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Optimization of Lipase-Catalyzed Synthesis of Ginsenoside Rb1 Esters Using Response Surface Methodology

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In the lipase (Novozyme 435)-catalyzed synthesis of ginsenoside Rb1 esters, different acyl donors were found to affect not only the degree of conversion but also the regioselectivity. The reaction of acyl donors with short carbon chain was more effective, showing higher conversion than those with long carbon chain. Among the three solvent systems, the reaction in *tert*-amyl alcohol showed the highest conversion rate, while the reaction in the mixed solvent of *t*-BuOH and pyridine (1:1) had the lowest conversion rate. To allow the increase of GRb1 lipophilicity, we decided to further study the optimal condition of synthesis of GRb1 with vinyl decanoate with 10 carbon chain fatty acids in *tert*-amyl alcohol. Response surface methodology (RSM) was employed to optimize the synthesis condition. From the ridge analysis with maximum responses, the maximum GRb1 conversion was predicted to be 61.51% in a combination of factors (40.2 h, 52.95 °C, substrate mole ratio 275.57, and enzyme amount 39.81 mg/mL). Further, the adequacy of the predicted model was examined by additional independent experiments at the predicted maximum synthesis conditions. Results showed that the RSM was effective to optimize a combination of factors for lipase-catalyzed synthesis of ginsenoside Rb1 with vinyl decanoate.

KEYWORDS: Lipase catalysis; synthesis; ginsenoside Rb1; GRb1 esters; Novozyme 435; response surface methodology

INTRODUCTION

As a traditional medicine in China and Korea, *Panax ginseng* (*Panax ginseng* C.A. Meyer) has been used for more than 2000 years due to its health-benefit effects. It has been reported that the pharmacological properties of ginseng are usually attributed to its triterpene glycosides, called ginsenosides (*1*). So far, more than 30 ginsenosides have been identified in *Panax ginseng*. According to the difference of the aglycone, most of them are classified into three types: 20(*S*)-protopanaxadiol type (e.g., ginsenosides Rb1, Rc, Rb2, and Rd), 20(*S*)-protopanaxatriol type (e.g., ginsenosides Rg1, Rf, and Re), and oleanolic acid type (e.g., ginsenoside Ro). Among them, ginsenoside Rb1 (GRb1) is considered as one of six major ginsenosides in the root of *Panax ginseng*. Previously, GRb1 was found to have beneficial effects on memory and learning by facilitating cholinergic function and increasing the synaptophysin level in the hippocampus (*2*). GRb1 also prevented ischemic neuronal death induced by transient cerebral ischemia (*3*), reduced oxidative stress caused by hydrogen peroxide (*4*), and promoted neurite lengths and neurite number of dopaminergic cells (*5, 6*). Although possessing many biological activities, GRb1 has limited application in the food industry and medical supplies due to the high hydrophilic polarity. It has been reported that GRb1 was slowly absorbed through digestive tract, and the oral bioavailability in rats was relatively low (*7*). The biological activities of polyhydroxylated compounds seem to depend not only on their chemical structure (*8*) but also on their degree of lipophilicity, which could enhance their uptake into cells or influence their interaction with proteins and enzymes (*9*−*13*). In order to increase the lipophilic solubility of GRb1, which would be expected to increase their tract and cellular absorption, esterification provides a route to obtain more nonpolar derivatives. It has been reported that acylation of cholestane saponins could increase the antitumor potency (*14*). Acylated saponin in low concentration also activated the metabolism of endothelial cells, which enhanced the permeability of the blood vessel walls for better adsorption of the saponin into tissues (*15*). Until now, several research groups have already reported the feasibility of the lipase-catalyzed acylation of various polyhydroxylated compounds, such as flavonoids, sugars, and glucosides in both toxic and less toxic organic media (*16*−*21*).

