Lipase-Catalyzed Esterification of Oleic Acid and Methanol in Hexane—A Kinetic Study

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The kinetics of immobilized lipase-catalyzed esterification of oleic acid and methanol in hexane were investigated. The reaction follows Michaelis–Menton kinetics as observed from the relationship of initial rate of the reaction, both as a function of enzyme and of substrate concentration. Inhibition by excess of methanol has been identified. The kinetic constants have been measured for the reaction in the absence of any significant external diffusional limitations. The kinetics of the enzymatic reaction are suggested to agree with a Ping-Pong Bi Bi mechanism.

KEY WORDS: Ester synthesis, esterification, immobilized lipase, kinetics, oleic acid, Ping-Pong Bi Bi mechanism.

Lipases (glycerol acyl hydrolases EC 3.1.1.3) are hydrolytic enzymes that break down triacylglycerols into free fatty acids and glycerol and exhibit maximum activity at the oil–water interface (1). They constitute a ubiquitous group of enzymes that does not require a co-factor for its catalytic activity. Under low water conditions, the hydrolysis reaction is reversible, i.e., the ester bond is synthesized rather than hydrolyzed. The ability of extracellular microbial lipases to remain catalytically active in predominantly organic reaction systems that contain very small amounts of water is now well known (2). There is an increasing interest in the development of lipase applications to the production of tailor-made oils and fats through interesterification reactions and to production of esters for natural flavors, fragrances and waxes (3).

We have optimized the immobilized lipase-catalyzed esterification of fatty acids and methanol (4). The lipase-catalyzed esterification reaction was then applied to recover sterols and tocopherols from vegetable oil deodorizer distillates (5). Information regarding the kinetics of the reaction is essential for understanding the reaction mechanism, as well as for rational design of esterification reactors for future scale-up. In this study we have examined the kinetics of the esterification of oleic acid and methanol in hexane, catalyzed by the nonspecific lipase from *Candida antarctica*. The reaction studied is:

$$\text{oleic acid} + \text{methanol} \xrightarrow{\text{lipase}} \text{methyl oleate} + \text{water} \quad [1]$$

MATERIALS AND METHODS

Enzyme and substrate. Randozyme SP-435 is a nonspecific lipase (40BIU; Novo Industri A/S, Copenhagan, Denmark) derived from a selected strain of *C. antarctica* fungus, immobilized on a macroporous acrylic resin. The immobilized lipase as received was dried under vacuum at 40°C for 48 h and stored over P_2O_5 in a desiccator for 48 h. The dried immobilized lipase was then placed in a sintered crucible over a saturated salt solution of magnesium chloride ($a_w = 0.334$) at 25°C in a mason jar, which was tightly sealed. Usually, 72 h was found to be sufficient for equilibration, and the moisture content was 0.75% (dry basis). Oleic acid (>99%) was obtained from Sigma Chemical Co. (St. Louis, MO). Methanol used for the reaction was Omnisolv-grade and contained 0.05% water.

Esterification reaction. The reactions were carried out at 25°C in a Gyrotory shaker (New Brunswick Scientific Company, New Brunswick, NJ) fitted with a thermostat. To study the kinetics of the reaction in hexane, appropriate concentrations of oleic acid in hexane were prepared, and 2.8 mL of the solution was added to a 4-mL septum-covered screw-cap reaction vial, along with the immobilized lipase. Methanol was added to the vials to initiate the reactions. Total volume in the reaction vial was maintained at 3 mL by adding required hexane. The reaction vials were fit into a styrofoam cup and placed horizontally on the mobile platform of the gyrotory shaker. Such an arrangement resulted in intimate mixing of the components. Samples (10–20 μL) were drawn with a 50-μL syringe at specific intervals of time and derivatized for subsequent gas-chromatographic analysis.

