Kinetic Study of Lipase Catalyzed Esterification Reactions in Water-in-Oil Microemulsions

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The kinetics of the esterification of lauric acid by (-)menthol, catalyzed by *Penicillium simplicissimum* lipase, was studied in water/bis-(2-ethylhexyl)sulfosuccinate sodium salt (AOT)/isooctane microemulsions. Due to their low water content, microemulsions assist in reversing the direction of lipase activity, favoring synthetic reactions. The kinetics of this synthesis follows a Ping-Pong Bi-Bi mechanism. The values of all apparent kinetic parameters were determined. The theoretical model for the expression of enzymic activity in reverse micelles, proposed by Verhaert et al. (Verhaert, R., Hilhorst, R., Vermüe, M., Schaafsma, T.J., Veeger, C. 1990. Eur. J. Biochem. 187: 59–72) was extended to express the lipase activity in an esterification reaction involving two hydrophobic substrates in microemulsion systems. The model takes into account the partitioning of the substrates between the various phases and allows the calculation of the intrinsic kinetic constants. The experimental results showing the dependence of the initial velocity on the hydration ratio, $w_o = [H_2O]/[AOT]$, of the reverse micelles, were in accordance with the theoretically predicted pattern.
Key words: lipase • microemulsions • reverse micelles • esterification • kinetics

INTRODUCTION

It is well established that many enzymes can be hosted in water-in-oil microemulsions or reverse micelles, retaining their catalytic activity.[18,23] The growing interest in this new area arises from the potential applications of these systems. Among the enzymes studied to date, lipases are most attractive due to their numerous biotechnological applications.[20]

Lipase (triacylglycerol acylhydrolase EC 3.1.1.3), under specific conditions, can catalyze a variety of synthetic reactions[11] involving hydrophobic substrates. These reactions are heterogeneous and take place at oil/water interfaces. To facilitate the reversal of the normal hydrolytic action of lipases, different approaches have been proposed, including the use of immobilized enzymes,[2] anhydrous organic solvents,[37] supercritical gases,[26] and reverse micelles.[3,6,9] In the case of reverse micelles, it has been shown that lipases in microemulsions can be successfully used to catalyze esterification reactions.[14,36] These fine dispersions of water in an organic medium, stabilized by amphiphilic molecules, present an interface through which the hydrophobic substrates are converted. The high reaction rates and conversion yields obtained with the use of microemulsions make these systems advantageous as compared with other heterogeneous media used for the same purpose.[29,30]

In this work a kinetic study of a typical esterification reaction, catalyzed by a lipase in microemulsion systems was undertaken to elucidate the mechanism and to determine the kinetic constants. The reaction studied was the following:

lauric acid + (-)menthol ⟶ (-)menthyl laurate + water

The lipase used was from *Penicillium simplicissimum* and the reaction was carried out in a AOT/isooctane/water microemulsion system.

To date, there are no extensive kinetic studies on lipase catalyzed esterification reactions involving two hydrophobic substrates in microemulsion systems.

MATERIALS AND METHODS

Materials

Lipase from *P. simplicissimum* was purified and characterized according to Sztajer et al.[31] The enzyme showed a single band in native and SDS-PAGE, and exhibited a specific activity of 142 units/mg of protein determined by titration of the free fatty acids released from triolein.

Bis-(2-ethylhexyl)sulfosuccinate sodium salt (AOT) was purchased from Sigma Chemical Co. Ninety-nine percent pure (-)menthol and isooctane were purchased from Merck, Germany. Lauric acid was a product of Sigma. All other reagents used were of the highest commercially available purity. Doubly distilled water was used throughout this study.

