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ORIGINAL PAPER

Enzyme-Catalyzed Synthesis of Monoacylglycerols Citrate: Kinetics and Thermodynamics

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Abstract The kinetics and thermodynamics of the esterification of citric acid (CA) and monoglycerides (MGs) for citrate esters of monoacylglycerols catalyzed by Novozym 435 in *tert*-butyl alcohol system was studied in this work. The relationship between initial reaction rate and temperature was established, based on the Arrhenius law. A linear relationship was established between the initial reaction rate and enzyme load up to 3 g/L, which demonstrated that the influence of external mass transfer limitations on the reaction could be eliminated. The reaction kinetics agreed with the Ping-Pong Bi–Bi mechanism with CA inhibition characterized by V_{max}, K_B, K_A, and K_{iA}, values of 0.7092 mmol/(min g), 0.0553, 0.0136 and 0.1948 mol/L, respectively. The model was used to simulate the reaction process. The values calculated from the kinetic mode agreed well with the experimental rate data under the different MG and CA concentrations.

Keywords Citrate esters of monoacylglycerols (CITREM) · Enzyme catalyzed esterification · Kinetics · Thermodynamics

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Introduction

Citric acid (CA) (2-hydroxypropane-l,2,3-tricarboxylic acid) was first isolated and crystallized from lemon juice by Karl Wilhelm Scheele in 1784. Commercial production of citric acid commenced in England in 1860 [1]. Citric acid is a commodity chemically produced and consumed throughout the world. It is used mainly in food, beverages, and pharmaceuticals. Although it is one of the oldest industrial fermentations, its world production is still in rapidly increasing. Global production of citric acid in 2007 was over 1.6 million tonnes [2].

However, CA is less effective in hydrophobic media because of its low solubility therein. Attachment of alkyl chains and other lipophilic moieties (e.g. partially deacylated triacylglycerols) to a CA molecule is an efficient way of solving the problem [3–6]. As eco-friendly products, lipid soluble derivatives of CA are biodegradable, nontoxic, and can be used as plasticizers [3], skin treatment products [4], surfactants [7], waterproofing products [8], etc. It is well-known that citrate esters of monoacylglycerols (CITREM), as a lipophilic derivative of CA, can be used as a food additive for a purpose of improving the solubility and the stability because of its acidity and emulsification [9–13].

Citrate esters are conventionally prepared by chemical means, by reacting an acylglycerol with citric acid or its anhydride in the presence of a chemical catalyst. The anhydride method can be carried out at lower temperatures. However, this process is more expensive because of the extra step necessary to synthesize the anhydride. When citric acid is used, temperatures above 130 °C must be avoided to prevent decomposition of the acid [14]. To overcome these disadvantages, the use of enzymes has opened a new avenue for producing citrate esters under

mild conditions. Recently, successful enzymatic synthesis of CITREM has been reported by Huang et al. [15].

Lipases are spontaneously soluble in aqueous solutions, but their natural substrates (i.e., lipids) are not. Although use of a proper organic solvent or an emulsifier helps to overcome the problem of intimate contact between substrate and enzyme, the practical use of lipases in such pseudohomogeneous reaction systems poses technological difficulties and economic difficulties [16]. Although most lipases are still used in soluble form, attempts to improve the economics of the technological processes and to decrease the contamination of the product have been more and more often implemented through immobilization [17]. Immobilized lipases are those lipases localized in a defined region of space, which is enclosed by an imaginary (or material) barrier that allows for physical separation of the enzyme from the bulk reaction medium and that is, at the same time, permeable to both reactant and product molecules [16, 17].

The purpose of the present study was to investigate the influence of the synthesis parameters, including reaction temperature, shaking speed, and substrate concentration on the reaction rate for the CITREM synthesis catalyzed by an immobilized lipase, and to calculate the kinetic and thermodynamic parameters of the reaction.

Materials and Methods

Materials

Novozym 435 (lipase B from *Candida antarctica*, CAL) was purchased from Novozymes A/S (Bagsvaerd, Denmark). Anhydrous CA was purchased from Shanghai Jingchun Reagent Co., Ltd. (Shanghai, China). MGs (purity, 90.67 %) with the content of palmitic acid, stearic acid, oleic acid, and linoleic acid of 5.93, 3.52, 62.85, 22.73 %, respectively (quantitated by GC) was also purchased from Shanghai Jingchun Reagent Co., Ltd. (Shanghai, China). *tert*-Butyl alcohol and phosphate were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Molecular sieve material (4 Å) were obtained from the Dalian Institute of Chemical Physics, Chinese Academy of Science (Dalian, Chinese). Methanol was of HPLC purity. All of the other reagents were analytical grade.

