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β-D-Glucosidase-Catalyzed Deglucosidation of Phenylpropanoid Amides of 5-Hydroxytryptamine Glucoside in Safflower Seed Extracts Optimized by Response Surface Methodology

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β-D-glucosidase-catalyzed deglucosidation of phenylpropanoid amides of 5-hydroxytryptamine (PAHAs) glucoside in safflower (*Carthamus tinctorius* L.) seed extracts, including N-(*p*-coumaroyl)serotonin glucoside (CSG) and *N*-feruloylserotonin glucoside (FSG), was optimized by response surface methodology (RSM). The Box−Behken design (BBD) was employed to evaluate the interactive effects of independent variables on the deglucosidation rates of CSG and FSG. The variables involved were pH (5.6−6.2), temperature (45−55 °C), and enzyme load (2.0−3.0%, relative to the weight of the total substrate). The substrate concentration was fixed at 3.3 g/L on the basis of factorial experiments. The optimum conditions obtained via RSM at a fixed time of 2 h were as follows: pH, 5.9; temperature, 48 °C; and enzyme load, 3.0%. Under these conditions, the actual deglucosidation rates of CSG and FSG were 75.5 and 42.2%, respectively, which agree well with the predicted values (75.3 and 41.9%) by RSM. The final incubation time (10 h) was determined by the time course of the deglucosidation under the above-mentioned optimum conditions, which gave the deglucosidation rates of both CSG and FSG above 90%. Simultaneously, 2-hydroxyarctiin, a typical cathartic β-glucoside, was also removed by 80.3%.

KEYWORDS: Safflower (*Carthamus tinctorius* L.) seeds; *N*-(*p*-coumaroyl)serotonin glucoside (CSG); *N*-feruloylserotonin glucoside (FSG); *N*-(*p*-coumaroyl)serotonin (CS); *N*-feruloylserotonin (FS); β-D-glucosidase; deglucosidation; response surface methodology

INTRODUCTION

Some bioactive plant compounds, although having various functions in drug and food systems, are greatly restricted for certain applications because of their low solubility, low stability, or side effects. Thus, appropriate modification or transformation of these compounds is essential to improve their functionalities. Conventional chemical conversion, because of the involvement of basic or acidic catalysts and organic solvents, always fails to meet the increasing requirements for drug and food safety (*1*), and it is currently associated with the formation of unwanted byproduct and colored or color-forming compounds, which lower product yield and require more demanding downstream processing (*2*). Enzymatic conversion, therefore, is now becoming widely used in this field for its low pollution, high selectivity, and mild reaction conditions.

Phenylpropanoid amides of 5-hydroxytryptamine (PAHAs) in safflower (*Carthamus tinctorius* L.) seeds are a class of neutral phenolic amides in conjugated polyamines, containing 7−10 members characterized as a serotonin moiety bound to a phenylpropanoid moiety via an amide bond (*3*−*5*). They are the major active compounds in safflower seeds, possessing radical-scavenging, antioxidative, antityrosinase, melanine inhibitory, pro-inflamatory cytokine inhibitory activities, etc. (*6*−*11*). *N*-(*p*-Coumaroyl)serotonin (CS) and *N*-feruloylserotonin (FS) are two main bioactive compounds with the simplest structures in PAHAs. The glucosides of CS and FS, i.e., *N*-(*p*-coumaroyl)serotonin glucoside (CSG) and *N*-feruloylserotonin glucoside (FSG), also occur in safflower seeds, which combined account for more than 20% of the total amount of PAHAs (chemical structures shown in **Figure 1**). However, their biological activities are obviously lower than those of CS and FS (*5*, *6*). Besides, some other glucosidic compounds with certain side effects, e.g., 2-hydroxyarctiin (cathartic effects) and matairsinol-mono-β-glucoside (bitter taste) (chemical structures shown in **Figure 2**), are co-extracted along with PAHAs, accounting for 5−20% of the total amount of the crude PAHA extracts (*3*, *12*−*14*). Therefore, an appropriate use of β-D-glucosidase-catalyzed hydrolysis is of great significance not only to transform CSG and FSG to CS and FS but also to eliminate the deleterious glucosides as mentioned above.