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Preparation of Microemulsions

Microemulsions were prepared by adding appropriate quantities of alcohol and fatty acid, in an isooctane solution containing 100 m*M* AOT. In this mixture, a dilute solution of lipase in 50 m*M* sodium acetate buffer (pH 5.0) was then added to give a final enzyme concentration of 0.05 mg/mL. The final water content, depending on the desired hydration ratio, was adjusted by addition of the required amount of buffer. A clear solution was obtained after gentle shaking for a few seconds. It was assumed that the consumption of the substrates during the reaction did not alter the microemulsion system. The water content of the AOT/isooctane solutions was controlled periodically after measuring it with a Metrohm Karl-Fischer titrator.

Activity Measurements

The reaction of the esterification of lauric acid and (-)menthol was carried out in 1 mL total volume of AOT/isooctane microemulsion. The reaction was performed at a temperature of 35°C, with a molar hydration ratio, $w_o = [H_2O]/[AOT] = 6$, while the pH of the dispersed aqueous phase was fixed at 5.0. These reaction conditions correspond to the optimum values of the parameters affecting the *P. simplicissimum* lipase catalytic activity for this type of reaction (unpublished results).

Initial reaction rates were determined in capped vials placed in a thermostatted bath at 35°C. Aliquots were withdrawn at selected time intervals and analyzed for fatty acid content. The rate of esterification was determined spectrophotometrically, by a procedure based on the Lowry and Tinsley[16] and Han and Rhee,[5] with minor modifications. The depletion of fatty acid was typically monitored as follows: 0.1-mL samples from the reaction mixture were added to screw-cap test tubes containing 4.7 mL of isooctane, 0.2 mL chloroform, and 1 mL of cupric acetate in pyridine (5% w/v, pH 6.0). After centrifugation at 2500 rpm for 1 min, the free fatty acids present in the upper organic phase could be determined by measuring the absorbance at 715 nm. Curves of product concentration as a function of time over several minutes, were linear, thus allowing a reliable determination of the initial slopes, by linear regression calculations.

Determination of Kinetic Parameters

The determination of the kinetic parameters of the esterification reaction of (-)menthol and lauric acid was performed by initial velocity analysis. (-)Menthol concentration was varied at different fixed concentrations of lauric acid. The concentrations of (-)menthol and lauric acid were varied over ranges of 0.8 to 4.2 and 0.2 to 2.2, respectively, times the observed values of the Michaelis constants. All kinetic measurements were carried out in duplicate.

RESULTS AND DISCUSSION

The effect of enzyme concentration on the esterification rate is shown in Figure 1. The linearity observed is consistent with a kinetically controlled enzymatic reaction; e.g., the enzyme is continuously fed with the substrates through the surfactant layer.

Figure 2 shows the variation of the initial velocity of the esterification reaction as a function of menthol concentration for various concentrations of lauric acid. As can be seen from the hyperbolic curves, the reaction follows Michaelis–Menten-type kinetics, further confirming that the enzymatic reaction is kinetically controlled.

Figure 3 shows the dependence of the initial rate of the esterification on the fatty acid concentration, for various (-)menthol concentrations tested. It can be seen that, when the concentration of lauric acid increases, the initial velocity increases up to an acid concentration of 37 m*M*. However, at higher lauric acid concentrations a decrease of the initial velocity for all (-)menthol concentrations tested was observed. The same behavior was observed in the case of the esterification of (-)menthol by other fatty acids, such as myristic acid, in the same microemulsion system (Fig. 4).

The decrease of lipase activity in the presence of high fatty acid concentrations could be related to a possible instability of the enzyme in the microemulsion system used. However, when the lipase stability was tested, it was found that it is not affected within the time interval of our measurements (at least 15 min). Furthermore, incubation of the enzyme in the AOT microemulsion at 35 °C, with previous addition of either menthol or lauric acid, before the second substrate is added, considerably increases enzyme stability for longer time periods (data not shown). Also, a possible effect of the change in the pH value, occurring due to the high lauric acid quantities, should be ruled out, because the reaction proved to be rather insensitive to relatively small pH variations. A 1-unit pH shift caused only a 4% decrease of the enzyme activity (results not shown). A plausible interpretation for the inhibitory effect of the fatty acid on the esterification might be the amphiphilic character of these

