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Optimization of Enzymatic Hydrolysis Conditions for Producing Soy Protein Hydrolysate with Maximum Lipolysis-Stimulating Activity

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ABSTRACT

The optimum hydrolysis conditions of 2.5% (w/v) soy protein isolate (SPI) with 1% (w/w of SPI) Flavourzyme® for increasing glycerol release in mature 3T3-L1 adipocytes were investigated by response surface methodology (RSM). Higher glycerol release indicated higher lipolysis-stimulating activity. The independent variables were hydrolysis time (HT) 19.2 - 220.8 min, pH 5.32 - 8.68 and reaction temperature (RT) 33.2 - 66.8°C. Based on the response surface and contour plots, the optimum hydrolysis of SPI with Flavourzyme® for maximizing the glycerol release in the cells occurred at pH = 7.12, RT = 48.77°C and HT = 124.85 min. The F-value for lack of fit was not significant ($p > 0.05$), so the second order model was appropriate for describing the response surface. In addition, the model had a satisfactory coefficient of R^2 (= 0.935) and was verified experimentally.

Key words: soy protein isolate, hydrolysates, lipolysis-stimulating activity, glycerol release, 3T3-L1 adipocytes

INTRODUCTION

Obesity is characterized by increased adipose tissue mass and is associated with high health risk(1). This phenomenon is due to the imbalance between energy intake and expenditure which may lead to adipocyte accumulation(2). Lipolysis, the process by which adipocytes degrade and release triglycerides, is a potential therapeutic target for obesity(3). Adipose tissue lipolysis is often considered a well established metabolic pathway. The catabolic process leads to the breakdown of triglycerides into nonesterified fatty acids and glycerol(4). Treatment of adipose tissue with functional ingredients with glycerol release from the tissue measured at the end of incubation has been widely used as the lipolytic index in many studies(5-7).

Enzymatic hydrolysis of soy protein has been reported to have several biological activities, such as angiotensin-I converting enzyme inhibitory activity(8,9), cholesterol-lowering activity(10), and anti-adipogenic activity(11). However, only a few studies focused on its lipolysis-stimulating activity. Based on our preliminary study, the hydrolysis of soy protein with several enzymes, especially Flavourzyme®, revealed the potential to increase its lipolytic activity in terms of glycerol release in mature 3T3-L1 adipocytes (data unpublished). Flavourzyme® is produced by fermentation of a selected strain of *Aspergillus oryzae* and contains both endoprotease and exopeptidase activity. Tsou *et al*. (2010b)(12) studied enhancement of the soy protein anti-adipogenic activity by limited hydrolysis with Flavourzyme®. They concluded that extensive enzymatic hydrolysis of soy protein with degree of hydrolysis (DH) greater that 8% did not guarantee any further enhancement of anti-adipogenic activity. Since limited enzymatic hydrolysis in terms of controlling DH of soy protein plays an important role in the production of protein hydrolysate for better functionality(10,12), suggesting hydrolysis conditions for the selected enzyme should be key for maximizing the lipolytic actiivity for protein hydrolysate.

The objective of our investigation was to optimize the hydrolysis conditions of soy protein with Flavourzyme® and to produce a specific hydrolysate for maximizing the glycerol release in mature 3T3-L1 adipocytes.

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MATERIALS AND METHODS

I. *Chemicals*

Soy protein isolate (SPI), New Soy 88®, was purchased from Gemfont Company (Taipei, Taiwan). Flavourzyme® Type A 1000L (1000LAPU/g) was obtained from Novo Nordisk A/S (Copenhagen, Denmark). Dulbecco's Modified Eagle Medium (DMEM) was obtained from Gibco (Grand Island, NY, USA). The fetal bovine serum (FBS) was obtained from Biological Industries (Kibbutz Beit Haemek, Israel), and the 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX) and insulin were purchased from Sigma (St. Louis, MO, USA).

II. *Experimental Design*

2.5% (w/v) SPI and 1% (w/w of SPI) Flavourzyme® were employed to obtain each hydrolysate at different hydrolysis conditions. Glycerol was measured after treating mature 3T3-L1 adipocytes with each hydrolysate. A 3-factor-5-level central composite rotatable design (CCRD) was employed in this study[13]. The factorial design consisted of 8 factorial points, 6 axial points, and 5 central points. The coded and actual levels of the three variables in Table 1 were selected to hydrolyze SPI with Flavourzyme in order to obtain hydrolysate for maximizing glycerol release in the mature 3T3-L1 adipocytes. The variables and their levels selected for the study of glycerol release were: hydrolysis pH (5.32 - 8.68); reaction temperature (RT) (33.2 - 66.8°C); and hydrolysis time (HT) (19.2 - 220.8 min).