Lipase activity was estimated by measuring the initial rate of the reaction to avoid possible complications arising from product inhibition, occurrence of the reverse reaction and depletion of the substrate. The initial rates were estimated from the slope of plots of methyl oleate produced *vs.* time and reported as $\mu mol \cdot min^{-1} \cdot g^{-1}$ of immobilized lipase.

Gas-chromatographic analysis. Methyl oleate produced during the course of the reaction was quantitated by a gas-chromatographic method as described previously (5). Samples drawn were derivatized with a 0.3–0.5-mL solution of Sylon BFT (Supelco Canada, Oakville, Ontario, Canada) and pyridine (1:7, vol/vol), heated at 105°C for 10 min. After cooling to room temperature, 1–2 μL was injected into the gas chromatograph. The number of moles of methyl oleate produced were calculated by converting the gas chromatography results from weight % to mole %.

RESULTS AND DISCUSSION

Figure 1 shows the relationship of the initial rate of esterification of oleic acid and methanol in hexane as a function of enzyme concentration for different substrate concentrations. The linearity of the relationship was established for a wide range of substrate concentrations (ranging from 8.3 to 800 mM oleic acid). The linear relationship is indicative of a kinetically controlled enzymatic reaction, and the system behaves like a homogeneous reaction system. This relationship holds when there are no limiting factors, such as a low substrate concentration, presence of activators or inhibitors or mass-transfer effects. The effect of oleic acid concentration on the initial rate of the reaction is shown in Figure 2. Such a substrate-saturation curve is typical of enzymes that follow Michaelis–Menton kinetics (6).

The immobilized lipase was stable when stored over the saturated salt solution in a sealed jar for over three months. Incubation in hexane for 4 h, which was the solvent of choice for the kinetic study, also did not have any

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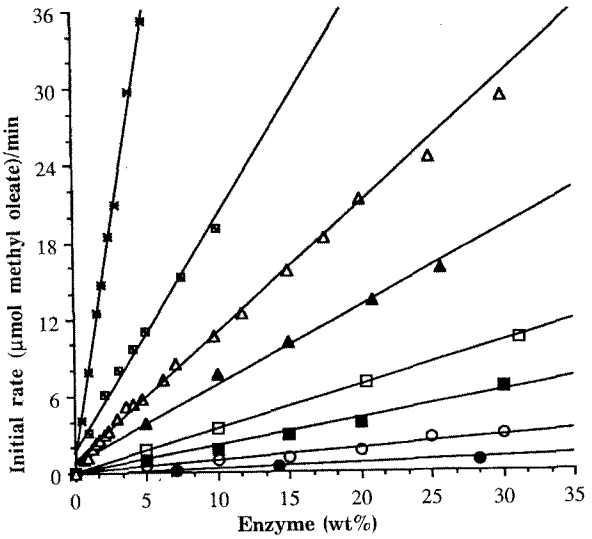


FIG. 1. Effect of enzyme concentration on the initial rate of esterification of oleic acid and methanol in hexane, catalyzed by the immobilized lipase Randozyme SP-435. The reactions were carried out at 25°C in 4-mL reaction vials that contained equimolar concentrations of oleic acid and methanol in 3 mL hexane, along with different amounts of the immobilized lipase. Amounts of oleic acid (mM): ●, 8.3; ○, 16.6; ■, 33.4; □, 66.7; ▲, 133.3; △, 200; ⊡, 400; ✖, 800.