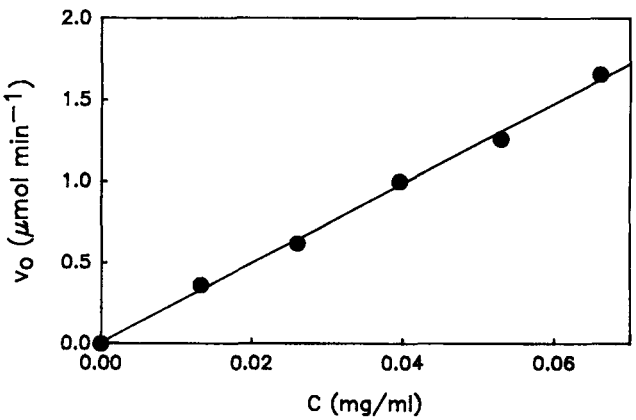


Figure 1. Initial velocity of esterification of 100 m*M* lauric acid by 50 m*M* of (-)menthol, as a function of enzyme concentration. The experimental conditions are described in Materials and Methods.

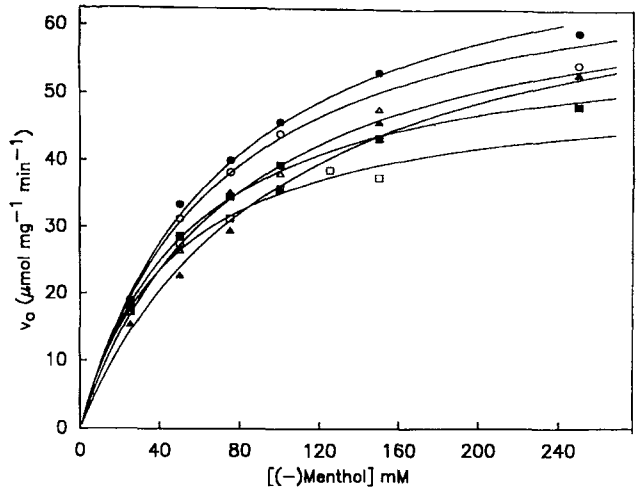


Figure 2. Initial velocity of esterification as a function of (-)menthol concentration at the following fixed lauric acid concentrations: (□): 15.2 mM; (■): 21.0 mM; (○): 31.5 mM; (●): 36.8 mM; (△): 43.8 mM; (▲): 75.0 mM. The experimental conditions are described in Materials and Methods.

molecules, which may result in their partitioning between the micellar interface and the bulk organic solvent.[34] The presence of high amounts of fatty acids within the micellar interface could influence the structure and rigidity of the surfactant layer,[7] thus modifying the partitioning of the other substrate. In our case, (-)menthol could be displaced from the vicinity of the interface, because this alcohol is not a cosurfactant. A similar inhibitory effect at high fatty acid concentrations was recently reported[8] for the esterification of diols in reverse micelles.

Determination of Apparent Kinetic Parameters

The double reciprocal plot of the initial velocity vs. reciprocal menthol concentrations at several lauric acid concentrations is shown in Figure 5. A set of parallel lines was obtained, indicating a Ping-Pong Bi-Bi mechanism.[28] A similar mechanism has been also proposed for lipase

