Hydrophilic Glyceryl Ferulates Preparation Catalyzed by Free Lipase B from Candida antartica

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Abstract: Ferulic acid (FA), 4-hydroxyl-3-methoxy-2-benzylacrylic acid, has antioxidant, anticancer and ultraviolet absorption activities. However, the low hydrophilicity of FA has limited its application. Glyceryl ferulate (FG), which is an all-natural hydrophilic derivative of FA, can be used as an antioxidant and UV filter in food and cosmetic formulations. However, the applications of FG in these fields are limited due to its low content in nature. In this work, free liquid lipase was firstly used as a catalyst for FG preparation. Several different free liquid lipases (Candida antartica lipase-B, Candida antartica lipase-A, Thermomyces lanuginosus (Lipozyme TL 100L)) were screened and compared. The effects of the transesterification parameters (time, temperature, enzyme load and substrate ratio) were optimized and evaluated by response surface methodology. A reaction thermodynamic investigation was also performed. The results showed that, among the tested free lipases, the maximum FG yield (84.8±1.5%) was achieved using free Candida antartica lipase-B. Under the optimized conditions (an atmospheric system, an enzyme load of 11.1% and a 20:1 molar ratio of glycerol to EF at 70℃ for 39.5 h), the FG yield and EF conversion were 84.8±1.5% and 95.7±1.2%, respectively. The activation energies of FG formation and EF conversion were 56.4 and 58.0kJ/mol, respectively.

Key words: hydrophilic glyceryl ferulate, transesterification, response surface methodology, free lipase, thermodynamic investigation

1 Introduction

Ferulic acid (FA) is also known as 4-hydroxy-3-methoxy cinnamic acid, is ubiquitously found in some plants, and has UV-absorbing, antioxidant, free radical scavenging and antitumor activities. However, the low hydrophilicity of FA has limited its application in pharmaceutical, cosmetic and other fields. To overcome this problem, modifications of FA using hydrophilic groups (for example, glycerol, hydrophilic aliphatic alcohols and monosaccharides) has attracted attention.

Glyceryl ferulate (FG) is a popular hydrophilic FA derivative. FG exhibited good UV adsorbing properties at 280–360 nm with a λmax at 322 nm. In previous reports, chemical catalysts and enzymes have been used for FG preparation. The presence of phenolic hydroxyl makes FA easy to oxidize and sensitive to heat. Therefore, compared with chemical catalysts, enzymes have been popular catalysts for the preparation of FA derivatives, due to their mild reaction conditions.

Among the enzymes used in the preparation of FA derivatives, lipases (triacylglycerol hydrolases E.C. 3.1.1.3) have been widely used in many reactions, due to their broad specificity for some substrates and high catalytic activities. The active site of lipases is composed of a serine, an aspartate or glutamate, and a histidine. In addition, lipases have open (active) and a closed (inactive) conformation in the presence of interfacial activation. Moreover, the open form of a lipase molecule can stabilize the open form of other lipases, which can create dimers with altered catalytic activities. The open form can increase the lipase activity. Therefore, many technologies have been used to maintain the open form of lipase by using the movement of the lid in the presence of hydrophobic surfaces, for example, genetic manipulation, immobilization and other physicochemical modifications. Due to their good operational stability, recovery and reusability, these immobilized enzymes have been commercially prepared and used in many reactions.

In our previous enzymatic preparation of FG, the immobilized Novozym 435 had the best catalytic activity for hy-
drophilic FG and glycerol dif erulate (DFG) preparation. Matsu et al. also used immobilized Candida antarctica lipase B as a biocatalyst to prepare FG. However, in these previous methods of FG preparation, the high cost of the immobilized Novozym 435 (2.273$/kg) and the adsorption of glycerol on the immobilized support limited their industrial applications. Recently, and due to their low cost, free liquid lipase Candida antarctica lipase-B, 142$/kg was also performed.

Moreover, no available information focusing on FG preparation were evaluated. To analyze the catalytic properties, and reaction variables (temperature, substrate ratio, time, and enzyme load) on hydrophilic FG preparation were evaluated. To analyze the catalytic property of free lipase, a thermodynamic reaction investigation was also performed.

2 Experimental

2.1 Materials

The free liquid lipases Candida antarctica lipase-A (CALA, 26.3 mg protein/mL, 420 LU/mg), Candida antarctica lipase-B (CALB, 15.1 mg protein/mL, 435 LU/mg), and Thermomyces lanuginosus (Lipozyme TL 100L, 35.4 mg protein/mL, 100 LU/mg) were provided from Novozymes A/S (Bagsvaerd, Denmark). One LU corresponds to the amount of enzyme that liberates one micromole of butyric acid per minute from a tributyrin substrate at 30°C and pH 7.0. Ethyl ferulate (EF, purity > 99%) and FA were provided from Suzhou Chang Tong Chemical Co., Ltd. (Suzhou, China).