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Table 1. Operating Variables, Levels, and Experimental Data Used in Central Composite Design (CCD)

	independent variables				
treatment	time (X_1)	temperature (X_2)	substrate ratio (X_3)	enzyme amount (X_4)	response (Y)
1	24(−1)	35(−1)	150(−1)	20(−1)	18.72
2	48(+1)	35(−1)	150(1)	20(−1)	28.39
3	24(−1)	55(+1)	150(−1)	20(−1)	26.67
4	48(+1)	55(+1)	150(−1)	20(−1)	37.37
5	24(−1)	35(−1)	250(+1)	20(−1)	20.78
6	48(+1)	35(−1)	250(+1)	20(−1)	35.79
7	24(−1)	55(+1)	250(+1)	20(−1)	38.67
8	48(+1)	55(+1)	250(+1)	20(−1)	43.63
9	24(−1)	35(−1)	150(−1)	40(+1)	19.68
10	48(+1)	35(−1)	150(−1)	40(+1)	32.37
11	24(−1)	55(+1)	150(−1)	40(+1)	32.62
12	48(+1)	55(+1)	150(−1)	40(+1)	38.17
13	24(−1)	35(−1)	250(+1)	40(+1)	24.80
14	48(+1)	35(−1)	250(+1)	40(+1)	37.28
15	24(−1)	55(+1)	250(+1)	40(+1)	51.10
16	48(+1)	55(+1)	250(+1)	40(+1)	57.53
17	12(−2)	45(0)	200(0)	30(0)	22.60
18	60(+2)	45(0)	200(0)	30(0)	42.71
19	36(0)	25(−2)	200(0)	30(0)	2.46
20	36(0)	65(+2)	200(0)	30(0)	42.79
21	36(0)	45(0)	100(−2)	30(0)	36.95
22	36(0)	45(0)	300(+2)	30(0)	50.45
23	36(0)	45(0)	200(0)	10(−2)	32.10
24	36(0)	45(0)	200(0)	50(+2)	49.29
25	36(0)	45(0)	200(0)	30(0)	45.14
26	36(0)	45(0)	200(0)	30(0)	38.79
27	36(0)	45(0)	200(0)	30(0)	42.32
28	36(0)	45(0)	200(0)	30(0)	40.82
29	36(0)	45(0)	200(0)	30(0)	38.04

Table 2. Regression Coefficients of Predicted Second-Order Polynomial Model for Response Variables

variable	coefficient (β)	standard error	p-value[a]
intercept	41.02	1.51	0.0001
X_1	4.90	0.69	0.0001
X_2	7.86	0.69	0.0001
X_3	4.27	0.69	0.0001
X_4	3.25	0.69	0.0003
X_{11}	−2.25	0.66	0.0043
X_{22}	−4.76	0.66	0.0001
X_{33}	0.51	0.66	0.4521
X_{44}	−0.24	0.66	0.7230
X_{12}	−1.39	0.84	0.1218
X_{13}	0.017	0.84	0.9843
X_{14}	−0.20	0.84	0.8164
X_{23}	2.29	0.84	0.0167
X_{24}	1.41	0.84	0.1154
X_{34}	1.26	0.84	0.1572

[a] $p < 0.01$, highly significant; $0.01 < p < 0.05$ significant; $p > 0.05$, not significant.

The purpose of the present study is to investigate lipase-catalyzed synthesis of GRb1 esters with acyl donors by *Candida antarctica* lipase B (Novozyme 435). Response surface methodology (RSM) was employed to optimize the synthesis conditions. The main advantage of RSM is the reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. In this study, the effects of important four reaction factors, reaction time (12, 24, 36, 48, 60 h), temperature (25, 35, 45, 55, 65 °C), substrate mole ratio (100, 150, 200, 250, 300), enzyme concentration (10, 20, 30, 40, 50 mg/mL), were selected for the reaction optimization. Then, the synthesis of GRb1 esters was reproduced under the optimized condition to ascertain whether this RSM was effective to optimize the synthesis conditions.