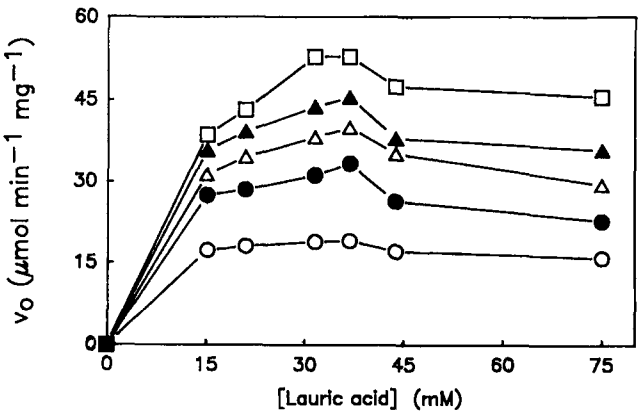


Figure 3. Initial velocity of esterification as a function of lauric acid concentration at the following fixed menthol concentrations: (○): 25 mM; (●): 50 mM; (△): 75 mM; (▲): 100 mM; (□): 150 mM. The experimental conditions are described in Materials and Methods.

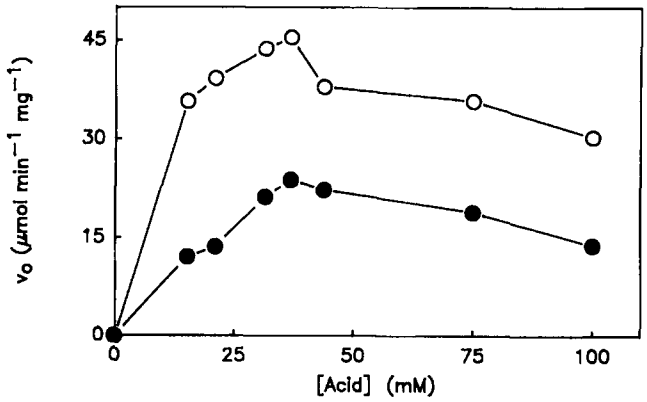


Figure 4. Dependence of the initial velocity of esterification of 100 mM (-)menthol on the concentration of lauric acid (○) and myristic acid (●). The experimental conditions are described in Materials and Methods.

catalyzed esterifications in other nonconventional media, such as esterification of oleic acid and ethanol in *n*-hexane,[2] esterification of dilaurin and lauric acid in cyclohexane,[24] transesterification of tributyrin, and heptanol in hexane.[37] According to this mechanism, lipase reacts with lauric acid to form the lipase–lauric acid complex. The complex is then transformed to a carboxylic–lipase intermediate and water is released. This is followed by an attack of (-)menthol on this intermediate to form the (-)menthyl laurate.

The initial velocity is then given by Eq. (1).

$$V_0 = \frac{V^*_{max}}{1 + \frac{K^*_{M,A}}{[A_{ov}]} + \frac{K^*_{M,B}}{[B_{ov}]}} \qquad (1)$$

where V_o is the initial velocity, V^*_{max} is the maximum velocity, $K^*_{M,A}$ and $K^*_{M,B}$ are the Michaelis constants for lauric

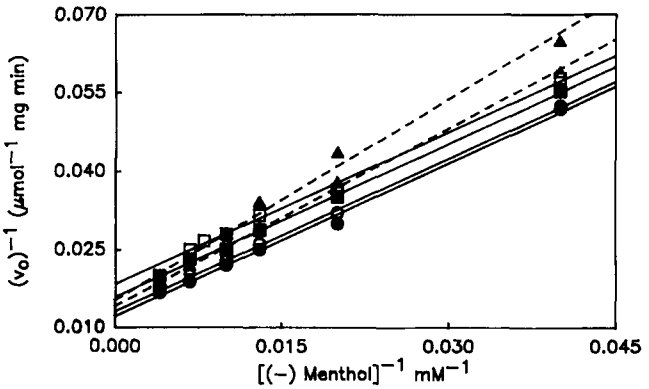


Figure 5. Double reciprocal plot of the initial velocity of esterification vs. the reciprocals of (-)menthol concentrations, at fixed lauric acid concentrations: (□): 15.2 mM; (■): 21.0 mM; (○): 31.5 mM; (●): 36.8 mM; (△): 43.8 mM; (▲): 75.0 mM, respectively. The lines were calculated by a linear regression fit with correlation coefficients >0.993. The values of the slopes were 0.970 (±0.042), 0.978 (±0.018), 0.976 (±0.026), and 0.977 (±0.036) for the four parallel lines (solid lines) corresponding to the first four lauric acid concentrations, and 1.134 (±0.031) and 1.280 (±0.079) for the two higher acid concentrations (dashed lines).