III. *Cell Culture*

The 3T3-L1 preadipocytes (Bioresource Collection and Research Center, Hsin-Chu, Taiwan) were seeded in 24-well plate at a density of 1×10^4 cells/well. All cells were grown in DMEM supplemented with 10% FBS at 37°C in a 5% CO_2 humidified atmosphere. To induce differentiation, 2-day postconfluent 3T3-L1 preadipocytes (Day 0) were stimulated for 48 h with 0.5 mM IBMX, 0.1 mM DEX, and 10 mg/mL insulin in DMEM supplemented with 10% FBS. Cells were then maintained in 10% FBS/DMEM medium with 10 μg/mL of insulin every 2 days (day 4 and 6). At approximately day 8 after the induction of differentiation, more than 95% of the preadipocytes had differentiated into adipocytes. The cells were then treated with 400 ppm Flavourzyme-SPI hydrolysate for 72 h in 10% FBS/DMEM. On day 11, the medium was collected to assay glycerol. In addition, 3T3-L1 cells were washed twice with ice-cold PBS (pH 7.2, 5.36 mM KCl, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 0.14 M NaCl, 0.34 mM Na_2HPO_4) and harvested into lysis buffer (5 M NaCl, 1 M pH 8.0 Tris-HCl, 0.5 M EDTA, 20% SDS, nonidet P-40 substitute, 6.03 mM sodium deoxycholate)[14]. Cells were disrupted by sonication and then centrifuged at 13,000 ×g for 7 min at 4°C. The supernatants were analyzed for protein concentration using a BCA™ protein assay kit[15] (Pierce, Rockford, IL, USA).

Table 1. Coded and actual levels of experimental variables

Variable	Coded level of variable				
	-1.68	-1	0	1	1.68
pH	5.32	6	7	8	8.68
Reaction temperature (RT, °C)	33.2	40	50	60	66.8
Hydrolysis Time (HT, min)	19.2	60	120	180	220.8

IV. *Assay for Glycerol*

Glycerol assay kit (GY105, Randox Laboratories, Antrim, UK) is a direct colorimetric procedure for the measurement of glycerol by utilizing a quinoneimine chromogen system in the presence of glycerol kinase (GK), glycerol phosphate oxidase (GPO) and peroxidase (POD). Thirty microliters of medium or sample and 1 mL of kit reagent containing GK, GPO, POD, ATP, 4-aminophenazone and 3,5-dichloro-2-hydroxybenzene sulphonic acid were mixed and incubated for 5 min at 37°C. The absorbance at 520 nm of the sample (A_{sample}), sample blank ($A_{sample\ blank}$) and standard ($A_{standard}$) against the reagent blank within 30 min was measured. Glycerol concentration was calculated as follows:

$$\text{Glycerol (μmol/L)} = (A_{sample} - A_{sample\ blank}) / A_{standard} \times \text{SGC} \quad (1)$$

where SGC is the standard glycerol concentration (μmol/L).

V. *Data Analysis*

The quadratic model for predicting the optimal point was expressed according to equation (2):

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (2)$$

where Y is a response variable of glycerol release; b_i are regression coefficients for linear effects; b_{ik} are regression coefficients for effects from the interaction; b_{ii} are regression coefficients for quadratic effects; and X_i are coded experimental levels of the variables. The response surface regression procedure of the statistical software package[16] (SAS Institute Inc., 2000) was used to analyze the experimental data. The fit of the models was evaluated by the determination coefficients (R^2) and adjusted R^2 (R^2_{adj}).

VI. *Verification of the Model*

Optimizations of hydrolysis conditions, including pH, HT and RT for maximizing glycerol release in the adipocytes were calculated by the predictive equation from the RSM. The actual glycerol release at optimum condition was determined after treating the mature adipocytes with each hydrolysate. The results were compared with the predicted value.

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RESULTS AND DISCUSSION

I. *Regression Models of Responses*

Table 2 shows the hydrolysis conditions with coded levels and their experimental results of glycerol release in the cells. Among the various treatment, the maximum glycerol release (362.68 nmol/mg protein) was from treatment #15 (pH 7.0, 50°C, and 120 min) and the minimum glycerol release (339.27 nmol/mg protein) was treatment # 4 (pH 6.0, 60°C,and 180 min). In addition, the basal glycerol release (334.74 nmol/mg protein) of the cells without adding any SPI hydrolysate was much lower than that added with hydrolysate. The result revealed benefit of the hydrolysate increasing lipolysis-stimulating activity in the cells. Table 3 illustrates the coefficients of the regression model for glycerol release. The results indicated that glycerol release depended on the linear term of pH ($p < 0.001$) and RT ($p < 0.01$), the quadratic terms of three variables ($p < 0.001$) and the interaction of pH and HT ($p < 0.01$). Based on the regression analysis, 93.5% ($R^2 = 0.935$) of the variations were explained by the model. The adjusted coefficient showed that 87% ($R^2_{adj} = 0.87$) of the variations could be explained by the fitted model. The high values of R^2 and R^2_{adj} of the models showed a close agreement between the experimental results and the theoretical values predicted by the model[17]. Furthermore, a coefficient of variation (CV) less than 1% indicated that the model was reproducible.