Response surface methodology (RSM) with appropriate experimental designs, e.g., Box−Behnken design (BBD), has been effectively applied to optimize the intended parameters in biochemical

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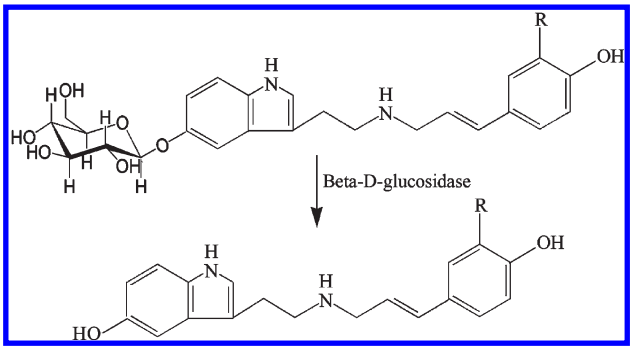

Figure 1. β-D-glucosidase-catalyzed deglucosidation of CSG (1, R = H) and FSG (2, R = OCH_3) to CS and FS.

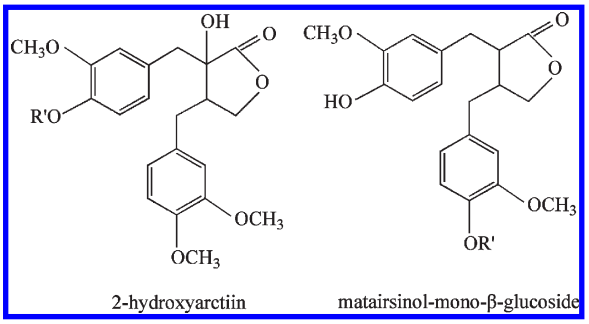

Figure 2. Chemical structures of 2-hydroxyarctiin and matairsinol-mono-β-glucoside (R′ = glucosyl).

reactions (*15*−*17*). This study deals with optimizing β-D-glucosidase-catalyzed deglucosidation of CSG and FSG and co-extracted yet unwanted glucoside(s) in safflower seed extracts via RSM. The purposes were to better understand the relationship between factors (pH, temperature, and enzyme load) and responses (deglucosidation rates of CSG and FSG) in the enzymatic deglucosidation and then to determine the optimum conditions for the deglucosidation process.

MATERIALS AND METHODS

Materials. β-D-Glucosidase powder was purchased from Sigma Corporation (St. Louis, MO) and was dissolved to 1 mg/mL by 0.05 mmol/L HAC/NaAC buffer (pH 5.0). Crude safflower seed extracts used as substrates containing 0.94% CSG, 0.53% FSG, 2.58% CS, and 3.82% FS by weight were prepared by the method reported (*19*). CSG, FSG, CS, and FS standards with purities of 96.5, 97.3, 98.6, and 97.8%, respectively, were prepared by the established methods (*5*), quantified by high-performance lipid chromatography (HPLC), and characterized by mass spectrometry (MS), nuclear magnetic resonance (NMR), and ultraviolet (UV) adsorption spectra. Methanol for formulating the mobile phase was of HPLC purity from Tedia Corporation. All of the other chemicals were of analytical reagent grade.

Analytical Methods. PAHAs were analyzed by the methods reported (*18*, *19*): methanol (A) and 0.4% acetic acid (B) were used as the mobile phase in a linear gradient from 15% A (v/v) to 50% A (v/v) in 20 min and then to 100% A in 10 min. The column (Sunfire-C18, 150 × 2.1 mm inner diameter, 5 μm) was conditioned for 10 min at 20 °C using the starting mobile phase after each run. The other chromatographic parameters were as follows: mobile phase flow rate, 0.3 mL/min; column temperature, 20 °C; injection volume, 5 μL; and a Waters model 2996 photodiode array detector (PAD) set at 310 nm. Except for the flow rate (1 mL/min) and the detection wavelength (279 nm) (*13*), the conditions to analyze 2-hydroxyarctin were the same as those for PAHAs. Statistical analysis showed relative standard deviations (RSDs) of the two analytical methods for PAHAs and 2-hydroxyarctin as 1.37 and 1.45%, respectively.