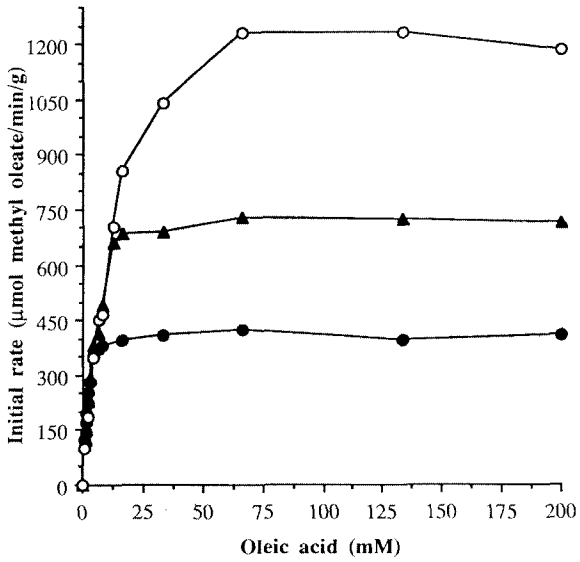


FIG. 2. Effect of oleic acid concentration on the initial rate of esterification of oleic acid and methanol in hexane. The reactions were carried out at 25°C in 4-mL reaction vials that contained different concentrations of oleic acid and 1.7 (●), 3.3 (▲) or 6.7 (○) mM methanol in 3 mL hexane, along with 4 mg of the immobilized lipase.

deleterious effect on the lipase activity. Selwyn's test (7) was performed to check the stability of the enzyme during the assay. Methyl oleate produced for varying enzyme concentrations at a fixed oleic acid concentration were estimated during the course of the reaction, and the relationship of $e_o . t = F(p)$ was tested, where e_o is the enzyme concentration, t the time and p is the methyl oleate produced at time t. A superimposable plot was obtained (not shown here) when the methyl oleate produced in the different reactions was plotted against enzyme concentration multiplied by time. The results indicate that the rate throughout the reaction period is proportional to the total enzyme concentration and that the immobilized lipase is not denatured during the assay.

The kinetics of the reaction were investigated by studying the effect of the concentration of both oleic acid and methanol on the initial rate of the reaction. Figure 3 shows the effect of methanol concentration on the initial rate of the reaction for fixed oleic acid concentrations. For a fixed concentration of oleic acid, the initial rate of the reaction increased with an increase in the concentration of methanol until a critical concentration of methanol was used. An increase in the concentration of methanol above the critical value, which is specific for the fixed concentration of oleic acid, resulted in a drop in the initial rate. A further decrease in the initial rate was observed with an increase in the concentration of methanol until a zero initial rate was reached at all concentrations of oleic acids (not shown here). It is known that hydrophilic solvents can strip water essential for catalytic activity of enzymes (8). To compensate for any loss in water from the immobilized lipase, reactions were carried out with higher initial moisture content (from 1 to 20%). Such an increase in moisture content of the enzyme did not have any significant effect on the initial rate of the reaction at high inhibiting concentrations of methanol. Therefore, it is possible that methanol is a substrate inhibitor for the lipase-catalyzed esterification. A typical upward curvature was obtained for the Lineweaver-Burk plot of the reciprocal initial rate *vs.* reciprocal concentration of methanol (not shown here). For high concentrations of methanol, especially when the concentration of oleic acid is low, the curve did go sharply upwards, indicating high substrate inhibition by methanol, competitive with oleic acid.

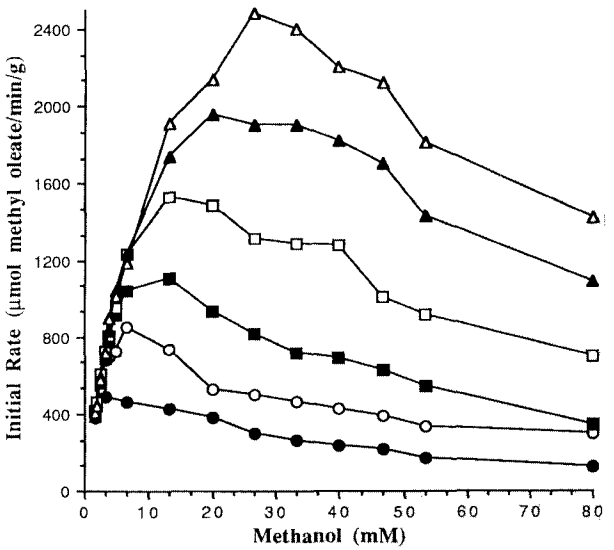


FIG. 3. Effect of methanol concentration on the initial rate of esterification of oleic acid and methanol in hexane. The reaction system (at 25°C) consisted of different fixed concentrations of oleic acid and different concentrations of methanol in 3 mL hexane, along with 4 mg of the immobilized lipase. Key as in Figure 1.

