The Optimization of Protease Enzyme Extraction From Streblus Asper (Kesinai)

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Abstract. Protease from Streblus asper (Kesinai) is an interesting rennet substitute and yet very few studies had been conducted so far. In the present study, the leaf extract of Kesinai had been discovered to investigate the ability of this milk coagulating enzyme. The development of the optimized conditions for enzyme extraction was analyzed by using Central Composite Design (CCD). The studied factors were ratio of sample to buffer, weight of sample (g) and homogenization time (min). It was found that a 30 g of S. asper leaves sample with the ratio of the sample to buffer of 1:1 and at a mixing rate of 2 minutes established the most desirable conditions for serine proteases extraction from the S. asper leaves sample.

1. Introduction

Animal rennet is the traditional and most common used enzyme in coagulating milk over the century. Owing to the limited availability of mammalian stomachs which has failed to meet the increasing demand of cheese production, and due to the religious reasons (e.g., Judaism and Islam), it led to the development of other sources of enzymes, ranging from plants, fungi, and microbial sources, that can substitute for animal rennet [1]. Although there are various of fungi and microbial sources available for protease enzyme production, there is only a few are considered as commercial producers according to the Governmental food safety organizations such as the European Food Safety Authority: “Qualified Presumption of Safety status denies the protease enzyme produced by the fungi” [2]. Hence, the vegetable rennet has become a preferable substitute for the rennet enzyme substitute [3].

The protease enzyme from S. asper Lour (Kesinai) is very consistent in manufacturing and it tends to reduce allergic properties of dairy products. Besides, plant protease is suitable for vegetarians and it might be used in the production of Kosher and halal cheeses. Therefore, plant protease offers an excellent option for biotechnology fields, food processing, medical application, daily production and pharmacology industries. In food science applications, enzymes have been utilize to improve the quality of products. Other than that, proteases are also applied in daily production such as cheese production, for coagulating milk to form cheese curd and improving the texture or flavor of the cheese products.

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Plant proteases have been discovered as milk coagulants by characterizing and isolated aspartic proteases from plant origin. Multiple plant based aspartic proteases have been reported to contain coagulating characteristics including proteases from Centaurea calcitrapa [4] green fruit of Ficus carica [5]; Actinidia chinesis [6] and fruits’ part of Solanum dubium [7]. Leave extract from S. asper has been prove to contain serine proteases, which could be a potential rennet substituent [8-9].

The high potential of these plants protease has significantly increased the interest among researchers to investigate our studied plant, Kesinai further. However, to the best of our knowledge, very limited studies have been reported on the optimization of protease enzyme extraction thus far. Plant proteases show the substrate specificity as well as high activity and stability over a wide range of pH and temperature values and in the presence of various metal ions, organic solvents, and inhibitors [8]. This paper evaluates the relationships among the optimized conditions, namely, ratio sample to buffer, weight of samples and homogenization time, in order to yield optimum protease activity from S. asper for milk coagulation

2. Materials and Methods

2.1. Material and Sample Preparation

The fresh samples of S. asper leaves were collected from Bintong, Wang Ulu, Perlis and washed several times with distilled water and left to dry at room temperature for half an hour prior to extraction procedures. All the chemicals used in the study were analytical graded and used without further purification.

2.2. Crude Enzyme Extraction

The protease extraction method was based on the modification of the method by Mehrnoush et. al [3] & Ruzaina et. al. [10]. The 50 g of fresh plant parts were homogenized by using a laboratory blender at room temperature and adding with potassium buffers. The homogenates were filtered through muslin cloth and the filtrate were centrifuged at 10000 rpm for 30 minutes at 4°C using high speed refrigerated centrifuge in order to discard the solid formed. The supernatants were collected and used as the crude enzyme extract. The crude enzyme was centrifuged twice to replace the ultrafiltration technique. The crude enzyme was stored at 4°C for further use.