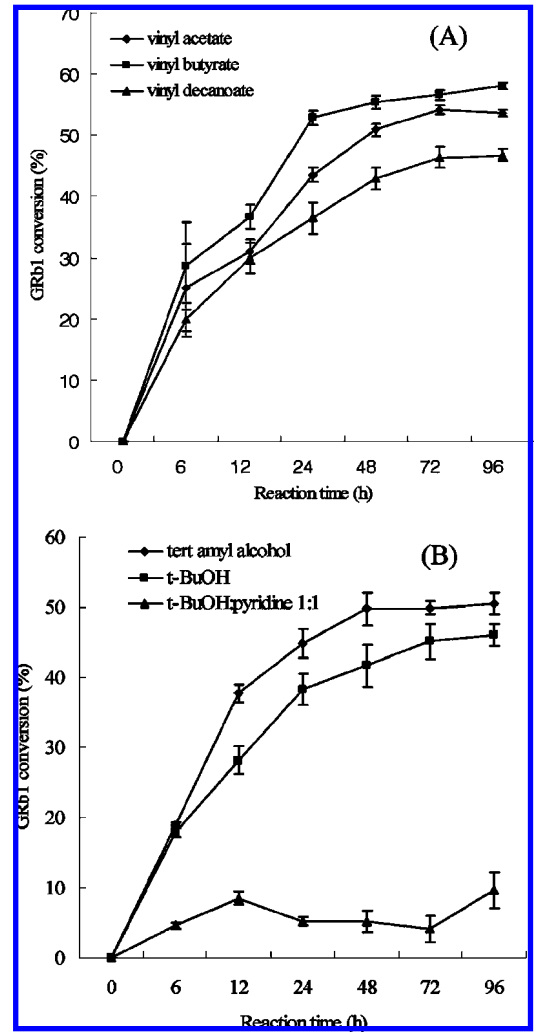

Figure 1. Effect of different acyl donors (**A**) and different organic solvent systems (**B**) on GRb1 conversion in *tert*-amyl alcohol by Novozyme 435 at 45 °C.

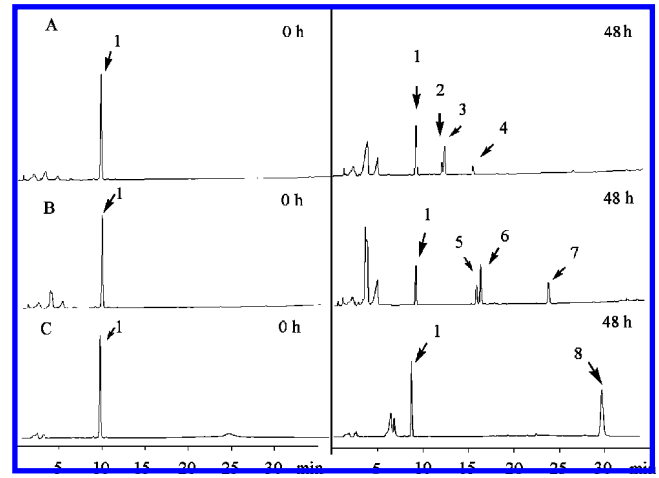

Figure 2. HPLC chromatograms of the lipase-catalyzed esterification of GRb1 with different acyl donors at 0 or 48 h: (**A**) reaction with vinyl acetate; (**B**) reaction with vinyl butyrate; (**C**) reaction with vinyl decanoate. Peak assignments: 1, GRb1; 2, 3, and 4, GRb1-acetate species; 5, 6, and 7, GRb1-butyrate species; 8, GRb1-decanoate species.

MATERIALS AND METHODS

Materials. *Candida antarctica* lipase type B (Novozyme 435) was purchased from Sigma Chemical Co. (St. Louis, MO). Ginsenoside Rb1 was obtained from Hongjiu Ginseng Co., Ltd. (Dalian, China). Vinyl

