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Impact of Processing Conditions on the Milk Clotting Activity of Crude Protease Extracted from Chinese Ginger

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Abstract. The impact of three processing parameters namely homogenization time, pH of buffer, and amount of buffer, involved in the liquid extraction of proteinases from ginger rhizomes, was investigated on milk coagulating activity by response surface methodology. An empirical quadratic model was applied to experimental data pertaining to the average enzymatic activity and equation describing the optimal conditions was obtained. The maximum milk clotting activity (274 U/mL) was obtained by homogenizing the ginger rhizomes in 76 mL of extraction buffer with a pH of 7.0 for 2.4 min. No significant ($p > 0.05$) difference was observed between the experimental and predicted values. These results are useful in the optimization of extraction procedures, which are of significant relevance to the production of standardized plant coagulants suitable for the cheese industry.

Keywords: Ginger protease, Plant coagulant, Response surface methodology

1. Introduction

Proteolytic enzymes or proteases are very important industrial enzymes, accounting for nearly 60% of the total worldwide enzyme production [1]. Plant-derived proteases such as papain, bromelain, ficin, and calotropins have potential application in food and biotechnological industries due to their broad substrate specificity, high stability in extreme conditions, good solubility and activity over a wide range of pH and temperature. Most of the plant derived-proteases have been classified as cysteine proteases [2].

Rennin, a milk-coagulating enzyme used in cheese making, is obtained from the stomach of the unweaned calf [3]. The worldwide increase of cheese consumption combined with rennet scarcity and ethical concerns have developed a global interest for natural milk-curdling enzymes from plant sources.

Ginger juice is extensively employed in the manufacture of Ginger milk curd (a traditional Chinese dessert), locally named as "Jiang zhi ning ru". This sweet food was originated about 100 years ago in the Shawan town of Panyu District, Guangzhou in the Guangdong Province in southern China. The juice is squeezed from a piece of ginger, filtered and put into a bowl. Sugar is added to milk and allowed to heat until it simmers. After cooling for a while, milk is poured quickly into the bowl of ginger juice which solidifies to form curd. The coagulating activity of the ginger juice is due to enzymes which also possess proteolytic activity.

There is not any information about the optimization of extraction conditions of milk coagulating cysteine protease from ginger rhizomes. In this study, a response surface methodology was employed for modelling the possible relationship between the extraction conditions, namely the homogenization time, pH of buffer and amount of extraction buffer on the milk coagulating property of extracted cysteine protease from ginger rhizomes. Research was conducted with the final goal of obtaining conditions that lead to maximum yields of

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extracted activity, which hopefully could be applied to the optimization of the extraction process, a requirement for industrial scale up and commercial exploitation of such alternative rennet.

2. Material and methods

2.1. Preparation of crude ginger protease extracts

Unless stated otherwise, all of the extraction procedures were carried out at 0-4°C. Ginger rhizomes (*Zingiber officinale* cv. Laiwu Shandong, 15 g) were peeled, diced, washed with deionized water, and then homogenized for 1, 2 or 3 min (as appropriate) using an HR 2839 blender (Philips, China) with 50, 75 or 100 mL 20 mM phosphate buffer (as appropriate) having pH 6.0, 7.0 or 8.0. The homogenate was filtered through cotton cloth. The filtrate was centrifuged at 12,000g for 20 min (Allegra 64R Centrifuge, Beckman Coulter, USA) to obtain crude enzyme extract.

2.2. Milk clotting activity

The milk clotting activity was determined following the procedure of Arima et al. [4]. Briefly, assay milk (2 ml, 10% skimmed milk in 0.01 M $CaCl_2$, pH 6.5) was incubated for 10 min at 37 °C, and then enzyme extract (0.2 ml) was added. Milk clotting time was determined by manually rotating the flask occasionally and checking for visible clot formation. One milk-clotting unit is defined as the amount of enzyme required to clot 10 ml of substrate in 40 min at 37 °C.

2.3. Experimental design

The impact of three independent variables, namely homogenization time (1–3 min, X1), pH of buffer (6–8, X2) and amount of buffer (50–100 mL, X3) on milk clotting activity of extracted protease from ginger rhizomes was investigated using RSM. The independent variables and their levels were selected on the basis of literature and preliminary experiments. A Box–Behnken design with three variables at three levels was used to determine the response pattern and then establish a model [5, 6]. The experimental design, data analysis and quadratic model building were performed using "Design Expert" software (Version 8.0.4, Stat-Ease Inc., Minneapolis, MN, USA).

$$Y = \beta_o + \sum_{i=1}^{n} B_i X_i + \sum_{i<j}^{n} B_{ij} X_i X_j + \sum_{j=1}^{n} B_{jj} X_j{}^2$$

where Y is response, B0 is a constant, Bi is the linear coefficient, Bij is the second-order interaction, and Bjj is the quadratic coefficients. The variable, Xi, is the un-coded independent variables.