2.3. Optimization of Protease Enzyme Extraction

The optimization of protease enzyme extraction was done by using Central Composite Design (CCD). The factors involved in this experimental design were the ratio of sample to buffer, weight of samples (g) and homogenization time (minutes). While the response of the design was protease activity (unit/mL). Table 1 showed the respective level of each factor.

| Factors                  | Unit | Symbol | Low level (-1) | High level (+1) |
|--------------------------|------|--------|----------------|-----------------|
| Ratio (Sample:buffer)    | A    | A      | 1              | 5               |
| Weight of sample         | g    | B      | 10             | 50              |
| Homogenization Time      | minutes | C   | 2              | 6               |

2.4 Protease Activity

The protease activity was determined according to the Sigma’s protocol [11]. Accurate amount of 15 mL casein was added in 15 mL vial and heated at 37°C in a water bath for 5 minutes. Then 1 mL of sample was added in the vial. Solutions was mixed and left for 10 minutes in 37°C water bath for a reaction to occur. Then, 5 mL of TCA was added to stop the reaction. The solution was incubated foranother 30 minutes at 37°C. After incubation, the solution was filtered using filter paper to remove
insoluble particles from the sample. A 2 mL of the sample solution was taken and added to 5 mL sodium carbonate solution and 1 mL Folin’s reagent in a vial. Sodium carbonate was added to regulate pH drop caused by addition of Folin’s reagent while Folin’s reagent itself was added to react primarily with free tyrosine. Then, the solution proceeded by determination of their absorbance using spectrophotometer. The protease enzyme activity was determined as follows:

\[
Protease \text{ activity (Unit/mL enzyme)} = \frac{\text{(µmol tyrosine)}}{(V_T)(V_C)}
\]  

(1)

Notes:
1. \(V_T\) = total assay volume (mL); \(V_E\)=volume of enzyme used (mL); \(V_C\)=volume used in colorimetric reaction (mL); \(t\)=time of reaction (minute)
2. One protease unit = 1.0 mole (181 g) of tyrosine per minute at pH7.5, 37ºC.

3. Result and Discussion

3.1. Optimization of Protease Enzyme Extraction

Table 2 showed all 48 experimental runs suggested by Design-Expert Software version 9 with their representative results. Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

| Run | Factor 1 Ratio (sample: buffer) | Factor 2 Weight of sample (g) | Factor 3 Homogenization time (min) | Response enzyme activity (Unit/mL) |
|-----|-------------------------------|-------------------------------|----------------------------------|----------------------------------|
| 1   | 5.00                          | 10.00                         | 6.00                             | 0.249                            |
| 2   | 3.00                          | 30.00                         | 2.00                             | 0.265                            |
| 3   | 5.00                          | 30.00                         | 4.00                             | 0.191                            |
| 4   | 3.00                          | 50.00                         | 4.00                             | 0.268                            |
| 5   | 1.00                          | 50.00                         | 2.00                             | 0.389                            |
| 6   | 5.00                          | 10.00                         | 2.00                             | 0.235                            |
| 7   | 1.00                          | 10.00                         | 2.00                             | 0.364                            |
| 8   | 1.00                          | 50.00                         | 6.00                             | 0.382                            |
| 9   | 5.00                          | 50.00                         | 6.00                             | 0.168                            |
| 10  | 1.00                          | 50.00                         | 2.00                             | 0.416                            |
| 11  | 3.00                          | 30.00                         | 4.00                             | 0.270                            |
| 12  | 3.00                          | 10.00                         | 4.00                             | 0.210                            |
| 13  | 3.00                          | 30.00                         | 6.00                             | 0.259                            |
| 14  | 3.00                          | 10.00                         | 4.00                             | 0.240                            |
| 15  | 1.00                          | 30.00                         | 4.00                             | 0.346                            |
| 16  | 3.00                          | 30.00                         | 6.00                             | 0.343                            |
| 17  | 1.00                          | 10.00                         | 6.00                             | 0.315                            |
| 18  | 1.00                          | 50.00                         | 6.00                             | 0.400                            |
| 19  | 3.00                          | 30.00                         | 4.00                             | 0.343                            |
| 20  | 3.00                          | 50.00                         | 4.00                             | 0.296                            |
| 21  | 5.00                          | 50.00                         | 2.00                             | 0.178                            |
| 22  | 3.00                          | 30.00                         | 4.00                             | 0.296                            |
| 23  | 1.00                          | 10.00                         | 2.00                             | 0.371                            |
| 24  | 5.00                          | 50.00                         | 2.00                             | 0.234                            |
| 25  | 5.00                          | 10.00                         | 2.00                             | 0.254                            |
| 26  | 5.00                          | 10.00                         | 2.00                             | 0.255                            |
The optimal result shows the highest protease activity of 0.42 ± 0.02 Unit/mL was obtained at the condition of 30 g of sample, 1:1 ratio of sample to buffer and 4 min mixing time. The lower the ratio of sample to buffer had resulted higher concentration of extracting protease thus higher enzyme activity. 4 min of mixing time is a suitable time for extraction of protease from *S. asper* as reported by [8]. However, due to the our previous screening result, mixing time showed an insignificant effect to the protease activity control.