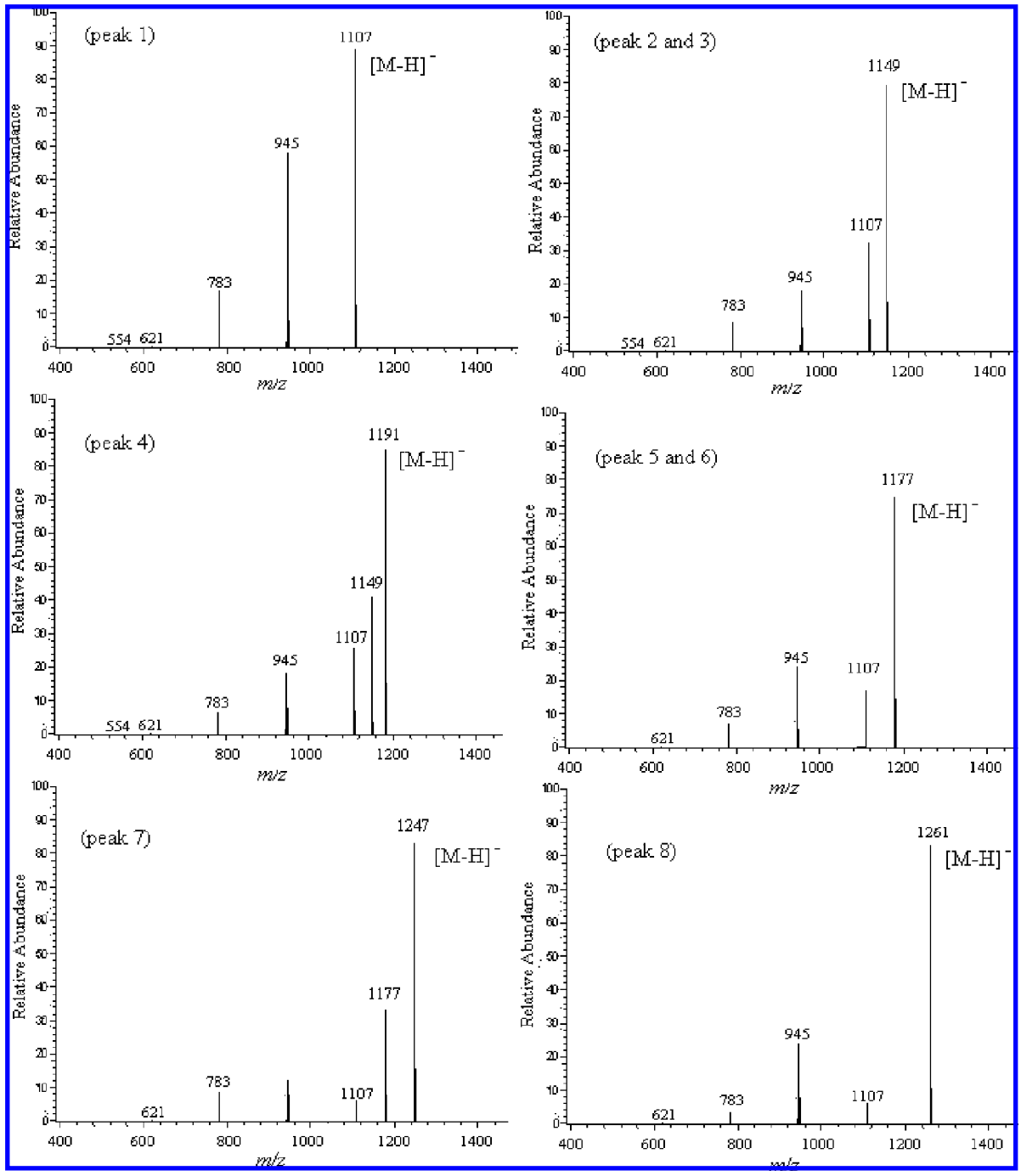

Figure 3. Mass spectra of GRb1 and new species in the esterification of GRb1 with acyl donors.

acetate, vinyl butyrate, and vinyl decanoate were provided from Sigma-Aldrich Chemical Co. (St. Louis, MO). Four angstrom molecular sieves were from ACROS Organics (Morris Plains, NJ). *tert*-Butanol (*t*-BuOH), *tert*-amyl alcohol, and pyridine were of analytical reagent grade.

Procedure of Enzymatic Synthesis. The enzymatic reactions were carried out in a temperature-controlled water shaker at 185 rpm. The procedure for the enzymatic esterification of GRb1 in *tert*-amyl alcohol is described as a representative. GRb1 (2 mg/mL), 4 Å molecular sieves (50 mg/mL), and acyl donor were added in *tert*-amyl alcohol. According to central composite design, the molar ratio of acyl donor to GRb1 ranged from 100 to 300; reactions were performed by adding Novozyme 435 enzyme and shaking the reaction mixture at temperatures ranging from 25 to 65 °C. Samples were withdrawn at different times ranging from 12 to 60 h, then filtered through a syringe filter (0.45 um) prior to HPLC-MS/MS analysis.