Enzymatic Deglucosidation. The crude safflower seed extracts were dissolved in HAC/NaAC buffer with corresponding pH to 3.3 g/L, which was determined by the factorial experiments. Aliquots of 30 mL of the resultant solution were each added into a triangular flask, and then the desired amount of β-D-glucosidase was also added. Hydrolysis was performed in a thermostatic shaking bath at 15 revolutions/min under a controlled temperature for 2 h. The mixture was immediately heated to and maintained at 100 °C for 15 min to stop the reaction. Finally, the mixture was filtered by a 0.45 μm membrane to remove the enzyme. The amounts of CSG, FSG, CS, FS, and 2-hydroxyarctiin were determined by HPLC.

Table 1. Experimental Design and Results of the Deglucosidation of CSG (Y_1) and FSG (Y_2)

	independent variables			responses	
treatment[a]	pH	temperature	enzyme load[b]	deglucosidation rate of CSG	deglucosidation rate of FSG
number	X_1	X_2 (°C)	X_3 (%)	Y_1 (%)	Y_2 (%)
1	−1 (5.6)[c]	1 (55)	0 (2.5)	68.2	35.3
2	1 (6.2)	1 (55)	0 (2.5)	67.3	34.2
3	0 (5.9)	1 (55)	−1 (2.0)	61.8	32.9
4	0 (5.9)	1 (55)	1 (3.0)	71.1	39.6
5	−1 (5.6)	−1 (45)	0 (2.5)	69.6	37.1
6	0 (5.9)	0 (50)	0 (2.5)	72.2	37.9
7	1 (6.2)	0 (50)	−1 (2.0)	62.1	32.4
8	1 (6.2)	0 (50)	1 (3.0)	73.8	41.3
9	0 (5.9)	−1 (45)	−1 (2.0)	62.5	32.7
10	−1 (5.6)	0 (50)	1 (3.0)	73.8	41.3
11	1 (6.2)	−1 (45)	0 (2.5)	70.4	36.7
12	−1 (5.6)	0 (50)	−1 (2.0)	65.1	32.5
13	0 (5.9)	0 (50)	0 (2.5)	71.4	38.3
14	0 (5.9)	0 (50)	0 (2.5)	72.0	38.1
15	0 (5.9)	−1 (45)	1 (3.0)	75.2	40.6

[a] Treatments were run in random order. [b] Enzyme load (%, relative to the weight of total substrates). [c] Numbers in parentheses represent the actual experimental amounts.

Experimental Design. A three-level, three-factor Box−Behken design was employed, in which 15 experiments were involved, and the deglucosidation rates of CSG and FSG were used as the indices in evaluating the kinetics of the β-D-glucosidase-catalyzed deglucosidation. The independent factors and levels in both coded and uncoded parameters and the experimental design with observed values for the responses are shown in **Table 1**. The factors and levels studied in deglucosidating CSG and FSG were determined on the basis of the factorial experiments, such as pH (5.6, 5.9, and 6.2), temperature (45, 50, and 55 °C), and enzyme load (2.0, 2.5, and 3.0%).

Statistical Analysis. RSM applied in this study is an efficient statistical technique for modeling and optimization of multiple variables to predict the best conditions with a minimum number of experiments (*20*), which adopts multiple regression and correlation analyses to test the effects of several independent variables on the dependent responses. Experimental data from the Box−Behken design were analyzed by means of RSM to fit the quadratic polynomial equation with the Design Expert software (version 7.1.3.1, State-Ease, Inc., Statistics Made Easy, Minneapolis, MN). The quadratic polynomial equation is shown below.

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j \qquad (1)$$

where Y is the predicted response, X_i and X_j are the coded independent variables, β_0 is the intercept, β_i is the linear-term coefficient, β_{ii} is the quadratic-term coefficient, and β_{ij} is the cross-term coefficient. All coefficients of the model were generated by multiple regressions, and the statistical significance and the fitting quality of the model were evaluated by the coefficients of determination (R^2) and analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Selection of Independent Variables and Their Levels. The effects of levels of several independent variables on the deglucosidation