General Considerations for Enzymatic Esterification Reactions

Esterification was conducted as described previously [15]. The reactions were performed in 10-mL stoppered tubes. CA and MGs were dissolved in 3 mL of *tert*-butyl alcohol. A molecular sieve was added to remove the water in the reaction system. The added amount of the molecular sieve was 120 g/L. Reaction temperature was controlled using a water shaking bath with different shaking speed. The quantities of enzyme added to reactions were the combined mass of the enzyme and its support. After the reaction was complete, CITREM products were recovered by removing the enzyme, the molecular sieve and the solvent through filtration and vacuum distillation, respectively.

Analysis

Reactants were analyzed according to our previous report [15]. The products were also isolated and identified as described previously [15]. Analysis of CA was performed using an HPLC system (Waters, Corporation, Milford, America).

CITREM was fractionated from the esterification reaction products by thin layer chromatography (TLC) on silica gel plates. Two new bands found in the plate were scraped off and eluted several times with methanol and the substances were subsequently analyzed by HPLC–electro spray ionization–mass spectroscopy (HPLC–ESI–MS) using a Waters Platform ZMD 4,000 mass spectrometer with a direct ESI interface.

Results and Discussion

The Effect of Temperature

Temperature significantly affects enzyme catalysis, because the temperature effect is related to the activity and stability of the enzyme. The effect of the reaction temperature on the conversion of citric acid during the reaction is shown in Fig. 1. It is obvious that the temperature has a considerable influence on the reaction rate; in fact, the reaction rate increased with increasing temperature. An increase in the reaction temperature resulted in an acceleration effect, according to the Arrhenius law, catalyzed by enzymes. At elevated temperatures, the operation is also enhanced, since a higher temperature decreases the viscosity of the solutions so as to reduce the mass transfer limitations [18]. Figure 1 shows that a 50 % conversion of CA was achieved after 20 h at 60 °C and 60 h at 40 °C, respectively. The reaction equilibrium was reached faster at the higher temperature. Similar results have also been reported [19]. However, temperatures that are too high (higher than 55 °C) will lead to a decrease in the final CA conversion, which is the result of higher lipase deactivation rates at higher temperatures. The other probable reason for the lower final CA conversion at higher temperature is that the equilibrium shifted to the left because of the fact that the reaction was endothermic.

The initial reaction rates, defined as the initial CA conversion per unit time (v_0), were calculated from six

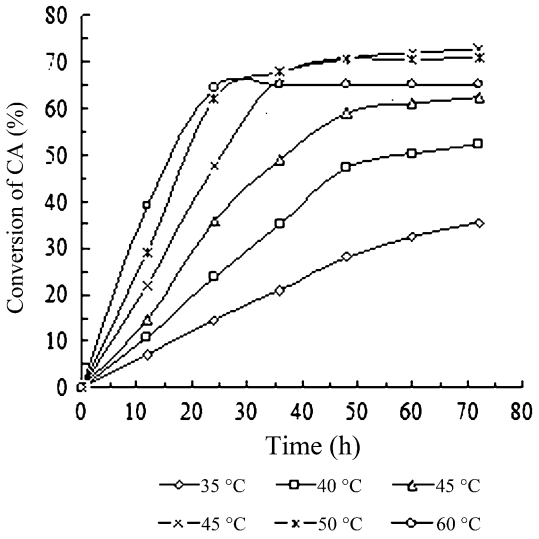

Fig. 1 Effect of reaction temperature on the conversion of citric acid during the reaction. CA concentration 0.12 mol/L, molecular-sieve 120 g/L, molar ratio of MG/CA 2:1, and enzyme load 9.0 %, shaking speed 210 rpm