For a fixed methanol concentration, the initial rate increased with an increase in oleic acid concentration, and no evidence of inhibition by oleic acid was found at all concentrations of methanol (Fig. 4). This was also true at higher fixed concentrations of methanol (up to 400 mM tested), that are not presented here. The Lineweaver-Burk (1/initial rate vs. 1/[oleic], double reciprocal) plots are shown in Figure 5. The lines are parallel at low fixed concentrations of methanol (0.8 and 1.7 mM). Reciprocal plots in which the slopes are unchanged, regardless of the concentration of the second substrate, are consistent with a mechanism in which the reaction proceeds through a modified form of the enzyme and a series of binary complexes. As the fixed methanol concentration was increased, the slopes of the lines increase with a decrease in 1/v axis intercept to a limit of 1/Vmax.

A straight line was obtained when the slopes of the double reciprocal plots were plotted against the methanol concentration (Fig. 6), characteristic of high substrate inhibition in two-step transfer reactions. These results are characteristic of the Ping-Pong Bi Bi mechanism with dead-end inhibition by methanol and are in agreement with the results for lipase-catalyzed esterification reported by Chulalaksananukul *et al.* (9).

The reaction scheme for the lipase-catalyzed esterification of oleic acid and methanol in hexane is shown in Scheme 1. (L, immobilized lipase; O, oleic acid; M, methanol; L-O, lipase-oleic complex; l, modified lipase; MO, methyl oleate; L-M, lipase-methanol dead-end complex; W, water; l-W, modified lipase-water complex; l-M, modified lipase-methanol complex; L-MO, lipase-methyl oleate complex; k_i, inhibitor constant for methanol.) The reaction sequence follows a Ping-Pong Bi Bi mechanism in which one of the substrates forms a dead-end complex with the free lipase. The lipase initially forms a noncovalent lipase-oleic acid complex, which then subsequently

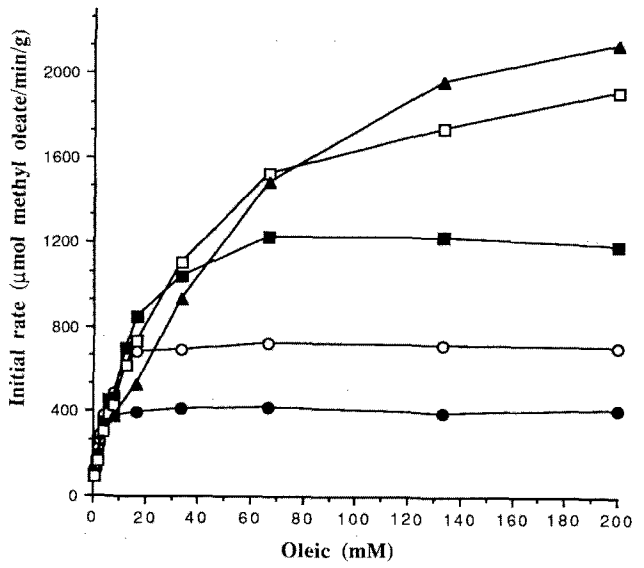


FIG. 4. Effect of oleic acid concentration on the initial rate of esterification of oleic acid and methanol in hexane. The reaction system (at 25°C) consisted of different fixed concentrations of methanol and different concentrations of oleic acid in 3 mL hexane, along with 4 mg of the immobilized lipase. Amounts of methanol (mM): ●, 1.7; ○, 3.3; ■, 6.7; □, 13.3; ▲, 20.0.