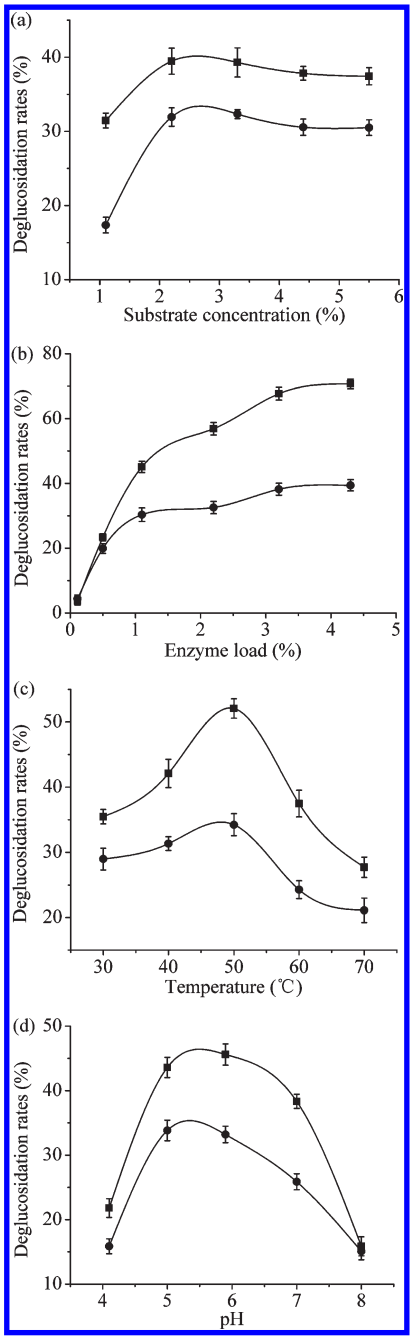

Figure 3. Effects of the substrate concentration, enzyme load (relative to the weight of total substrates), temperature, and pH on the deglucosidation rates of CSG (■) and FSG (●): (a) enzyme load, 1.1%; pH, 5.0; temperature, 35 °C, (b) substrate concentration, 3.3 g/L; pH, 5.0; temperature, 35 °C, (c) enzyme load, 1.1%; substrate concentration, 3.3 g/L; pH, 5.0, and (d) enzyme load, 1.1%; substrate concentration, 3.3 g/L; temperature, 35 °C.

rates of CSG and FSG are shown in **Figure 3**. The deglucosidation rates of both CSG and FSG show increasing−decreasing patterns with temperature and pH increasing and increasing−flat patterns with substrate concentration and enzyme load increasing. Among these variables, the substrate concentration had a minor influence on the deglucosidation rates; therefore, it was fixed at 3.3 g/L based on the experimental results. In addition, the reaction time was fixed at 2 h on the basis of preliminary experiments. The maximum deglucosidation rates of CSG and FSG were obtained at 50 °C, pH 5.9, and enzyme load of 3.0%. The most cost-effective conditions for the deglucosidation rates of CSG and FSG via enzymatic deglucosidation should be the lowest glucosidase load and the least energy consumption. Therefore, the lower, middle, and upper levels of the three independent variables were chosen in **Table 1**.

Table 2. ANOVA for Quadratic Models Pertaining to the Deglucosidation of CSG (Y_1) and FSG (Y_2)

	SS		df		MS		F value		$p > F$[a]	
source	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2
model	267.78	139.25	9	9	29.75	15.72	56.37	47.64	0.0002	<0.0001
X_1	1.80	1.80	1	1	1.80	1.80	3.42	5.45	0.1237	0.0270
X_2	10.81	4.65	1	1	10.81	4.65	20.48	14.09	0.0062	0.0042
X_3	271.36	120.90	1	1	271.36	120.90	411.80	366.36	<0.0001	<0.0001
X_1X_2	0.72	0.12	1	1	0.72	0.12	1.37	0.36	0.2947	0.4568
X_1X_3	1.32	1.10	1	1	1.32	1.10	2.51	3.33	0.1743	0.0602
X_2X_3	2.89	1.21	1	1	2.89	1.21	5.48	3.67	0.0664	0.0523
X_1^2	4.14	6.48	1	1	4.14	6.48	7.84	19.64	0.0380	0.0020
X_2^2	13.80	3.33	1	1	13.80	3.33	26.15	10.09	0.0037	0.0085
X_3^2	19.25	0.75	1	1	19.25	0.75	36.47	2.27	0.0018	0.1030
residual	2.64	1.66	5	5	0.53	0.33				
lack of fit	2.29	0.86	3	3	0.76	0.76	4.41	4.75	0.1904	0.1246
pure error	0.35	0.80	2	2	0.17	0.16				
correlation total	270.42	140.20	14	14						
Y_1: CV % = 1.25, adj R^2 = 0.9611							Y_2: CV % = 1.42, adj R^2 = 0.9702			

[a] $p < 0.05$ indicates statistical significance.