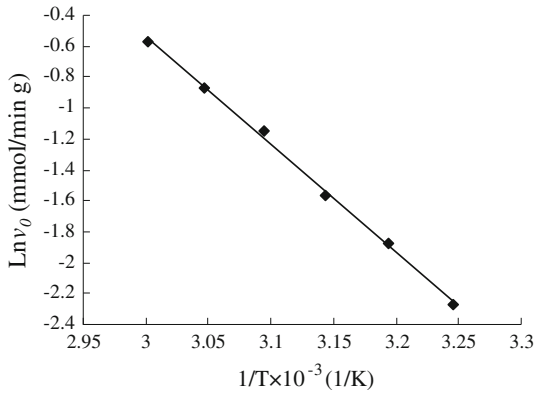

Fig. 2 Arrhenius plots for the estimation of the thermodynamic parameters. CA concentration 0.12 mol/L, molecular-sieve 120 g/L, molar ratio of MG/CA 2:1, and shaking speed 210 rpm

experimental points of the CA conversion-time profile corresponding to the first 12 h of the reaction, where the profiles were found to be approximately linear. The Arrhenius plot of the resulting data is shown in Fig. 2. The activation energy (E_a) was calculated from the slope by plotting $\ln v_0$ versus $1/T$, which was 57.87 kJ/mol ($R^2 = 0.9982$). The higher activation energy can be attributed to the CA has electron-donating and steric hindrance effects, which lead to a lower reaction rate than other lipase-catalyzed esterification reactions [20].

The Effect of Shaking Speed

The shaking speed was varied between 50 and 250 rpm to study the external mass transfer resistance. As shown in

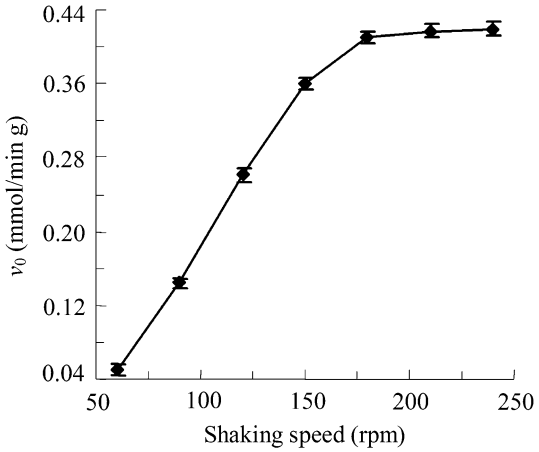

Fig. 3 Effect of shaking speed on the initial rate. CA concentration 0.12 mol/L, molecular-sieve 120 g/L, molar ratio of MG/CA 2:1, temperature 54 °C, and enzyme load 10 mg/L

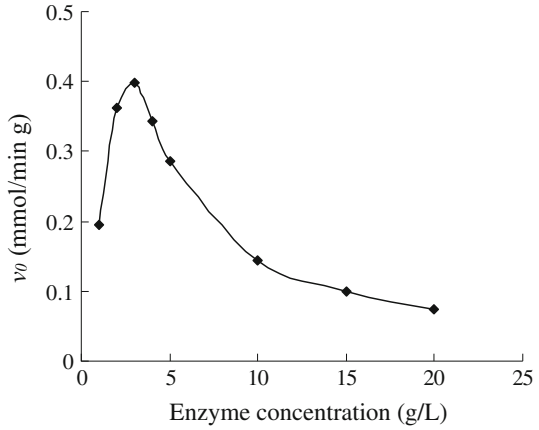

Fig. 4 Effect of enzyme concentration on the initial rate. CA concentration 0.12 mol/L, molecular-sieve 120 g/L, molar ratio of MG/CA 2:1, temperature 54 °C, shaking speed 210 rpm

Fig. 3, the initial reaction rate rose with the shaking rate up to 180 rpm and remained constant at higher shaking speeds. The profile can be related to the presence of external mass transfer limitations at lower speeds, and the mass transfer limitations was eliminated at higher shaking speeds in the reaction. Transfer of reactants between the organic phase and the biocatalyst surface is a function of the interfacial area, dependent on the shaking speed.

The Effect of Enzyme Load

As shown in Fig. 4, the initial reaction rate was drastically raised by increasing the enzyme load (<3 g/L). The relationship between the initial reaction rate and enzyme load (<3 g/L) showed linear behavior, which indicates that it was a kinetically controlled enzymatic reaction without mass transfer limitations. The initial reaction rate was