3. Results and discussion

3.1. Optimization procedure

In order to determine the optimum levels of the independent variables, multiple response optimizations were carried out [7]. According to Montgomery [8], the three dimensional (3D) response surface plots are highly recommended for the graphical demonstration of the interaction effect of the independent variables on the response variables. The effect of homogenization time, pH of buffer and amount of buffer on the milk clotting activity of ginger protease extracts from ginger rhizome and their interactions, are shown in Fig. 1. The factors significantly affected the response individually and interactively ($p < 0.05$). The effect of interaction between homogenization time and pH of buffer was more significant ($p = 0.0031$) than the interaction between homogenization time and amount of buffer ($p = 0.0395$) and pH of buffer and amount of buffer ($p = 0.0045$). Based on the linear effect homogenization time and pH of extraction buffer were more significant ($p < 0.0001$) than amount of buffer for milk clotting activity response (Table 1).

An increase in homogenization time resulted in a higher milk clotting activity. Increasing the time of homogenization of the ginger rhizome is expected to improve extraction due to more extensive disruption of cell membrane (due to the more intimate contact promoted between the solid and the liquid phases) with concomitant easier and faster release of proteinases into the extraction medium [9]. The activity reached a maximum when the time of homogenization was at a certain level with no significant further improvement thereafter. Homogenization time above the optimum level had a negative effect on the activity of ginger

protease. A possible explanation for this phenomenon is the denaturation of proteins, and hence the enzyme inactivation due to intensive agitation. The pH of the extraction solution utilized has the most important linear effect on the final specific activity of the extracted enzyme. The total activity of ginger protease was enhanced at a pH of 7.0. It may be inferred that the large linear effect of pH on the final specific activity of the enzyme extracted arises primarily from the change in the intrinsic activity of the enzyme as a response to pH rather than from changes in the extraction yield brought about by pH [10]. Amount of buffer had a similar effect on the milk clotting activity of ginger protease as homogenization time and pH of the extraction medium. A volume of 76 ml of phosphate buffer was the best for extraction. There was a decrease in total activity when a lower volume of buffer was used for extraction, due possibly to an insufficient amount of buffer to penetrate the solid mass. In addition, increasing the buffer content above the optimum volume caused a rapid decrease in total activity of the extracted protease which ultimately resulted in undue dilution of solution [9].

3.2. Model fitting

By use of analysis of variance (ANOVA), the established model was found to be significant ($p < 0.0001$) and could be used to predict milk clotting activity of ginger protease (Table 1).