Model Fitting and ANOVA. The data were analyzed by employing a multiple regression technique, and the two quadratic polynomial models, which represented an empirical relationship between the tested variables and responses, were obtained as shown below.

$$Y_1\ (\%) = 71.87 - 0.36X_1 - 1.05X_2 + 5.21X_3 - 0.20X_1X_2 + 0.57X_1X_3 - 0.85X_2X_3 - 0.95X_1^2 - 1.82X_2^2 - 2.40X_3^2 \quad (2)$$

$$Y_2\ (\%) = 38.10 - 0.34X_1 - 0.50X_2 + 3.76X_3 + 0.10X_1X_2 - 0.52X_1X_3 - 0.30X_2X_3 - 1.06X_1^2 - 0.94X_2^2 - 0.71X_3^2 \quad (3)$$

where Y_1 and Y_2 are the predicted values for the deglucosidation rates of CSG (%) and FSG (%), respectively, and X_1, X_2, and X_3 the coded variables as described in **Table 1**.

According to ANOVA of the second-order regression models, the models are statistically significant and adequate to explain most of the variability. p values less than 0.01 reveal that the regression models are significant at a 99% confidence level. p values of the lack of fit for the two models are 0.1904 and 0.1246, respectively, which indicate that the models are stable and can better predict the variation of the deglucosidation rates. The adjusted coefficients of determination given in **Table 2** show that the regression models can adequately represent the relationship between the variables and responses. The values of 0.9611 for model 1 and 0.9702 for model 2 reflect total variation in the responses studied at 96.11 and 97.02%, indicating a satisfactory goodness of the regression models. Further analysis via a comparison between the predicted and observed values (**Figure 4**) shows a close agreement between them, which also indicates a good fit of both models.

The main effects of the variables on the deglucosidation rates of CSG and FSG together with their significance are shown in **Figure 5**. The linear terms of X_2 and X_3 and quadratic terms of X_1^2, X_2^2, and X_3^2 for model 1 are significant with their $p < 0.05$, as well as the linear terms of X_1, X_2, and X_3 and quadratic terms of X_1^2 and X_2^2 for model 2. Hence, the results indicate that the variables have significant effects on the reaction, whereas to

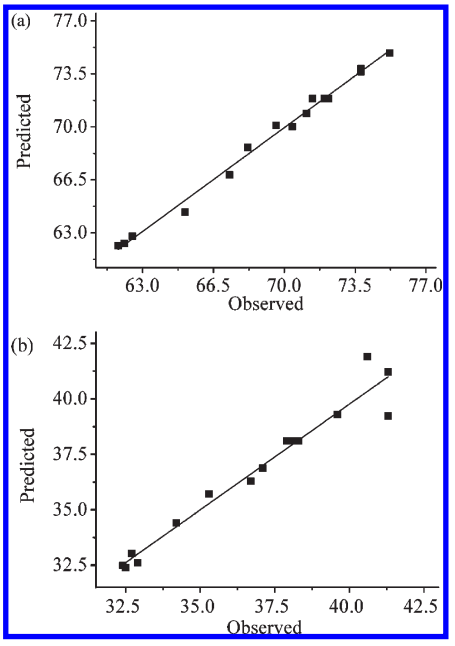

Figure 4. Relationship between the observed and predicted deglucosidation rates: (a) deglucosidation rate of CSG, with R^2 = 0.9912, and (b) deglucosidation rate of FSG, with R^2 = 0.9505.

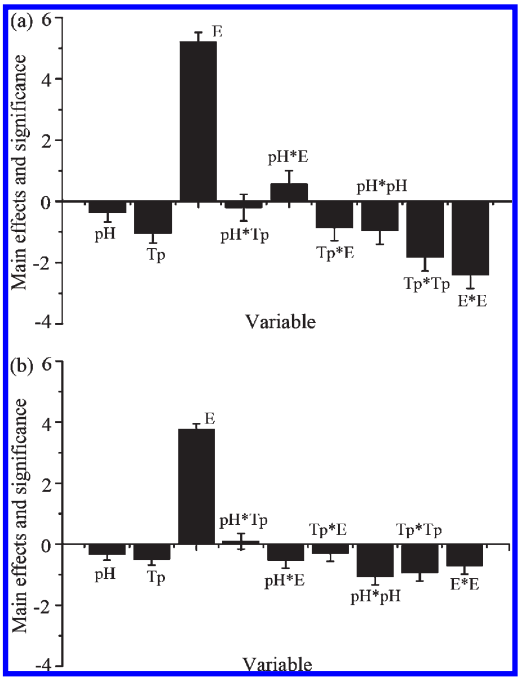

Figure 5. Effects of variables and their significance for the deglucosidation rates: (a) deglucosidation rate of CSG and (b) deglucosidation rate of FSG.