### 3.2. Analysis of variance (ANOVA) of Protease Enzyme Extraction

The ANOVA was revised to eliminate those insignificant model terms which had the p-value greater than 0.05. In this case, model term of C, interaction BC and AC were eliminated in order to improve the model. The modified ANOVA result of the response was presented in Table 3 below. To indicate the significance of the model and the effect of factors in the process is by calculating the probability P value express in (Prob> F). For p-values less than 0.05 which provides at least 95% confidence level for the model was significant to the process [12]. The main and quadratic effects of A, AB, A² were significant model terms while the effect B and B² indicated the least significant effect on the response variable. Other than that, the lack of fit, which determined the fitness of the model, show a value of 0.85 implied the lack of fit was not significant relative to the pure error, therefore, ensuring a satisfactory fit between response surface models and experimental data.

The reduce fit model equations of protease activity were expressed as follows:

\[
Protease \text{ Activity} = 0.29 - 0.070 * A + 8.933E - 003 * B - 0.028 * A * B + 0.029 * A^2 - 0.017 * B^2
\]  
\[
Protease \text{ Activity} = 0.34187 - 0.058345 * A + 5.10547E - 003 * B - 6.92708E - 004 * A * B + 7.36553E - 003 * A^2 - 4.30114E - 005 * B
\]  

\[
(2)
\]

\[
(3)
\]
Table 3. ANOVA analysis for optimization of protease enzyme.

| Source        | Sum of Squares | df | Mean Square | F-Value | p-value | Prob > F |
|---------------|----------------|----|-------------|---------|---------|----------|
| Model         | 0.18           | 5  | 0.035       | 31.05   | < 0.0001| Significant |
| A-Ratio       | 0.15           | 1  | 0.15        | 129.90  | < 0.0001|           |
| B-Sample      | 2.394E-003     | 1  | 2.394E-003  | 2.12    | 0.1525  |           |
| AB            | 0.018          | 1  | 0.018       | 16.34   | 0.0002  |           |
| A^2           | 7.639E-003     | 1  | 7.639E-003  | 6.78    | 0.0127  |           |
| B^2           | 2.605E-003     | 1  | 2.605E-003  | 2.31    | 0.1360  |           |
| Residual      | 0.047          | 42 | 1.127E-003  |         |         | Not significant |
| Lack of Fit   | 8.905E-003     | 9  | 9.894E-004  | 0.85    |         |           |
| Pure Error    | 0.038          | 33 | 1.165E-003  |         |         |           |
| Cor Total     | 0.22           | 47 |             |         |         |           |

Based on the adjusted fit model, the standard deviation and mean of the model were 0.034 and 0.29 respectively. The values were relatively small thus indicate that the experimental results were close to the true values. Fisher test (F test) and the R^2 were used to verify the accuracy of the model. The ANOVA of the quadratic model shows the R^2 which closer to 1, indicates the better correlationship between the experimental and predicted results [13]. The probability value (Prob>F) should less than 0.05 to prove that the quadratic model is significant, for at least 95 % confidence level. Similar to R^2 value should greater than 80 % for biochemical research. After revised, the R^2 shows reduced to 0.79, which reasonable agreement with the requirement of R^2 for biochemical studies.

3.3. Enzyme Activity of Protease

The enzyme activity of proteases of S. asper was significantly (p < 0.05) influenced by the main effects of ratio of sample to buffer, A and weight of samples, as shown in Figure 1. According to Mehrnoush et. al [8], the independent effect of buffer pH and content resulted the most significant effect for enzymatic control. From this study, the p-value of buffer pH and content were 0.000 and 0.001 respectively. Thereby, the p-values statistically less than 0.05 proved the significant effect to the response. The result shows that the 1:1 ratio of sample to buffer had yielded the highest protease activity while the weight of the sample was optimized within 30-50 g. The high amount of buffer would dilute the solution causing the decrease in the enzyme activity of the protease. Therefore, the lower the content of the buffer will concentrate the leaf extract and thus guarantee the higher content of enzyme protease.

4. Conclusion

Our optimization study showed that 30 g of sample, 1:1 ratio sample to buffer and 2.0 minutes of homogenization time, yielded the highest protease enzyme activity. Meanwhile, homogenization time showed insignificant effect throughout the optimization processes. Therefore, this study proved that the S. asper is a potential source to produce protease enzyme that can be substituted with animal source.
Figure 1. Interaction between factors A (Sample) and B (Ratio) on protease activity.

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