Condition of HPLC-MS/MS Analysis. Quantitative analysis of GRb1 and GRb1 esters was performed by Hewlett-Packard HPLC series 1100 (Agilent Technologies, Little Falls, DE) equipped with quaternary pump, vacuum degasser, autosampler, and UV detector. The HPLC system was connected to Agilent Chemstation software. Phenomenex C_{18} column (250 mm × 4.6 mm, 4 μm, Phenomenex Co., Torrance, CA) was used for a separation. Gradient elution was described as follows: from 30% to 40% acetonitrile (CH_3CN) in water (H_2O) in the initial 15 min, from 40% to 52% CH_3CN in the following 5 min, and maintained at 52% CH_3CN for another 20 min; after that, 100% CH_3CN was used to wash column for 20 min. The flow rate was 1.0 mL/min. The detection wavelength was set at 203 nm. The GRb1 conversion was defined as (the area of GRb1 before reaction − the area of GRb1 after reaction)/(the area of GRb1 before reaction) × 100%. The HPLC-MS/MS analysis was carried out with an Agilent 1100 series LC/ESI-MSD SL (single quadrupole) spectrometer (Agilent Technologies). HPLC conditions were the same as described above. Conditions for the negative ion mode of MS were as follows: capillary voltage, −2.0 kV; dry nitrogen gas, 5.0 L/min; heater temperature of nitrogen gas, 350 °C; nebulizer gas pressure, 40 psi; scan range, 400−1500 *m/z*.

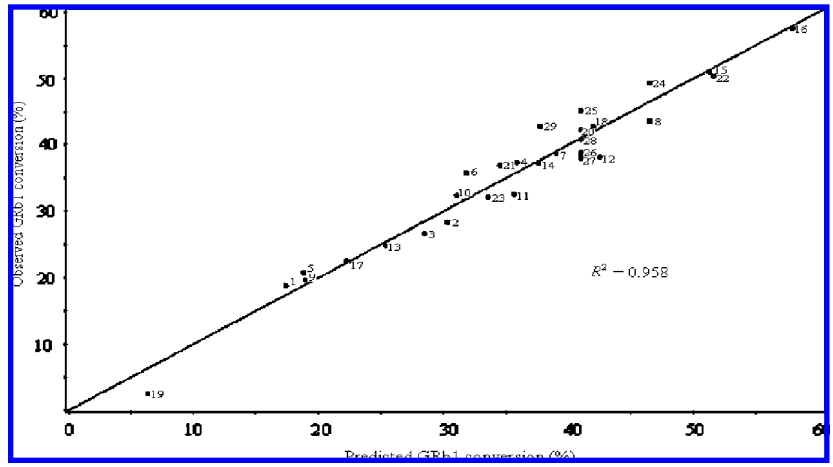

Figure 4. Relationships between observed response and predicted values for the esterification of GRb1 with vinyl decanoate. The numbers indicate experiment numbers presented in **Table 1**.

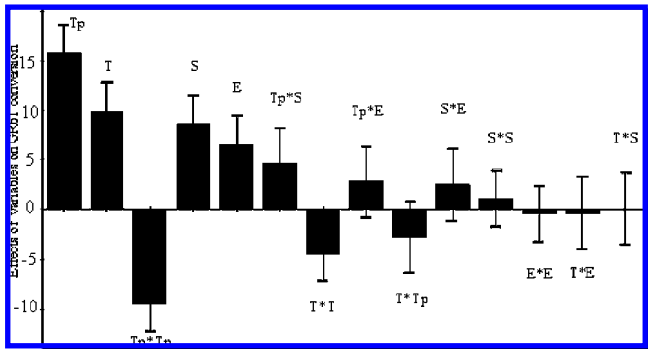

Figure 5. Main effects of variables and their significance for the esterification of GRb1 with vinyl decanoate. T, time; Tp, temperature; S, substrate mole ratio; E, enzyme concentration.