2.2 Free liquid lipase-catalyzed transesterification

Glycerol (10 mmol) reacted with EF (1 mmol) under atmospheric pressure in 25 mL flasks at 70°C for 24 h. Free liquid lipases were used as biocatalysts. The total reaction volume was approximately 1 mL. The reaction mixture was stirred with a magnetic stirrer at 300 rpm. These standard conditions were used unless otherwise stated in the text. The samples were withdrawn and separated according to a previous report.

\[ \text{FG Yield (\%)} = \frac{C_{\text{FP}}}{C_{\text{EF}}} \times 100 \]  

Where \( C_{\text{FP}} \) (mol/L) and \( C_{\text{EF}} \) (mol/L) are the concentrations of FG and EF, respectively; \( C_{\text{FP}} \) (mol/L) is the initial concentration of EF.

2.3 Products analysis

According to previous methods, the samples were analyzed using a HPLC with a UV detector. Methanol (solvent A) and 0.5% (v/v) glacial acetic acid in water (solvent B) were used to elute the samples using a C18 column (5 μm, 250 mm × 4.6 mm) at 1 mL/min. Solvent A first increased from 20% to 85% over 24 min, then decreased to 20% in 6 min and was maintained at 20% for 4 min at 325 nm and 35°C. Then, the products were identified according to previous methods.

2.4 RSM design

A three-factor-three-level design was carried out in this study. The different levels and factors were selected as follows: reaction temperature (50°C, 70°C, and 90°C), reaction time (6 h, 24 h, and 42 h) and lipase load (5%, 15%, and 25%).

A total of 17 tests were performed using a Box-Behnken design to evaluate the coefficients. According to the quadratic polynomial equation, the mathematical relationship of the Box-Behnken design can be expressed as follows:

\[ Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \]  

From the above formula, Y represents one response (FG yield or EF conversion), \( X_i \) and \( X_j \) are transesterification parameters, and \( \beta_0, \beta_i, \beta_{ij} \) are the intercept, linear, quadratic, and interaction terms, respectively.

2.5 Statistical analysis

Triplicate experiments were performed for each investigated parameter. The results are expressed as the average ± S.E.M. Statistical analysis was performed using Service Solution (SPSS) version 19.0. The differences of the mean values were evaluated by analysis of variance (two-way ANOVA), and statistical significance was determined at a 95% level of probability.

3 Results and Discussion

3.1 Enzyme screening

In this study, three commercial free lipases were used and compared for the transesterification of glycerol with EF. When Candida antarctica lipase-A (CALA) and Thermomyces lanuginosus (Lipozyme TL 100L) were used as biocatalysts, no FG was formed. Compared with CALA and Lipozyme TL100L, Candida antarctica lipase-B (CALB) showed the best activity for the reaction, which can be as-
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cribed to the higher enzyme activity of CALB (435 LU/mg) than those of CALA (420 LU/mg) and Lipozyme TL 100L (100 LU/mg). Figure 1 shows that, when free CALB was used as catalyst and with time increasing from 2 h to 24 h, the FG yield and EF conversion gradually increased to 52.7 ± 1.7% and 70.8 ± 2.5%, respectively. After 24 h, the highest FG yield (52.7 ± 1.7%) was obtained, which is much greater than that of the transesterification of glycerol with triolein (TO) at 72 h with Novozym 435 (29% yield). This result may explain the elimination of the external mass transfer limitation of the reaction substrates to the active site of lipase using free liquid lipase CALB as a catalyst.

3.2 Effect of different feruloyl donors

Figure 2 shows that when FA was used as a feruloyl donor, FA conversion and FG yield were lower than 30% and 10%, respectively. However, when EF was used as a feruloyl donor, FG yield and EF conversion increased with the reaction progress. EF conversion was 88.6 ± 2.3% at 24 h, which was higher than FA as the feruloyl donor (< 30%). These results can be attributed to the higher melting and lower solubility of FA (174°C) than EF (64°C) in this reaction system, which results in a large mass transfer limitation in the system with FA as the feruloyl donor. Therefore, considering the melting point and solubility of the feruloyl donor, EF was used as the feruloyl donor in subsequent experiments.