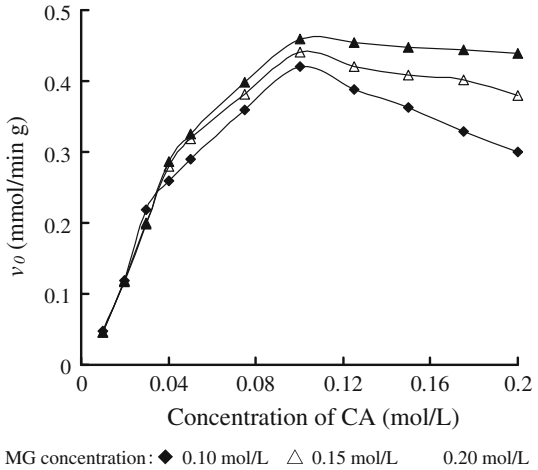

Fig. 5 Effect of substrate concentration on the initial rate. CA concentration 0.12 mol/L, molecular-sieve 120 g/L, enzyme load 10 mg/L, temperature 54 °C, shaking speed 210 rpm

drastically reduced with increasing enzyme load (>3 g/L), which was probably caused by saturation with enzyme. Similar reaction characteristics have been reported in other lipase-catalyzed esterification reactions [20, 21].

The Effect of Substrate Concentration

The influence of CA concentration on the initial reaction rate was investigated at three fixed MG molecular concentration levels. The reaction rate increased with the increase in MG molecular concentration. The reaction rate showed increasing–decreasing patterns with increasing CA molecular concentration at the three fixed MG molecular concentration levels (Fig. 5). The maximum initial reaction rate was obtained at a CA concentration of 0.10 mol/L. The decrease in the initial reaction rate with increasing CA concentrations at higher concentration (>0.10 mol/L) can be assumed to be caused by an excess of CA inhibiting the catalytic function of the enzyme. Higher CA concentration could have caused higher acidity of the reaction mixture, which inhibited the catalytic activity of the enzyme; the other probable reason was the enzyme inhibiting effect of electron donation [22] and the competitive inhibition of the hydroxyl of CA [23].

Kinetic Model

Esterification of fatty acids with alcohols catalyzed by a lipase is often modeled using the Ping Pong Bi–Bi kinetic mechanism with inhibition by substrates [20, 21, 24, 25]. From the above results, it is shown that the enzyme was inhibited by CA at higher CA concentrations. The expression for the initial reaction rate in the case of this

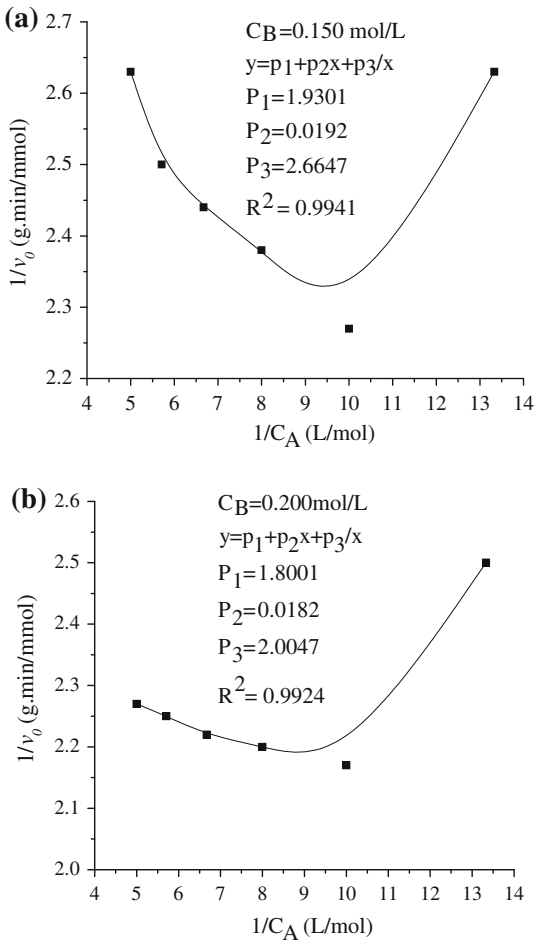

Fig. 6 Lineweaver–Burk plots **a** MG concentration 0.150 mol/L, CA concentration 0.12 mol/L, molecular-sieve 120 g/L, enzyme load 10 mg/L, temperature 54 °C, shaking speed 210 rpm. **b** MG concentration 0.200 mol/L, CA concentration 0.12 mol/L, molecular-sieve 120 g/L, enzyme load 10 mg/L, temperature 54 °C, shaking speed 210 rpm

study, following the acid inhibition Ping-Pong Bi–Bi kinetic mechanism is proposed as:

$$v_0 = \frac{V_{\max} C_A C_B}{C_A C_B + K_A C_B + K_B C_A \left[1 + \frac{C_A}{K_{iA}}\right]} \quad (1)$$

where v_0 is the initial reaction rate, $V_{\max}$ is the maximum reaction rate, C_A and C_B are the concentrations of CA and MG, K_A and K_B are the Ping-Pong constants for CA and MG, K_{iA} is the inhibition constant for CA [26]. The concentration of MG was regarded as a constant. The initial reaction rate equation can be expressed simply as:

$$\frac{1}{v_0} = \frac{1}{V_{\max}}\left(1 + \frac{K_B}{C_B}\right) + \frac{K_A}{V_{\max} C_A} + \frac{K_B C_A}{V_{\max} C_B K_{iA}} \quad (2)$$

Set $1/v_0$, $1/C_A$, $\frac{1}{V_{\max}}(1 + \frac{K_B}{C_B})$, $\frac{K_A}{V_{\max}}$, and $\frac{K_B}{V_{\max} C_B K_{iA}}$ as y, x, p_1, p_2, and p_3, respectively. The equation becomes

Table 1 Modeled kinetic constants

Parameters	Values
V_{max} (mmol/min g)	0.7092 ± 0.0016
K_B (mol/L)	0.0553 ± 0.00024
K_A (mol/L)	0.0133 ± 0.00011
K_{iA} (mol/L)	0.1948 ± 0.00042

V_{max} is the maximum reaction rate, K_A and K_B is the Ping-Pong constants for CA and MG, K_{iA} is the inhibition constant for CA

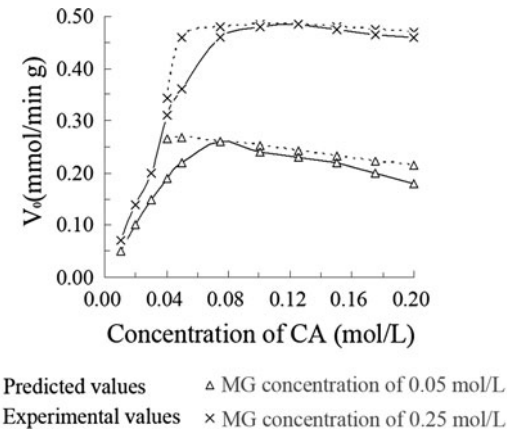

Fig. 7 Validation of the dynamic model as established for the esterification reaction, molecular-sieve 120 g/L, enzyme load 10 mg/L, temperature 54 °C, and shaking speed 210 rpm

$$y = p_1 + p_2 x + p_3/x \tag{3}$$

The Lineweaver–Burk plot of the reciprocal initial rate versus the reciprocal concentration of CA shows good agreement between experimental and modeled data with two different MG concentrations (0.150 and 0.200 mol/L, respectively) (Fig. 6). The non-linear appearance of the experimental data in the Lineweaver–Burk plot is further corroboration of the hypothesis that the enzyme was inhibited by CA. A similar phenomenon has also been detected in other reactions [27].

The kinetic parameters (Table 1) were calculated from the values of p_1, p_2, and p_3 (Fig. 6). The corresponding equation with the MG concentration ranged from 0.15 to 0.20 mol/L was as follows:

$$v_0 = \frac{0.7092 C_A C_B}{C_A C_B + 0.0136 C_B + 0.0553 C_A [1 + \frac{C_A}{0.1948}]} \tag{4}$$

To verify the kinetic model of enzymatically catalyzed esterification (Eq. 4), it was used to simulate the reaction process with the MG concentrations fixed at either 0.05 or 0.25 mol/L. As shown in Fig. 7, the values calculated from the kinetic mode agreed well with the experimental rate data under the different MG and CA concentrations. The experimental rate values were smaller than the simulated values at higher CA concentrations, which was probably caused by the inhibition of CA.

Conclusion

The esterification of citric acid (CA) and monoglycerides (MGs) for citrate esters of monoacylglycerols (CITREM) catalyzed by Novozym 435 in a *tert*-butyl alcohol system was studied. The relationship between initial reaction rate and temperature was established, based on the Arrhenius law. The activation energy (E_a) was 57.87 kJ/mol. Analyses of the kinetic data indicated that the esterification of the reaction can be described by a Ping-Pong Bi–Bi mechanism with citric acid inhibition. This model was used to simulate the kinetic experimental data and the initial reaction rates with good results.

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