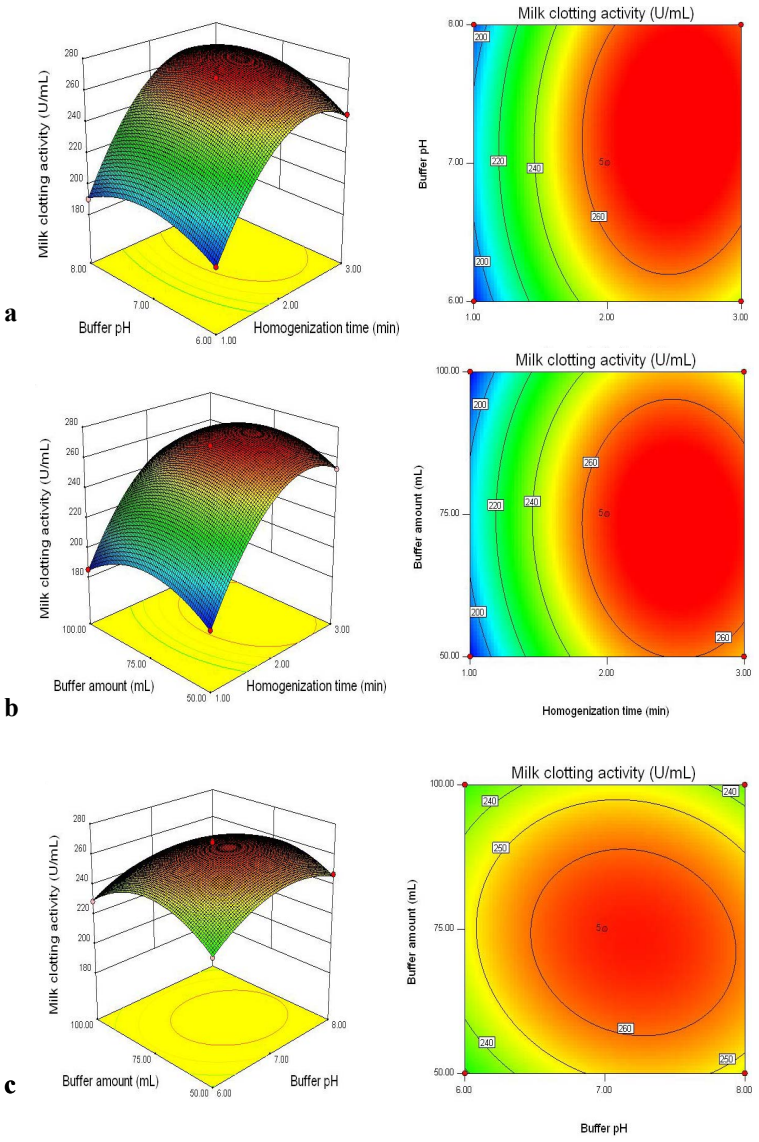

Fig. 1: Fitted response surface and corresponding contour. (a): MCA vs. homogenization time and buffer pH at fixed amount of buffer 75mL; (b): MCA vs. homogenization time and buffer amount at fixed buffer pH 7; (c): MCA vs. buffer amount and buffer pH at fixed homogenization time 2 min.

The lack of fit of the model was p = 0.5413 which is not significant indicating that the model equations was adequate for predicting the response under any combination of values of the variables. Coefficient (R^2) of determination is defined as the ratio of the explained variation to the total variation, and is a measurement of the degree of fitness [11]. A small value of R^2 indicates a poor relevance of the dependent variables in the model. The model can fit well with the actual data when R^2 approaches unity [12]. The high coefficient of determination value R^2 = 0.9989 obtained indicated that the model as fitted can explain 99.8% of the variability thus confirming the adequacy of the model.

Table 1 Analysis of variance (ANOVA) for response surface quadratic polynomial model

Source	Sum of squares	d.f	Mean squares	F- value	Prob (p) > F
Model	15541.51	9	1726.83	688.77	<0.0001
X_1	8064.50	1	8064.50	3216.61	<0.0001
X_2	231.13	1	231.13	92.19	<0.0001
X_3	55.13	1	55.13	21.99	0.0022
X_1^2	4251.16	1	4251.16	1695.62	<0.0001
X_2^2	828.21	1	828.21	330.34	<0.0001
X_3^2	1368.00	1	1368.00	545.64	<0.0001
X_1X_2	49.0	1	49.0	19.54	0.0031
X_1X_3	16.00	1	16.00	6.38	0.0395
X_2X_3	42.25	1	42.25	16.85	0.0045
Residual	17.55	7	2.51		
Lack of fit	6.75	3	2.25	0.83	0.5413
Pure error	10.80	4	2.70		
Total	15559.06	16			

R^2 = 0.9989, R^2_{adj} = 0.9974, R^2_{pred} = 0.9920

3.3. Verification of the model

Within the scope of the variables investigated in Box–Behnken design, additional experiments with different processing conditions were conducted to confirm the adequacy of the model equations. The conditions and the results of the confirmatory experiments are presented in Table 2. The correlation coefficient (R^2) between the observed and predicted values of MCA was 0.90. The good correlation between the values observed in the experiments and the values predicted by the mathematical equations confirmed that the response model was adequate to reflect the expected optimization within the range of variables employed.

Table 2 Observed and predicted values for validation of the experimental model

Trial no	Conditions			Observed value[a]	Predicted value
	Homogenization time (min)	Buffer pH	Buffer amount (mL)		
1	1.8	7.2	80	256 ± 2.5	258.26
2	2.0	6.8	77	263 ± 2.8	264.89
3	2.2	6.6	80	265 ± 1.6	266.14
4	2.6	6.4	70	265 ± 3.5	264.53
5	2.4	6.4	68	260 ± 2.9	264.30

[a]Values are indicated as mean + SD (n = 3)

4. Acknowledgements

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