0273-2289/96/6103—0411$08.00

Reaction Kinetics of the Esterification of Lauric Acid in Iso-Octane Using an Immobilized Biocatalyst

F. VÁZQUEZ LIMA,[1,2] D. L. PYLE,[1] AND J. A. ASENJO*,[1]

[1]*Biotechnology and Biochemical Engineering Group, University of Reading, England;* [2]*Present address: Centre for Biochemical Engineering and Biotechnology, Department of Chemical Engineering, University of Chile, Beauchef 861, Santiago, Chile*

Received May 2, 1995; Accepted August 11, 1995

ABSTRACT

The kinetics of the esterification of lauric acid with geraniol catalyzed by a commercially immobilized lipase preparation from *Mucor miehei*, Lipozyme, was studied in well-stirred flasks under conditions of no external mass transfer limitations. It was shown that the reaction is inhibited by lauric acid and the reaction mechanism can be described as a Ping-Pong Bi-Bi with Dead-End inhibition caused by lauric acid.

Index Entries: Enzyme kinetics; lipase; esterification; lauric acid.

INTRODUCTION

The reaction mechanisms and kinetic behavior are among the key factors that affect the performance of biocatalysts. In the case of lipases, which catalyze multisubstrate-multiproduct reactions, complex kinetic mechanisms have been proposed to provide descriptions of the reactions taking place. Kinetic studies involving pancreatic lipase (*1,2*), lipase from *Candida cylindracea* (*3*), and lipase from *Aspergillus niger* (*4*), support the hypothesis that the hydrolytic action of lipases follows a two-step reaction mechanism, usually referred to as Ping Pong Bi-Bi (*5*). Sugiura &

*Author to whom all correspondence and reprint requests should be addressed.

Isobe (*6*) suggest that in the case of a lipase from *Chromobacterium,* the observed rate expressions correspond to an Ordered Bi-Bi mechanism (*5*). On the other hand, Wang (*7*) proposed an Ordered Uni-Bi mechanism (*5*) to describe the hydrolysis catalyzed by a human milk bile salt-activated lipase. Therefore, it is evident that there is no generally accepted mechanism to describe the mode of action of lipases.

Lipases are among the most promising enzymes to be used in organic solvents. They catalyze the hydrolysis of fats to give glycerides and glycerol. Their natural substrates are triglycerides of long-chain fatty acids which are insoluble in water. Lipases hydrolyze the ester bonds at the interface between the aqueous phase in which the enzyme is soluble, and the insoluble substrate phase (*8,9*). The reaction is reversible and therefore, the enzymes can also be used as catalysts in the formation of esters from alcohols and fatty acids. In aqueous solutions, the equilibrium is strongly shifted toward the starting reagents and esters cannot be synthesized. To overcome this difficulty reaction media containing very small amounts of water or comprised of organic solvents can be used to bring about a chemical equilibrium shift toward the ester (*8,9*). Our previous work (*8,9*) shows that, under the conditions studied in this paper, the enzymatic esterification of lauric acid with geraniol catalyzed by the immobilized preparation Lipozyme, is not controlled by external or internal mass transfer limitations; that water produced during the reaction has an inhibitory effect, this being predominantly a physical one, because of its accumulation around the enzyme, and that the enzyme is inhibited by lauric acid.

In this paper we report our investigation on the kinetics of the esterification reaction of lauric acid with geraniol catalyzed by Lipozyme, and the type of inhibition exerted by lauric acid.

THEORETICAL BACKGROUND: REACTIONS OF TWO SUBSTRATES

The Michaelis-Menten equation was originally derived for the kinetics of single-substrate reactions. However, a reaction with two substrates can be said to obey Michaelis-Menten kinetics if its rate depends on the concentration of the two substrates, so that if one substrate concentration is varied while the other is held constant, the reaction behaves like a single-substrate reaction obeying the Michaelis-Menten equation.

There are several kinetic mechanisms that can be used to explain two substrate reactions. One of them is the Ping Pong Bi-Bi mechanism in which a product is released between the addition of two substrates (*5*). These mechanisms are common in group transfer or substituted enzyme reactions. If the following reaction is considered:

$$A + B \rightleftharpoons P + Q$$

the reaction mechanism can be represented by the ping-pong or double-displacement mechanism:

$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\bullet}A \rightleftharpoons F{\bullet}P \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P + F$$

$$F + B \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} F{\bullet}B \rightleftharpoons E{\bullet}Q \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + Q$$

A first binds to the enzyme E, forming a binary complex E•A. An intramolecular reorganization takes place, the bond F-P being formed and the E-A bond being broken.