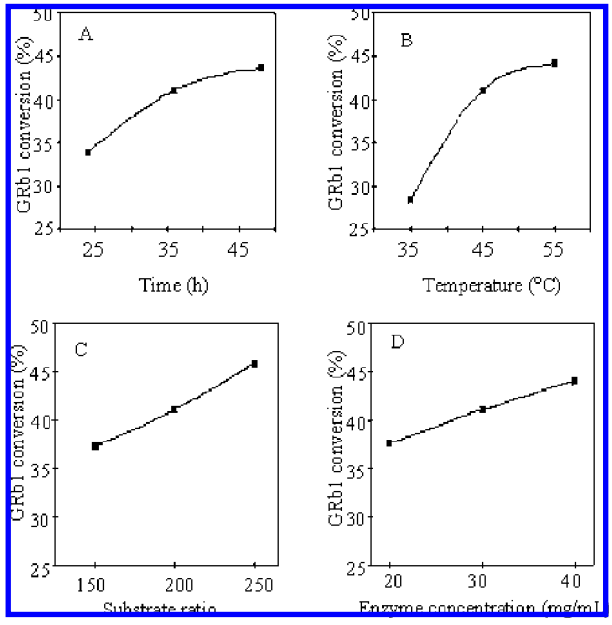

Figure 6. Prediction plot for esterification of GRb1 with vinyl decanoate by effect of reaction time (**A**), temperature (**B**), substrate mole ratio (**C**), and enzyme concentration (**D**).

Experimental Design. A five-level, four-factor central composite design (CCD) was applied in this experiment, consisting of 24 experiments in none central point and 5 center point replicates for a total of 29 runs. The variables selected were X_1 (time), X_2 (temperature), X_3 (substrate mole ratio), and X_4 (enzyme concentration) based on preliminary experiments. Several reports showed that time, temperature, substrate ratio, and enzyme amount were the major factors to influence the acylation of natural compounds by Novozyme 435 (*22*−*24*). The settings for the independent variables were as follows (−1/+1 value): time (h), 24/48; temperature (°C), 35/55; substrate mole ratio (acyl donors/GRb1), 150/250; enzyme concentration (mg/mL), 20/40. **Table 1** shows the independent factors (X_i), levels (−2, −1, 0, 1, 2), and actual experiments in terms of coded levels. A second order polynomial eq 1, which included all interaction terms, was used to calculate the predicted response:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \qquad (1)$$

where Y represents the response variable; β_0, β_i, β_{ii}, and β_{ij} were the interception coefficient, the coefficient of the linear effect, the coefficient of quadratic effect, and the coefficient of interaction effect, respectively. All data were analyzed by analysis of variance (ANOVA) for the response surface quadratic model of Design Expert 7.0.0 (Stat-Ease, Inc., Minneapolis, MN), and the response contour plot and predicted plot were generated by Design Expert 7.0.0. The ridge analysis of response surface regression procedure of the SAS program (SAS, 2000) was used to determine the estimated ridge of maximum or minimum response when the results expressed a saddle point in response surfaces.

RESULTS AND DISCUSION

Screening of Acyl Donors and Reaction Solvents. The effects of three acyl donors with different carbon chains (vinyl acetate, vinyl butyrate, and vinyl decanoate) and three reaction solvents (*tert*-amyl alcohol, *t*-BuOH, and the mixed solvent 1:1 *t*-BuOH/pyridine) on the GRb1 conversion rate were studied. **Figure 1A** shows the effect of three acyl donors on the GRb1 conversion for 96 h reaction. The reaction with vinyl butyrate shows the highest conversion, while the lowest conversion rate was observed in the reaction with vinyl decanoate. It seems that acyl donors with short carbon chains were more effective than those with long carbon chains. A similar observation was found by Pedersen et al. (*17*) in which the formation of sucrose esters with butanoic acid was higher than that with decanoic acid. In addition, most formation of GRb1 esters with vinyl acetate, vinyl butyrate, and vinyl decanoate occurred within the first 24 h; thereafter the reactions gradually approached equilibrium.

In the literature, *tert*-amyl alcohol, *t*-BuOH, acetone, acetonitrile, and the mixed solvent *t*-BuOH/pyridine were usually used in the enzymatic acylation of natural glycosides due to their high capacity for dissolving natural glycosides (*17, 18, 25, 26*). In this study, *tert*-amyl alcohol and *t*-BuOH only completely dissolved GRb1 upon heating at 80 °C, while the mixture of *t*-BuOH and pyridine