support the hierarchy, other variables, despite their insignificance, are not eliminated from the models.

Response Surface Plots Analysis. The relationship between the variables and responses can be better understood by examining the three-dimensional response surface plots, as shown in **Figures 6** and **7**, whose regression coefficients are generated from the predicted models shown in **Table 2**.

Figures 6a and **7a** show the effects of pH, temperature, and their mutual interaction on the deglucosidation rates of CSG and FSG when the enzyme load is at its medium level (2.5%). The maximum deglucosidation rates of both CSG and FSG appear in

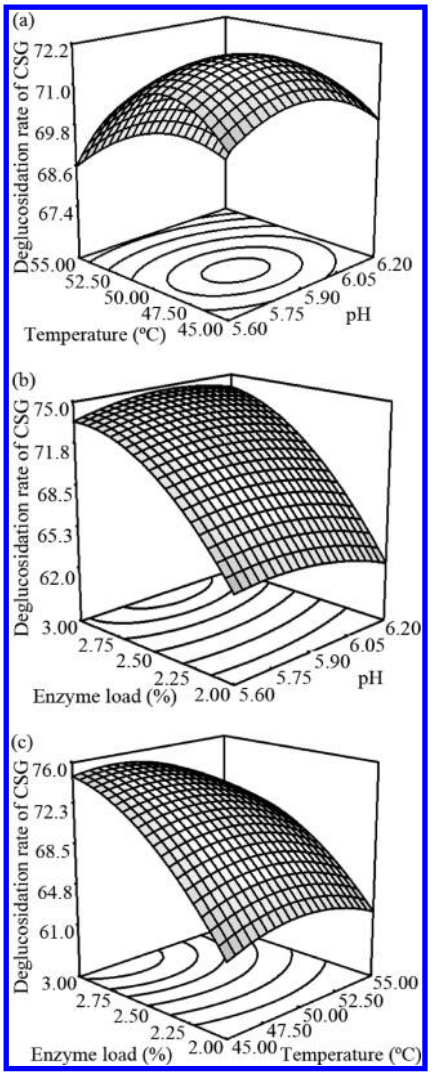

Figure 6. Response surface plots showing the effects of any two factors on the deglucosidation rate of CSG: (a) temperature and pH, with enzyme load (relative to the weight of total substrates) fixed at 2.5%, (b) enzyme load and pH, with temperature fixed at 50 °C, and (c) enzyme load and temperature, with pH fixed at 5.9.

a temperature range of 48−49 °C and a pH range of 5.8−5.9. Generally, a suitable increment in the reaction temperature (45.0−48.5 °C) increases the deglucosidation rates of CSG and FSG by reducing the mass-transfer limitations and making substrates more available to the enzyme (*21*). However, a higher temperature exceeding the optimal range of 48.5−48.7 °C has negative effects on the stability of the enzyme, resulting in a decrease of the deglucosidation rates of CSG and FSG. The pH has influences on the ionization of prototropy groups in the active sites of the enzyme and the combination of the active groups of the enzyme to the substrates. When the pH exceeds the optimal range of 5.8−5.9, the deglucosidation rates begin to decrease. **Figures 6b** and **7b** show the effects of the variation of enzyme load and pH and their mutual interaction on the enzymatic hydrolysis at a fixed temperature of 50 °C. Higher enzyme load results in higher deglucosidation rates of FSG and CSG at the same pH, but the increase becomes smoother when the enzyme load exceeds 2.2%. At a fixed pH of 5.9, increasing the enzyme load from 2.0 to 2.2% gives an increase of 3.6%, whereas increasing the enzyme load from 2.2 to 2.4% gives an increase of 2.7%, in the deglucosidation rates. The effects of pH on the deglucosidation rates at the fixed temperature are similar to those at the fixed

