peptides ($Y_3$) were significant. On the basis of the
experimental results, the optimum responses were obtained as the DPPH free radical scavenging ability of 76.85%, reducing power (absorbance value) of 0.1 Abs, and Fe2+ chelating ability of 17.19% under the operating parameters in solid-liquid ratio of 1:10, enzyme concentration of 10%, and hydrolysis time of 8 hr.

5. References

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