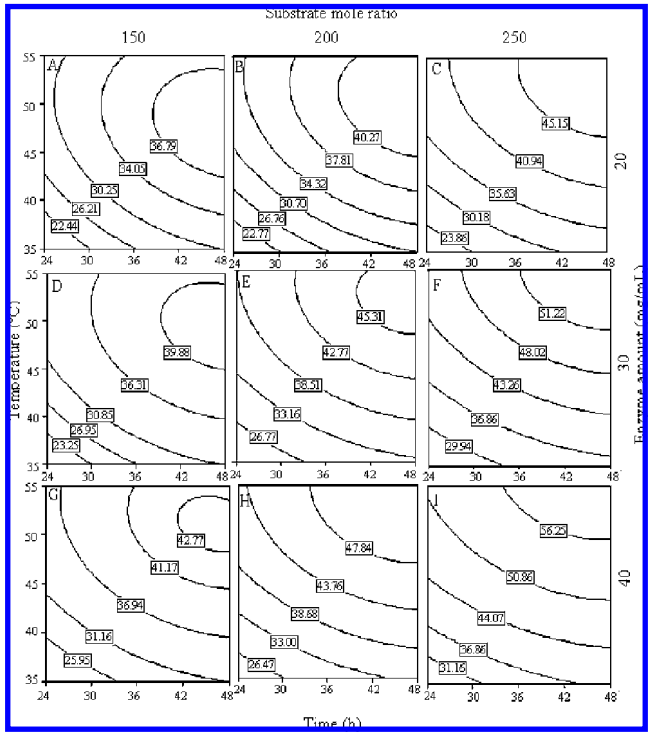

Figure 7. Contour plots showing the effect of reaction time and temperature on the conversion of GRb1 ester by holding constant the substrate mole ratio (150, 200, 250) and enzyme amount (20, 30, 40 mg/mL). The numbers inside the contour plots indicate conversions at given reaction conditions.

dissolved GRb1 even without heating. **Figure 1B** shows the effect of these three solvents; *tert*-amyl alcohol, *t*-BuOH, and the mixed solvent (1:1) *t*-BuOH/pyridine on the esterification of GRb1 with vinyl decanoate. As observed, the organic medium indeed influenced the conversion of GRb1 ester. The esterification reaction in *tert*-amyl alcohol showed the highest conversion rate, while the reaction in the mixed solvent *t*-BuOH/pyridine showed very low conversion rate. The final conversions in *tert*-amyl alcohol and *t*-BuOH after 96 h were 50.54% and 46.02%, respectively. However, in the mixed solvent *t*-BuOH/pyridine, the conversion was only 9.68% after 96 h.

The reactions were monitored by HPLC-MS/MS. The HPLC chromatograms (**Figure 2**) suggested that one species was produced in the reaction with vinyl decanoate, while more than one species were generated in the reaction with vinyl acetate and vinyl butyrate. The deprotonated molecules $[M - H]^-$ of peaks 1, 2, 3, and 4 at *m*/*z* 1107, 1149, 1149, and 1191 suggested that two GRb1 monoacetate and one GRb1 diacetate were formed in the reaction with vinyl acetate; peaks 5, 6, and 7 at *m*/*z* 1177, 1177, and 1247 suggested that two GRb1 monobutyrate and one dibutyrate were generated in the reaction with vinyl butyrate; peak 8 at *m*/*z* 1261 suggested that one GRb1 monodecanoate was produced in the reaction with vinyl decanoate (**Figure 3**). Our results showed good agreement with those of Gebhardt et al. who reported two monoacetylginsenoside Rb1 and one di-*O*-acetylginsenoside Rb1 were obtained from the process of acylation of GRb1 by Novozyme 435 (*17*). To allow the increase of GRb1 lipophilicity, we decided to further study the optimal esterification conditions for GRb1 with vinyl decanoate in *tert*-amyl alcohol using response surface methodology (RSM).

Analysis of Model. One of objectives of this study was the development of the RSM model for better understanding the relationships of the variables for a lipase-catalyzed direct esterification reaction of GRb1 with vinyl decanoate. The experimental factor settings were based on composite central design with the four factors and three levels together with two star points. The actual variable settings and the response are presented in **Table 1**. Among the various treatments, the highest conversion (57.53%) was treatment 16 (48 h, 55 °C, substrate mole ratio 250, and 40 mg/mL enzyme), and the lowest conversion (only 2.46%) was treatment 19 (36 h, 25 °C, substrate mole ratio 200, and 30 mg/mL enzyme). Further, ANOVA for the response surface quadratic model was employed to fit the second-order polynomial eq 1. From **Table 2**, regression coefficients were determined by employing the least-squares technique to predict quadratic polynomial models for GRb1 conversion. Among the coefficients, some factors with *p*-value <0.05 (β_1, β_2, β_3, β_4, β_{11}, β_{22}, and β_{23}) were selected, and others with *p*-value >0.05 (β_{33}, β_{44}, β_{12}, β_{13}, β_{14}, β_{24}, and β_{34}) were removed from the model since those with big *p*-values were less important factors. The fitted second-order polynomial eq 2 is given below:

$$Y = 41.02 + 4.90X_1 + 7.86X_2 + 4.27X_3 + 3.25X_4 - 2.25X_{11} - 4.76X_{22} + 2.29X_{23} \quad (2)$$