Table 2. Coded level combinations for a three-variable central composite orthogonal and rotatable design (CCD)

Treatment#[a]	Coded level of variable[b]			
	pH	RT (°C)	HT (min)	Glycerol Release (nmol/mg protein)
1	-1	-1	-1	352.26
2	-1	-1	1	340.56
3	-1	1	-1	344.06
4	-1	1	1	339.27
5	1	-1	-1	345.20
6	1	-1	1	352.79
7	1	1	-1	344.86
8	1	1	1	351.15
9	0	0	-1.68	346.98
10	0	0	1.68	350.52
11	0	-1.68	0	349.60
12	0	1.68	0	346.99
13	-1.68	0	0	344.09
14	1.68	0	0	343.23
15	0	0	0	362.68
16	0	0	0	358.55
17	0	0	0	358.14
18	0	0	0	361.93
19	0	0	0	357.60

[a]The treatments were run in a random order.
[b]RT, reaction temperature; HT, hydrolysis time

II. *Effect of Variables on Glycerol Release*

Table 4 shows the analysis of variance (ANOVA) for a second order polynomial model fitted to the response surface. The F-value for lack of fit was not significant, so

Table 3. Coefficients of the quadratic regression model for glycerol release in the mature 3T3-L1 adipocytes

Effect[a]	Regression coefficient	*T* test[b]
Intercept	$b_0 = 55.43$	1.03
X_1 = pH	$b_1 = 68.35$	6.29**
X_2 = RT	$b_2 = 3.12$	3.26*
X_3 = HT	$b_3 = -0.24$	-1.74
Quadratic		
X_{11}	$b_{11} = -5.67$	-8.20**
X_{22}	$b_{22} = -0.04$	-5.82**
X_{33}	$b_{33} = -0.0011$	-5.59**
Interaction		
$X_1 \times X_2$	$b_{12} = 0.094$	1.04
$X_1 \times X_3$	$b_{13} = 0.063$	4.21*
$X_2 \times X_3$	$b_{23} = 0.0012$	0.77
$R^2 = 0.935$		
$R^2_{adj} = 0.870$		
CV% = 0.7296		

[a] RT, reaction temperature; HT, hydrolysis time
[b] *$p < 0.01$, **$p < 0.001$

Table 4. Analysis of variance for a second order polynominal model fitted to response surface

Source	Degree of freedom	Sum of squares	Means of squares	F-value	Prob > F
Total model	9	844.06	93.78	14.38	0.0002
Linear	3	38.92	12.97	1.99	0.1862
Quadratic	3	687.75	229.25	34.69	< 0.0001
Cross product	3	126.38	42.13	6.46	0.0127
Total error	9	58.69	6.52		
Lack of fit	5	36.69	7.34	1.33	0.4014
Pure error	4	22.00	5.50		