3.3 Effect of the pressure of the reaction system

In this work, ethanol was formed as by-product of the transesterification of glycerol with EF. The effect of ethanol removal was also investigated. Two reactions were performed under a vacuum system and an atmospheric system. The EF conversion of the vacuum pressure was 40.2 ± 2.4% at 12 h, which was similar to that at 24 h (41.7 ± 1.8%) (Fig. 3). However, under atmospheric pressure, EF conversion (79.2 ± 2.2%) was 2 times that of the vacuum pressure. In addition, the EF conversion of the atmospheric pressure slowly increased to 88.6% at 24 h, which was much higher than 48.0 ± 3.0% of the transesterification of castor oil (CO) with EF using ionic liquids as the reaction medium. In theory, the removal of by-product (ethanol) at vacuum (0.1 MPa) can drive reaction toward more EF conversion. However, when free liquid lipase was used as the catalyst, the EF conversions at atmospheric pressure were higher than those at vacuum pressure, which may be ascribed to the presence of water in the atmospheric system. However, the presence of ex-
cessive water can result in more FA formation (~15% FA yield with 5% water added). When the lyophilized lipase was used, little FA formed (~3%); however, a relatively low EF conversion (~90%) and FG yield (~80%) were obtained. The effect of water on esterification can also be found in other free liquid lipase-catalyzed reactions. Therefore, atmospheric pressure was used as the most favorable reaction system for the transesterification with EF and glycerol to prepare FG.

3.4 Effects of transesterification variables.

Figure 4 shows that the reaction rate and EF conversion both increased from 50°C to 80°C, which was attributed to the fact that at high temperature, the lid covering the active sites of the lipase is displaced to allow the substrate to access the active sites. When the temperature exceeds 80°C, the EF conversion and reaction rate dramatically decrease (Fig. 4A), which can be interpreted by the fact that the enzyme is deactivated at a high temperature. Maximum EF conversion (95.5 ± 2.4%) was obtained at 70°C and 24 h. Therefore, 70°C was selected as the optimal temperature, which is lower than the optimal temperature (90°C) of the immobilized lipase Novozym 435. These temperatures were due to immobilization, which can stabilize the open form of the enzyme and improve the thermal stability of the enzyme. Similar regarding enzyme stability due to immobilization can be found in other reactions.

Figure 4B shows the effect of temperature on FG yield. In the solvent-free system, FG yield gradually increased with the reaction temperature from 50°C to 70°C. The maximum FG yield (72.8 ± 1.8%) was obtained at 70°C and 24 h. However, FG yield sharply decreased at 90°C due to the deactivation of free liquid CALB at high temperatures. In the products, 1-FG was the main product, and a very small amount of 2-FG (<1.5%) was found, which suggests that compared with the fatty acyls in monoacylglycerols, feruloyl migration in 1-FG was very difficult. This difficult migration was ascribed to the electronic donation and the large steric hindrance of feruloyl. However, with the increase of temperature, an increasing amount of glyceryl 1,3-diferulate (1,3-DFG) was formed and no 1,2-DFG was observed, which resulted from the electronic donation and the large steric hindrance of the feruloyl. The maximum amount of 1,3-DFG, 5~6%, was obtained at 70°C. These results were different from fatty acyls in diacylglycerols.

The initial reaction rates, defined as the initial EF conversion per unit time ($V_0$, mol/(L·min)), were calculated from six experimental points of the conversion-time profile corresponding to the first 1 h of the reaction (15.0% or less EF conversion), where the profiles were found to be approximately linear. From the slope of a straight line passing through these points, it is possible to calculate the activation energy. Under the different temperatures tested and according to $\ln V_0$ versus $1/T$, good linearity was obtained (Fig. 4C). The Arrhenius equation can be expressed as: $\ln V_0 = 15.5 - 6970/T$ for the transesterification of glycerol with EF, and the activation energy ($E_a$) was 58.0kJ/mol, which was higher than that of normal enzymatic reactions (0.97-34.5kJ/mol). The high $E_a$ of the reaction is ascribed to the electronic donation, the large steric hindrance and inhibition of EF, and the high sensitivity of free liquid CALB to temperature. The $E_a$ of FG formation was 56.4kJ/mol.

A series of experiments were conducted using free liquid CALB as the biocatalyst with different enzyme loads (Figs. 4D and 4E). When the enzyme load was less than 20% and ranged from 5% to 20%, EF conversion increased, and the maximum EF conversion was 96.1 ± 1.8%, which is higher than in the presence of the immobilized Novozym 435 as the biocatalyst (79.2% ± 2.4%) [41]. When the enzyme load was higher than 20%, the EF conversion and FG yield showed no significant difference.