ANOVA results of the response surface showed no significant lack of fit ($p = 0.3470$), a very small *p*-value of total model (0.0001), and satisfactory levels of coefficient of determination (R^2, 0.96; data not shown). Further, the observed GRb1 conversion correlated well with the predicted values, showing linear distribution with $R^2 = 0.958$ (**Figure 4**). These values indicated that this experimental model was adequate and reproducible.

Effect of Variables. ANOVA results showed that the GRb1 conversion was significantly affected by the first-order (linear) ($p = 0.0001$) and second-order ($p = 0.0001$) of the variables. **Figure 5** shows the main effect of variables on the response. It was found that all first-order variables had a significantly positive effect on the GRb1 conversion among which temperature was the most significant one. Among the second-order variables, time (X_{11}) and temperature (X_{22}) with high significance ($p = 0.0001$ and 0.0043, respectively) showed negative effect on the GRb1 conversion. Most interactions between variables had a very minor influence on the GRb1 conversion since the *p*-values were larger than 0.05, except X_{23} (the interaction parameter of temperature and substrate mole ratio) with positive effect on the reaction. From **Figure 6**, it was easier to understand the effect of all the four linear factors on the GRb1 conversion. The yield of GRb1 conversion increased with increase of each factor. At early reaction time, the reaction increased quickly and gradually approached equilibrium at 40 h. A similar condition was observed with increasing temperature. On the other hand, high substrate mole ratio and high enzyme amount were always in favor of high yield of conversion since high substrate mole ratio and high enzyme amount could greatly move the reaction toward the esterification.

The relationships between reaction variables and response can be better understood by examining the planned series of contour plots (**Figure 7**) generated from the predicted model (eq 2) by holding constant the substrate mole ratio (150, 200, 250) and enzyme amount (20, 30, 40 mg/mL). Temperature and time were the most important variables for GRb1 conversion with small *p* values and therefore were considered to be indicators of effective performance. Panels in the same row represent the same enzyme amount, and panels in the same column represent the same substrate mole ratio. Such an application could be adopted to study the synthesis variables simultaneously in a five-dimensional space. Overall, all nine contour plots in **Figure 7** exhibited similar behavior in which predicted GRb1 conversion increased with increasing enzyme amount and substrate mole ratio.

Attaining Optimization. The optimum synthesis of GRb1 ester was determined by the ridge maximum analysis and canonical analysis. Canonical analysis is one of the multivariate linear statistical analyses used to locate the stationary point of the response surface and to determine whether it represents a maximum, minimum, or saddle point (*27*). The canonical analysis based on the stationary point resulted in the equation

$$Y = 36.03 + 4.71W_1^2 - 2.36W_2^2 - 8.45W_3^2 - 20.81W_4^2 \quad (3)$$

where W_1, W_2, W_3, and W_4 are the eigenvalues based on coded data and Y is the GRb1 conversion. The predicted response surface of the stationary point was a saddle because eigenvalues were mixed positive and negative (*27*). Therefore, a ridge analysis was performed further to determine the estimated ridge of maximum or minimum response. From the ridge analysis with maximum responses, the maximum GRb1 conversion was predicted to be 61.51% in the combination of variables 40.2 h, 52.95 °C, substrate mole ratio 275.57, and enzyme amount 39.81 mg/mL.

Model Verification. An additional independent experiment with the predicted maximum synthesis conditions was done to verify the adequacy of the predicted model. The predicted value was 61.51% GRb1 conversion, and the actual experimental value was 62.92%, which indicated that the optimum condition was appropriate. Therefore, the RSM in this study was effective to optimize a lipase-catalyzed synthesis of ginsenoside Rb1 with vinyl decanoate by *Candida antarctica* lipase B (Novozyme 435).

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