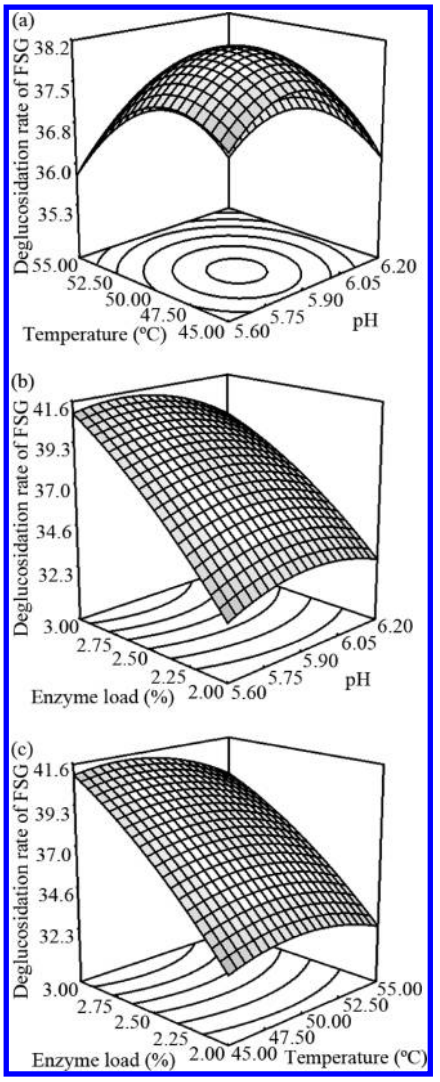

Figure 7. Response surface plots showing the effects of any two factors on the deglucosidation rate of FSG: (a) temperature and pH, with enzyme load (relative to the weight of total substrates) fixed at 2.5%, (b) enzyme load and pH, with temperature fixed at 50 °C, and (c) enzyme load and temperature, with pH fixed at 5.9.

enzyme load, as mentioned above. The deglucosidation rates begin to decrease as the pH exceeds its optimal point of 5.9, which indicates that a higher pH, between 5.9 and 6.2, has substantial negative influences on the structure of the enzyme. The effects of the temperature versus enzyme load at pH 5.9 on the deglucosidation rates presented in **Figures 6c** and **7c** are similar to those of pH versus enzyme load.

According to **Figures 6** and **7**, the independent variables (pH, temperature, and enzyme load) have greater influences on the deglucosidation of CSG than those of FSG. This phenomenon is probably due to the structural difference between CSG and FSG, which affects their combination with the active sites of β-D-glucosidase.

Optimization of the Reaction and Model Verification. Apart from evaluation of the effects of the process parameters and their interactions on the responses, RSM can predict the desirable combinations of the levels of variables that give higher deglucosidation rates of CSG and FSG. According to the overlying contour plot (**Figure 8**) generated from the two regression models, the maximum deglucosidation rates of CSG and FSG appear in a temperature range of 45.0−50.5 °C and an enzyme load range of 2.9−3.0%, with the reaction pH fixed at its medium level of

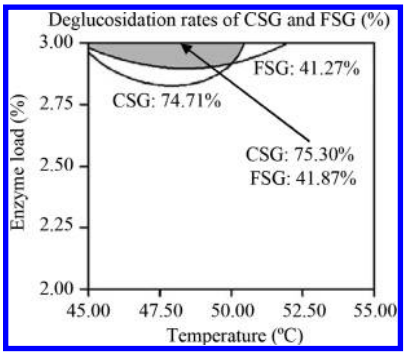

Figure 8. Graphic optimization by overlying the contours of two responses at pH 5.9.

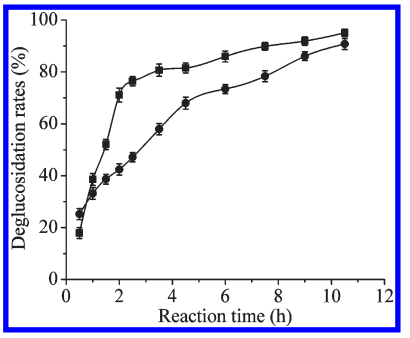

Figure 9. Time course of the enzymatic deglucosidation at the optimum conditions (enzyme load, 3.0%; pH, 5.9; temperature, 48 °C; substrate concentration, 3.3 g/L).