acid and menthol, respectively, and $[A_{ov}]$ and $[B_{ov}]$ the overall lauric acid and (-)menthol concentrations, respectively, based on total organic plus aqueous volumes. The asterisks indicate that these kinetic parameters represent apparent ones, because the reaction does not take place in a homogeneous medium. In the case of the microemulsion system used, the real kinetic constants are calculated by the model discussed below.

When the reciprocal values of $V_{max,app}$, determined from Figure 5, are plotted against the reciprocal concentrations of lauric acid, a straight line is obtained (Fig. 6). From the slope and the ordinate intercept, $K^*_{M,A}$ and V^*_{max} calculated were 19.52 mM and 124.2 μmol mg^{-1} min^{-1}, respectively. Similarly by plotting the reciprocal values of $K_{M,B,app}$ vs. the reciprocal concentrations of lauric acid, $K^*_{M,B}$ value of 124.8 mM can be calculated (Fig. 7).

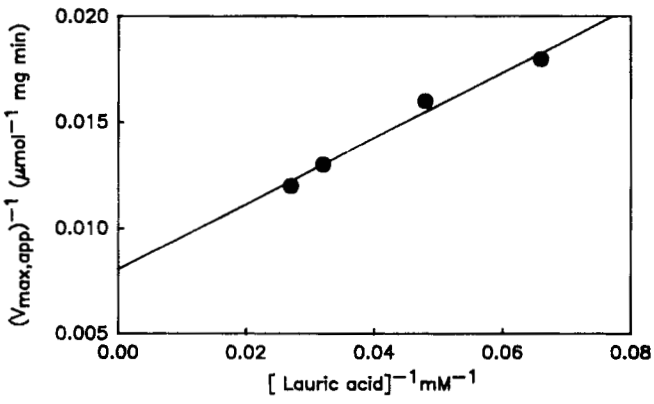


Figure 6. Double reciprocal plot of the apparent maximum velocity determined in Figure 5, as a function of the reciprocals of lauric acid concentrations.

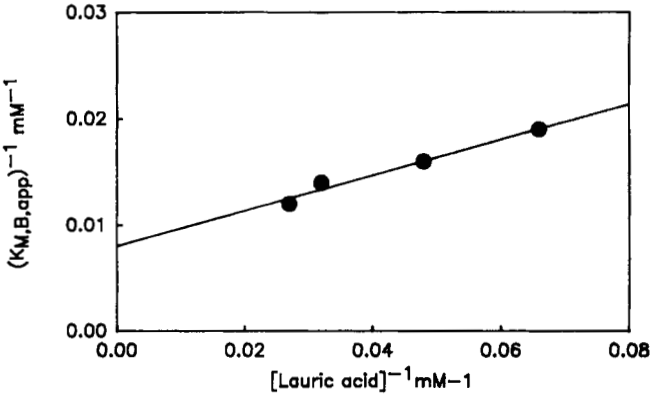


Figure 7. Double reciprocal plot of the apparent Michaelis constant for (-)menthol determined in Figure 5, as a function of the reciprocals of lauric acid concentrations.

Application of a Theoretical Model

A number of theoretical models have been proposed to explain the expression of enzymic activity in microemulsions.[1,12,22,25,32] Most of these models deal with enzymes catalyzing reactions of hydrophilic substrates. The model proposed by Verhaert et al.[32] takes into account the partitioning of the substrates between the different microphases in relation to the microemulsion properties, such as the concentration of reverse micelles, the rate of intermicellar exchange, the hydration ratio w_o, and the volume fraction of water.