With a molar ratio ranging from 1:1 to 50:1, FG yield and EF conversion both increased (Figs. 4F and 4G). When the substrate molar ratio was higher than 10:1, the reaction rate increased. A similar effect of the substrate molar ratio can also be found in other reactions. The maximum EF conversion (~100%) was acquired with a 50:1 substrate ratio, which was similar to the 25:1 substrate ratio (97.7 ± 2.0%). Therefore, 25:1 was selected as the optimal condition.

3.5 Model Fitting

In the response surface design, a model between the response and factors was established to reflect the optimal EF conversion and FG yield (Table 1). Multiple regression
techniques were used for data analysis and are demonstrated by the models (Table 2). The quadratic polynomial regression models of EF conversion and FG yield were very significant (p < 0.0001). The models were reliable and verified the prediction relationship between EF conversion (or FG yield) with transesterification variables. The coefficients of the regression models (R²) were 0.9897 and 0.9781. These results also illustrate that the models were suitable for the evaluation of the relationships between the transesterification variables and the responses. Therefore, two regression equations were successfully obtained as follows:

\[
\text{EF conversion} \% = 94.60 + 8.01X_1 - 18.25X_2 + 9.55X_3 - 1.58X_1X_2 - 5.07X_1X_3 + 2.10X_2X_3 - 1.98X_1^2 - 31.15X_2^2 - 7.46X_3^2
\]

\[
\text{FG yield} \% = 84.07 + 7.17X_1 - 14.40X_2 + 3.44X_3 - 1.25X_1X_2 - 5.80X_1X_3 + 4.48X_2X_3 - 1.58X_1^2 - 29.06X_2^2 - 8.45X_3^2
\]

Figures 5A, 5C and 5E show the interaction influence of the reaction parameters on EF conversion. The maximum EF conversion appeared at a 15%-25% lipase load and from 60-70°C for > 24 h. Figures 5B, 5D and 5F show the interaction influence of the reaction parameters on the FG

Fig. 4  Effect of temperature on EF conversion (A) and FG yield (B) with a 20% enzyme load and 10:1 substrate ratio. The relationship between the initial reaction rate and reaction temperature (C). The reaction conditions were the same as in Fig. 4A. The effect of the enzyme load on EF conversion (D) and FG yield (E) with a 10:1 substrate ratio at 70°C. The effect of the substrate ratio on EF conversion (F) and FG yield (G) with a 20% enzyme load at 70°C. Free liquid lipase CALB was used as the catalyst for all reactions.
yield. The maximum FG yield was obtained with a 10-20% enzyme load at 58-70°C for 33-42 h. The influence of the reaction parameters for transesterification was temperature > lipase load > time (Fig. 5), which was different from FG yield (lipase load > time > temperature). These results are due to the higher Ea of EF transesterification (58.0 kJ/mol) than FG formation (56.4 kJ/mol), and a reaction with a high Ea is more sensitive to a temperature change.

3.6 Reaction optimization and model verification

The reaction conditions were optimized by RSM as follows: a substrate molar ratio of 20:1 and a lipase load of 11.1% at 70°C for 39.5 h. Under the optimized conditions, the maximum EF conversion (95.7 ± 1.2%) and FG yield (84.8 ± 1.5%) were obtained, which were consistent with the predicted results (EF conversion 94.6 ± 1.6% and FG yield 84.1 ± 2.0%). These results indicate that the models were reliable. These results also suggest that, compared with previous reports (8, 9, 47), free liquid lipase B from Candida antarctica as a catalyst can improve the hydrophilic FG preparation.

Compared with Novozym 435 (96%) (36, 42), the FG yield of free lipase B from Candida antartica was lower. However, compared with immobilized Novozym 435, there are some advantages of the free lipase: (i) the cost of the free lipase ($142/kg) was much lower than that of immobilized Novozym 435 ($2,273/kg); (ii) for immobilized Novozym 435, a vacuum system was required; however, for free lipase, the atmospheric pressure was best; (iii) free liquid lipase B from Candida antarctica as a biocatalyst can eliminate the effect of the external mass transfer limitation and improve the reaction rate; and (iv) free lipase showed a
Table 1  Experimental design and results of the transesterification of EF with glycerol affected by the reaction temperature, reaction time and enzyme load.