5.9. Under these conditions, the deglucosidation rates of CSG and FSG are predicted to exceed 74.7 and 41.3%, respectively. Thus, the optimum conditions for the deglucosidation of CSG and FSG were obtained: temperature, 48 °C; pH, 5.9; and enzyme load, 3.0%. The predicted deglucosidation rates of CSG and FSG (75.3 and 41.9%, respectively) agree well with the observed values (75.5 and 42.2%, respectively) in the verification tests in triplicate, which indicates the validation of both models.

Time Course To Determine the Final Incubation Time. Under the optimized conditions as described above, the deglucosidation rates increase with the extension of the reaction time, whereas the increase becomes much slower after 2 h for CSG and 4.5 h for FSG. At the turning point, the deglucosidation rates of CSG and FSG are 71.2 and 68.5%, respectively (**Figure 9**). To obtain the deglucosidation rates of both CSG and FSG above 90% and to improve the production efficiency, the deglucosidation time was finally selected at 10 h. Therefore, the final optimum conditions were as follows: temperature, 48 °C; pH, 5.9; enzyme load, 3.0%; substrate concentration, 3.3 g/L; and deglucosidation time, 10 h. Under these conditions, the deglucosidation rates of CSG and FSG reached 93.7 and 90.2%, respectively. The HPLC profiles of CSG, FSG, CS, and FS in extracts before and after the deglucosidation are provided in **Figure 10a**. Meanwhile, cathartic 2-hydroxyarctiin, an unwanted co-extract observed in this study, was removed by 80.3% compared to that in the crude extracts before the deglucosidation. The HPLC profiles of 2-hydroxyarctiin in extracts before and after the deglucosidation are provided in **Figure 10b**.

In conclusion, β-D-glucosidase-catalyzed deglucosidation of CSG and FSG to CS and FS was successfully modeled and optimized by RSM. The enzymatic deglucosidation conditions were finally optimized via analysis of the factorial experiments, modeling of the experimental data, and analysis of the time course as follows: temperature, 48 °C; pH, 5.9; enzyme load, 3.0%;

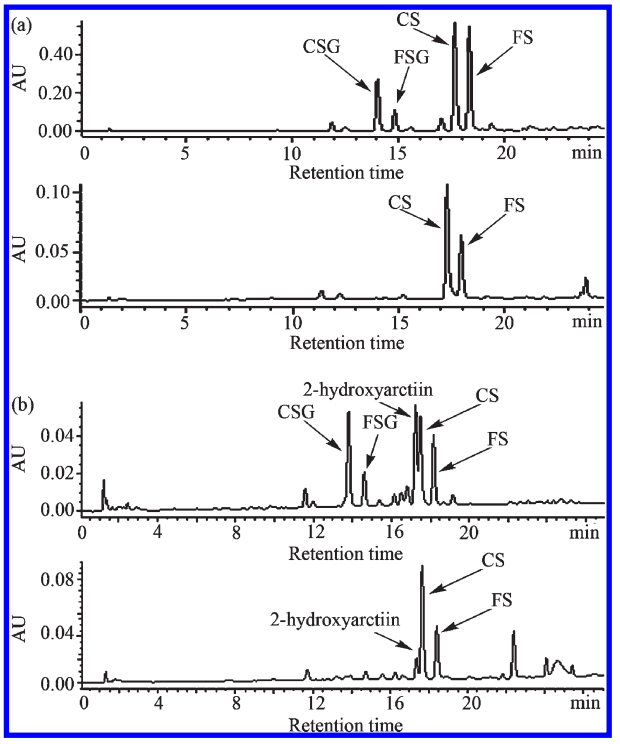

Figure 10. HPLC analysis of the crude extracts before and after the enzymatic deglucosidation: (a) HPLC profiles of the starting sample (top) and the enzymatic deglucosidation mixture (bottom) under 310 nm with PAD and (b) HPLC profiles of 2-hydroxyarctiin in the extracts before (top) and after (bottom) the deglucosidation under 279 nm with PAD.

substrate concentration, 3.3 g/L; and reaction time, 10 h. Under the optimum conditions, the optimized deglucosidation rates of both CSG and FSG were above 90%. Meanwhile, the unwanted co-extract, 2-hydroxyarctiin, was greatly removed via enzymatic deglucosidation.

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LITERATURE CITED

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