In the case of the present study, considering that the substrates diffuse from the oil continuous phase to the interface, where they are incorporated in the reverse micelles and finally bind to the enzyme in the water core of the reverse micelles, the reaction network for this Ping-Pong Bi-Bi mechanism is illustrated in Scheme 1. This model is similar to that proposed by Verhaert et al.,[32] except that it has been extended to involve two hydrophobic substrates. In our case, using the general equation for the kinetic model obeying a Ping-Pong Bi-Bi mechanism and following an approach similar to that of Verhaert et al., the steady-state equation for the initial activity is:

$$V_0 = \frac{V}{1 + \alpha_1 \dfrac{K_{M,A}}{[A_m]} + \alpha_2 \dfrac{K_{M,B}}{[B_m]} + \alpha_3} \qquad (2)$$

where

$$\alpha_1 = 1 + P_A \frac{[A_m]}{[A_{ov}]}$$

$$\alpha_2 = 1 + P_B \frac{[B_m]}{[B_{ov}]}$$

$$\alpha_3 = \frac{V}{[E_{ov}]}\left(\frac{1}{k_i^B[B_{ov}]} + \frac{1}{k_o^Q[Q_M]} + \frac{1}{k_i^A[A_{ov}]} + \frac{1}{k_{ex}\varepsilon_o^P[M]}\right)$$

In Eq. (2), $K_{M,A}$ and $K_{M,B}$ are the real Michaelis constants of lauric acid and menthol, respectively, V is the intrinsic maximum velocity, $[E_{ov}]$ is the enzyme concentration with respect to the overall microemulsion volume, $[M]$ is the molar concentration of reverse micelles, ε_o^P is the efficiency of the exchange of product P out of an enzyme-filled reverse micelle, and P_A and P_B are the partition coefficients of the substrates between the continuous and the dispersed phases, defined as the following:

$$P_A = \frac{k_i^A}{k_o^A}$$

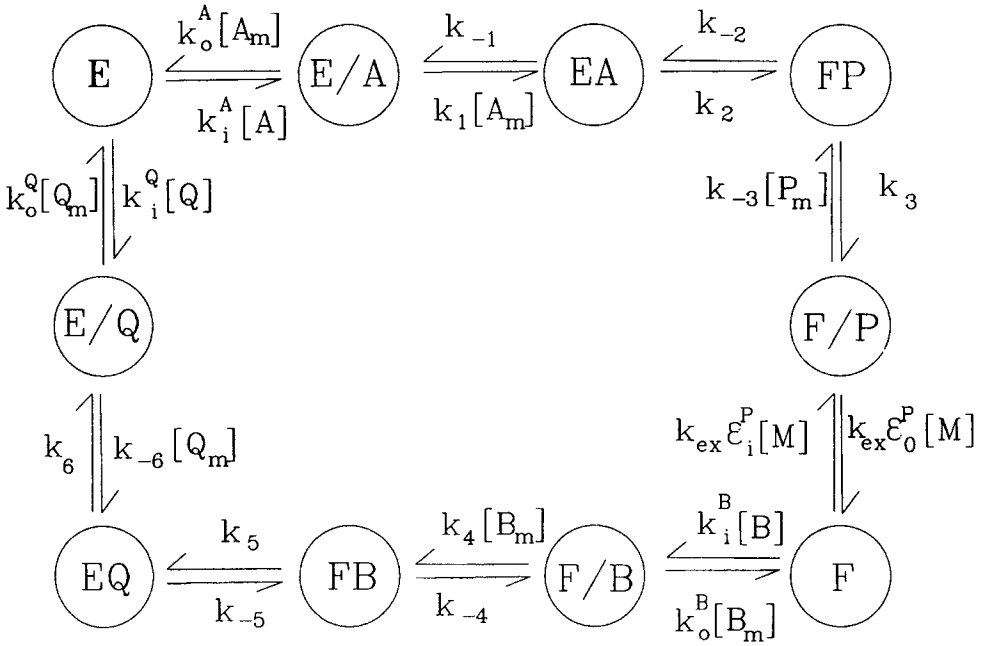
and

$$P_B = \frac{k_i^B}{k_o^B}$$

All other symbols are explained in the legend of Scheme 1.