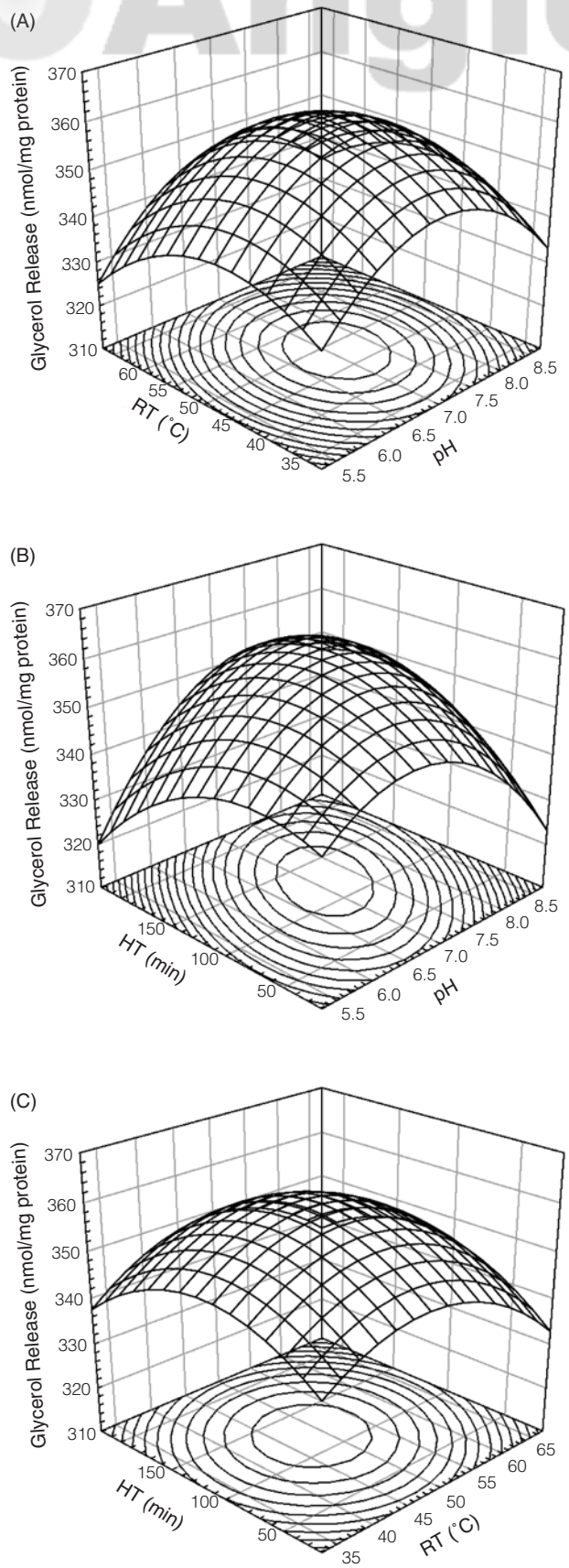

Figure 1. Response surface plot showing the effect on glycerol release in mature 3T3-L1 adipocytes: (A) reaction temperature (RT) and pH, (B) hydrolysis time (RT) and pH and (C) HT and RT.

Table 5. Results of verification tests for fitted model

Response variable	Glycerol release (nmol/mg protein)
Predicted value	359.69
Experimental value (mean)	359.92
Sample size	6
95% confidence interval	(340.48 , 379.36)

the second order model was appropriate for describing the response surface. The contour and three-dimensional plots presented in Figure 1 was produced for each pair of factors, whereas the third factor was taken as a constant at its middle level. Figure 1A shows the effect of RT and pH on glycerol release in the cells. The maximum glycerol release was obtained with RT and pH located in the medium levels. Both higher RT and pH resulted in the decrease of glycerol release. This could be due to the fact that Flavourzyme contained both endoprotease and exopeptidase activity[18], which had optimal pH in the range of 5.0 - 7.0 and optimal temperature around 50°C during hydrolysis of soy protein isolate. Higher RT and pH might cause a denaturation or transformation of the enzyme, which resulted in producing less optimal SPI hydrolysate to reveal the glycerol release activity in the cells.

Figure 1B illustrates the effect of HT and pH on the glycerol release in the cells. The maximum glycerol release was obtained with HT between 100 - 150 min and pH between 7.0 - 7.5. Higher HT and pH tended to result in a decrease of glycerol release. Tsou *et al.*'s (2010b) study on the effect of limited hydrolysis of soy protein with Flavourzyme® on anti-adipogenic activity also indicated that extensive hydrolysis or a longer hydrolysis time resulted in a decrease of the activity[12]. They also concluded that limited hydrolysis in terms of controlling DH as well as hydrolysis time was required to maintain the structure or the sequence of active peptides and to ensure functionality. Figure 1C shows the effect of HT and RT on glycerol release in the cells. The maximum glycerol release was obtained with HT between 100 - 150 min and RT between 45 - 50°C.

III. *Maximum Conditions and Verification of the Model*

Analysis of the surface response revealed that the stationary point for the production of Flavouzyme-SPI hydrolysate to cause glycerol release in the cells was a true maximum. The maximum predicated value of glycerol release was 359.69 nmol/mg protein under the following hydrolysis conditions: RT at 48.8°C, pH at 7.12 and HT at 124.9 min. Based on the regression analysis, the model had a satisfactory coefficient of $R^2 = 0.935$. In addition, the verification studies also proved that the predicted value of glycerol release for the model could be realistically achieved within a 95% confidence interval of experimental values (Table 5).

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CONCLUSIONS

This study attempted to optimize the hydrolysis conditions of SPI with Flavourzyme into the specific hydrolysate for the increasing glycerol release in mature 3T3-L1 adipocytes using the RSM. The reaction was well described by second-order polynomials. RT, pH and HT showed a significant quadratic effect on glyceride release. The interaction RT-pH had a significant effect on the SPI hydrolysis for glycerol release. A maximum glycerol release of 359.69 nmol/mg protein was expected at RT 48.77°C, with pH of 7.12 and HT of 124.85 min. The high value of R^2 and R^2_{adj} of the models showed close agreement between the experimental results and the theoretical values predicted by the model.

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