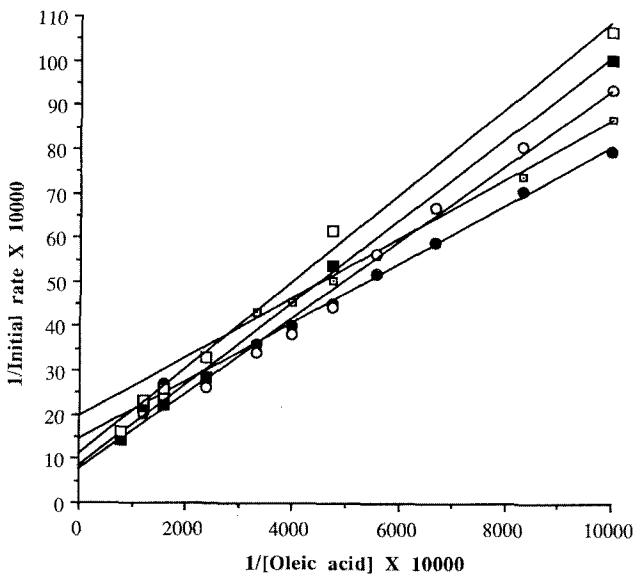


FIG. 5. Double-reciprocal plot of the initial rate of esterification at varying oleic acid concentrations. The reactions were carried out at 25°C with different concentrations of the substrates and 4 mg of the immobilized lipase. Amounts of methanol (mM): ⊡, 0.8; ●, 1.7; ○, 3.3; ■, 6.7; □, 13.3.

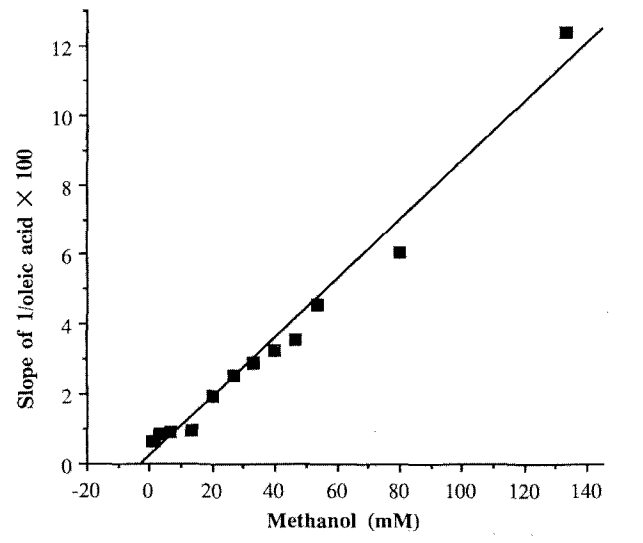
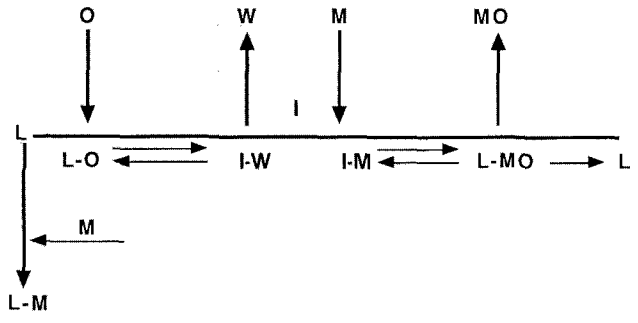


FIG. 6. Slope of 1/[oleic acid] vs. methanol concentration (slopes were estimated from Fig. 5).