It is known that the characteristic time for intramicellar diffusion is of the order of 10^{-6} to 10^{-10} s[27] and $k_{ex} \simeq 10^7\ M^{-1}\ s^{-1}$.[33] Thus, in general

$$k_i^A[A_{ov}],\ k_i^B[B_{ov}],\ k_o^Q[Q_{ov}] >> V/[E_{ov}] \qquad (3)$$



Scheme 1. Presentation of a Ping-Pong Bi-Bi reaction in microemulsions proposed by Verhaert et al., but involving two hydrophobic substrates. *A* and *B* stand for the substrates lauric acid and (-)menthol, respectively; *P* and *Q* are the products, water and menthyl laurate; and *E* and *F* are the two forms of lipase during a catalytic cycle. k_n, $k_{-n}(n = 1-6)$ are the rate constants of the different steps. k_i^j, k_o^j, $(j = A, B, Q)$ are the first-order constant for rate transfer of *A*, *B*, and *Q*, respectively, from the organic phase into the reverse micelles, and from the water pool to the organic phase. k_{ex} is the exchange rate between reverse micellar droplets. $\varepsilon_{i,o}^P$ is the efficiency of the exchange of product *P* into or out of an enzyme-filled reversed micelle. $[M]$ is the molar concentration of the reverse micelles. $[A_m]$, $[B_m]$, and $[Q_m]$ are the average concentrations of substrates *A* and *B* or product *Q* in a *A*-, *B*-, Q-filled reverse micelle.

and

$$k_{ex}\varepsilon_o^P[M] >> V/[E_{ov}], \tag{4}$$

the term α_3 is negligible. Furthermore, because Poisson statistics apply to the partition of solutes in the reverse micelles, the number of droplets filled with the hydrophobic substrates and product (ester) is low and the average micellar concentrations can be employed:

$$[A_m] = [B_m] = [Q_m] = [M]/\phi \tag{5}$$

where ϕ is the volume fraction of the dispersed water phase.

Based on the assumptions of Eqs. (3–5), Eq. (2) can be converted to:

$$V_o = \frac{V}{1 + \frac{K_{M,A}}{[A]_{ov}}P_A + \frac{K_{M,B}}{[B]_{ov}}P_B + \frac{\phi}{[M]}(K_{M,A} + K_{M,B})} \tag{6}$$

From this equation, and taking into account Eq. (1), one can determine the real values of the Michaelis constants, $K_{M,A}$ and $K_{M,B}$, by using the values of the partition coefficients, P_A and P_B. As the first approximation, we can use the values 512 and 280 for the partition coefficients of lauric acid and (-)menthol, respectively, as estimated by the values of the partition coefficients of lauric acid between octane and water[15] and of decanol, an analog of menthol, between octane and water.[13] Using these values in Eq. (6), the V_{max} of the reaction was found to be 124 μmol mg^{-1} min^{-1}. However, the values of the partition coefficients are certainly overestimated, because in the case where $w_o = 6$, the properties of the dispersed water vary considerably as compared with bulk water.[19] It has been reported[21,35] that the polarity of the water core for $w_o < 7$ corresponds to that of chloroform. In this extreme case, the partition coefficients of lauric acid and (-)menthol should be considered as approximately 4.55 and 4.30, respectively, according to the method of determination of the *P* values in different solvent systems, as proposed by Leo et al.[15] Under these conditions, the value of V_{max} calculated from Eq. (6), was found to be 136.4 μmol mg^{-1} min^{-1}.