| Treatment no. | $X_1$ time (h) | $X_2$ temperature (°C) | $X_3$ enzyme load$^b$ (%) | EF conversion (%) | FG yield (%) |
|--------------|----------------|------------------------|---------------------------|------------------|--------------|
| 1            | 6(-1)          | 70(0)                  | 5(-1)                     | 63.2 ± 1.8       | 57.7 ± 1.6   |
| 2            | 42(1)          | 70(0)                  | 25(1)                     | 97.0 ± 2.1       | 78.8 ± 1.9   |
| 3            | 24(0)          | 50(-1)                 | 5(-1)                     | 63.52 ± 1.6      | 57.9 ± 1.8   |
| 4            | 6(-1)          | 50(-1)                 | 15(0)                     | 72.8 ± 2.1       | 63.5 ± 1.9   |
| 5            | 24(0)          | 70(0)                  | 15(0)                     | 94.4 ± 2.9       | 86.7 ± 2.4   |
| 6            | 6(-1)          | 70(0)                  | 25(1)                     | 87.2 ± 1.5       | 71.2 ± 1.8   |
| 7            | 6(0)           | 90(1)                  | 5(-1)                     | 24.1 ± 1.0       | 23.3 ± 1.2   |
| 8            | 6(-1)          | 90(1)                  | 15(0)                     | 38.1 ± 1.3       | 34.0 ± 1.6   |
| 9            | 24(0)          | 50(-1)                 | 25(1)                     | 83.6 ± 1.4       | 60.8 ± 1.7   |
| 10           | 24(0)          | 70(0)                  | 15(0)                     | 94.7 ± 2.7       | 85.5 ± 2.5   |
| 11           | 24(0)          | 70(0)                  | 15(0)                     | 94.7 ± 1.9       | 84.0 ± 2.1   |
| 12           | 24(0)          | 70(0)                  | 15(0)                     | 94.8 ± 2.2       | 84.1 ± 1.8   |
| 13           | 24(0)          | 70(0)                  | 15(0)                     | 94.4 ± 1.6       | 80.1 ± 1.5   |
| 14           | 24(0)          | 90(1)                  | 25(1)                     | 52.7 ± 1.0       | 44.2 ± 1.2   |
| 15           | 42(1)          | 90(1)                  | 15(0)                     | 47.0 ± 1.5       | 40.9 ± 1.6   |
| 16           | 42(1)          | 50(-1)                 | 15(0)                     | 88.0 ± 1.6       | 75.4 ± 1.9   |
| 17           | 42(1)          | 70(0)                  | 5(-1)                     | 93.3 ± 2.4       | 88.5 ± 2.1   |

$^a$ Numbers were run in random order.
$^b$ Enzyme load (% relative to the weight of total substrates).

Table 2  ANOVA of the quadratic models for EF conversion and FG yield.

| Source            | Sum of squares | Degree of freedom | Mean square | F value | Prob > F |
|-------------------|----------------|-------------------|-------------|---------|----------|
| EF conversion     |                |                   |             |         |          |
| Model             | 8543.37        | 9                 | 949.26      | 74.41   | <0.0001  |
| Residual          | 89.30          | 7                 | 12.76       |         |          |
| Lack of Fit       | 89.17          | 3                 | 29.72       | 918.26  | <0.0001  |
| Pure error        | 0.13           | 4                 | 0.032       |         |          |
| Total             | 8632.67        | 16                |             |         |          |
| $R^2 = 0.9897$    |                |                   |             |         |          |
| FG yield          |                |                   |             |         |          |
| Model             | 6419.47        | 9                 | 713.27      | 34.76   | <0.0001  |
| Residual          | 143.75         | 7                 | 20.54       |         |          |
| Lack of Fit       | 118.74         | 3                 | 39.58       | 6.33    | 0.0533   |
| Pure error        | 25.00          | 4                 | 6.25        |         |          |
| Total             | 6563.22        | 16                |             |         |          |
| $R^2 = 0.9781$    |                |                   |             |         |          |
Fig. 5 Interaction effect of the reaction time and enzyme load on EF conversion (A) and FG yield (B) at 70°C. The interaction effect of the reaction time and temperature on EF conversion (C) and FG yield (D) with a 15% enzyme load. The interaction effect of the enzyme load and temperature on EF conversion (E) and FG yield (F) at 24 h.

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good selectivity for DFG formation.

4 Conclusion
In this work, hydrophilic FG was successfully prepared using free liquid lipase B from Candida antarctica as a biocatalyst. The reaction conditions were optimized as an 11.1% enzyme load and 20:1 substrate molar ratio at 70°C for 39.5 h, and the highest EF conversion (95.7 ± 1.2%) and FG yield (84.8 ± 1.5%) were achieved under optimal conditions. Due to the sensitivity of free liquid CALB to reaction temperature, a relatively high Ea of FG formation and Utilization of Cereal Resource.

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