SCHEME 1

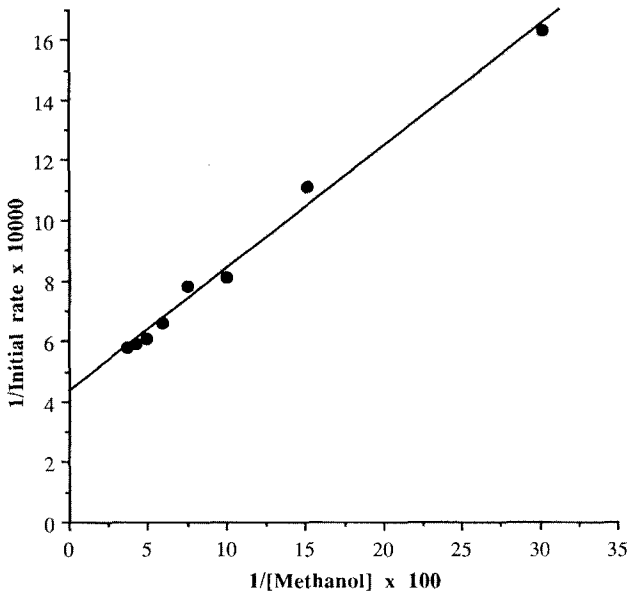


FIG. 7. The reciprocal of the initial rate of the esterification reaction vs. the reciprocal of methanol concentration. The reactions were carried out at 25°C in 3 mL hexane containing different concentrations of oleic acid and methanol, along with 4 mg of the immobilized lipase. The concentration of the oleic acid was five times the concentration of methanol.

transforms by a unimolecular isomerization reaction to the acyl-enzyme intermediate with the concomitant release of water. The lipase may also combine with methanol to form the lipase–methanol dead-end complex. The reason for the formation of a dead-end complex is that the modified lipase, which is the acyl-enzyme complex formed after the release of water in the first step of the reaction, may be structurally similar to the free lipase (10). Thus, it is possible that some of the free lipase could interact with methanol to form the dead-end complex. The modified lipase now reacts with methanol to form the modified lipase–methanol complex, which is also isomerized by a unimolecular reaction to a lipase–methyl oleate complex, which then yields the product methyl oleate and the free lipase.

The rate equation for the reaction by the nomenclature of Cleland (11) is then given by:

$$\frac{v}{V_{max}} = \frac{[O] \cdot [M]}{K_{m(O)} \cdot [M] \cdot \{1 + [M]/K_i\} + K_{m(M)} \cdot [O] + [O]\,[M]} \qquad [2]$$

where $[O]$ and $[M]$ are the concentrations of oleic acid and methanol, V_{max} is the maximum rate of the reaction, $K_{m(O)}$ and $K_{m(M)}$ are the Michaelis constants for oleic acid and methanol, and K_i is the inhibitor constant of methanol.

The values of V_{max}, $K_{m(O)}$, $K_{m(M)}$ and K_i were computed by nonlinear regression by means of the Sigma plot software on 281 experimental points to accurately estimate the constants. These values were 4.9 mmol · min^{-1} · g^{-1}, 13.3, 16.4 and 3.0 mM, respectively.

An additional plotting technique for confirmation of the Ping-Pong mechanism utilizes different concentrations of oleic acid and methanol in a constant ratio below the concentrations of methanol where substrate inhibition occurs (12). By substituting for $[O] = y \cdot [M]$ in Equation 2, where y is a constant, and by rearranging the equation we get:

$$1/v = 1/(V_{max} \cdot [M]) \cdot \{K_{m(O)}/y + K_{m(M)}\} + 1/V_{max} \cdot \{K_{m(O)}/(y \cdot K_i\} + 1\} \qquad [3]$$

Competitive substrate inhibition does not show as nonlinear reciprocal plots if both substrates are varied together in a constant ratio. Concentrations of oleic acid and methanol were chosen after carefully analyzing the rate curves obtained at different substrate concentrations. The ratio of the concentration of oleic acid to methanol was taken as five, and at all concentrations of oleic acid it was ensured that there was no inhibitory effect of methanol. A linear relationship was obtained for the reciprocal rate of the reaction and reciprocal methanol concentration (Fig. 7), as predicted by Equation 3.

In conclusion, we have studied the kinetics of immobilized lipase-catalyzed esterification of oleic acid and methanol in hexane. It is suggested that the reaction follows a Ping-Pong Bi Bi mechanism in which one of the substrates, methanol, is a substrate inhibitor.

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