Eq. (6) can be further transformed by introducing w_o, and by using simple geometrical terms.[32] From the following expressions,[10]

$$\phi = w_o\frac{M_w[\text{AOT}]}{1000\rho_w} \tag{7}$$

$$[M] = \frac{3M_w[\text{H}_2\text{O}]}{4\pi N\rho_w r^3} \tag{8}$$

where M_w is the molecular mass of water, ρ_w is the density of water (g/mL), N is Avogadro's constant, and r the radius

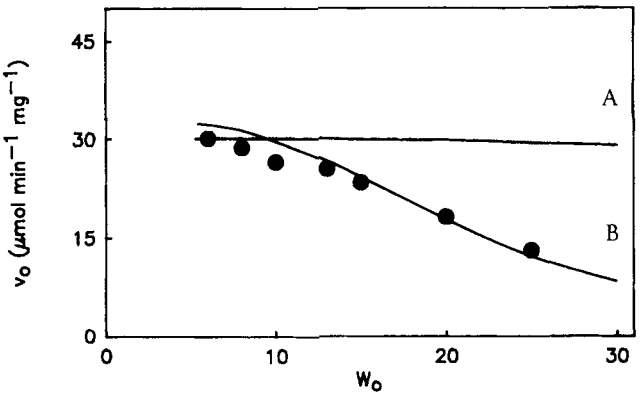


Figure 8. Dependence of the initial velocity of esterification of (-)menthol by lauric acid, as a function of the hydration ratio of the reverse micelles $w_o = [H_2O]/[AOT]$. The experimental values are represented by the symbols (●). The curves are calculated from Eq. (10) using the following sets of parameter values: *A* (----) (aqueous environment): $P_A = 512$; $P_B = 280$; $K_{M,A} = 0.038$ m*M*; $K_{M,B} = 0.446$ m*M*; $V = 124.44$ μmol mg^{-1} min^{-1}. *B* (——) (low polarity environment) $P_A = 4.55$; $P_B = 4.30$; $K_{M,A} = 4.29$ m*M*; $K_{M,B} = 29.02$ m*M*; $V = 136.4$ μmol mg^{-1} min^{-1}. $[A_{ov}] = 31.5$ m*M*; $[B_{ov}] = 50.0$ m*M*.

of the reverse micelle (cm), or

$$\frac{\phi}{[M]} = \frac{4\pi}{3}\frac{N}{1000}r^3 \tag{9}$$

by combining Eqs. (6) and (9) and assuming that $r(\text{cm}) = 0.175 \times 10^{-7}\ w_o$,[4,17] we obtain the following expression:

$$v_o = \frac{V}{1 + \dfrac{K_{M,A}P_A}{[A_{ov}]} + \dfrac{K_{M,B}P_B}{[B_{ov}]} + \alpha_4 w_o^3(K_{M,A} + K_{M,B})}$$

where

$$\alpha_4 = 4\frac{\pi}{3}\frac{N}{1000}(0.175 \times 10^{-7})^3 = 1.35 \times 10^{-5}\ \text{m}M^{-1} \tag{10}$$

Figure 8 shows the variation of the initial velocity of the esterification of (-)menthol and lauric acid measured in the AOT microemulsion systems as a function of w_o. The curves were calculated from Eq. (10) using two extreme sets for the parameter values, one corresponding to an aqueous microenvironment (upper line), and one corresponding to a less polar medium such as chloroform (lower line). As can be seen, our experimental results better fit to the case where the values of the partition coefficients, and therefore of the real K_M and V values, correspond to a lower polarity water core microenvironment.

In conclusion, the esterification reaction of lauric acid with (-)menthol catalyzed by *P. simplicissimum* lipase in AOT/isooctane microemulsions follows a Ping-Pong Bi-Bi mechanism. The partitioning of the two hydrophobic substrates between the continuous organic phase and the reverse micelles must be taken into account to determine the real kinetic constants.

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