The first product P, then leaves before the second substrate arrives. B cannot bind to the enzyme E, but can bind to the modified enzyme F. Since only one substrate is present on the enzyme at any one time, there may only be a single binding site. Another intramolecular rearrangement takes place, the bond E-Q being formed and the bond F-B being broken. The second product, Q, is then liberated, leaving the enzyme in its original form (*10*).

To determine the rate constants involved in the ping-pong bi-bi mechanism, the following expressions have been obtained for the initial forward rate in the absence of products (*5*):

$$v / v_{max} = ([A][B]) / (K_{m_B}[A] + K_{m_A}[B] + [A][B]) \quad (1)$$

In terms of elementary-step rate constants, the expressions for the K_m values and v_{max} are (*3,5*):

$$K_{m_A} = [k_4(k_2 + k_{-1})] / [k_1(k_2 + k_4)] \quad (2)$$

$$K_{m_B} = [k_2(k_{-3} + k_4)] / [k_3(k_2 + k_4)] \quad (3)$$

$$v_{max} = ([E]_0 k_1 k_2 k_3 k_4 1) / [k_1 k_3 (k_2 + k_4)] \quad (4)$$

Rearranged to show each substrate as the varied substrate, Eq. 1 becomes:

$$v / v_{max} = [A] / \{K_{m_A} + [A](1 + K_{m_B} / [B])\} \quad (5)$$

and

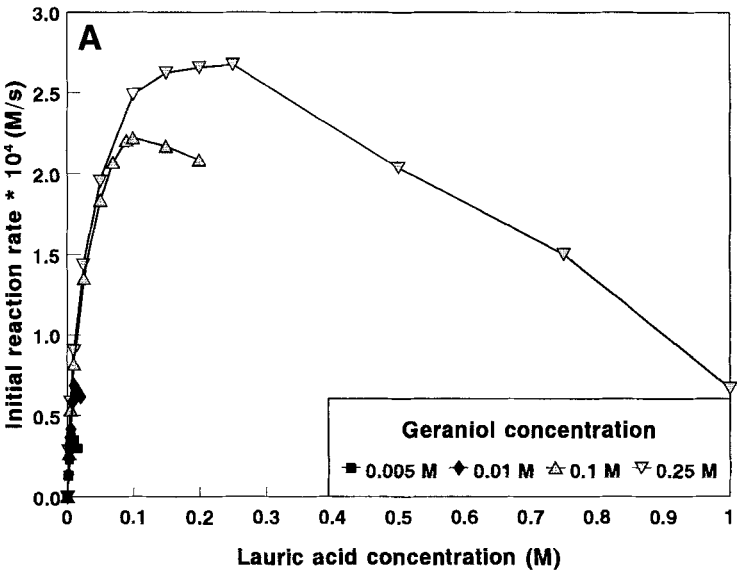
$$v / v_{max} = [B] / \{K_{m_B} + [B](1 + K_{m_A} / [A])\} \quad (6)$$

When considered individually, Eqs. 5 and 6 have the form of the classical Michaelis-Menten equation.

MATERIALS AND METHODS

Enzyme

A commercially immobilized lipase was used throughout this work. This enzyme, Lipozyme (Novo Industri A/S, Bagsvaerd, Denmark), is a



preparation of a *Mucor miehei* lipase immobilized on a macroporous anion exchange resin. The lipase is specific toward 1,3 ester bond triglycerides and, has a broad fatty acid specificity (*11*).

Chemicals

Analytical grade geraniol and lauric acid (dodecanoic acid) were purchased from Sigma (Poole, England); analytical grade hexane, cyclohexane, and iso-octane (2-2-4, trimethyl pentane) were supplied by BDH (London, UK).

Experimental Procedure

The kinetics of the reaction was determined by performing experiments where the initial concentration of one of the substrates was kept constant while varying the initial concentration of the other. Experiments were carried out in 125 mL conical flasks with a working volume of 50 mL, an agitation rate of 400 rpm, iso-octane as solvent and at 55°C. It has already been shown that under such conditions the system is not under mass transfer limitations (*8,9*). All experiments were carried out with fresh immobilized enzyme. Samples were taken at 0, 15, 30, and 60 s intervals and analyzed for the ester by gas chromatography as described previously (*21*).

RESULTS AND DISCUSSION

Figs. 1A and B show the effect of the concentration of lauric acid on the initial reaction rate when the initial geraniol concentration was fixed.

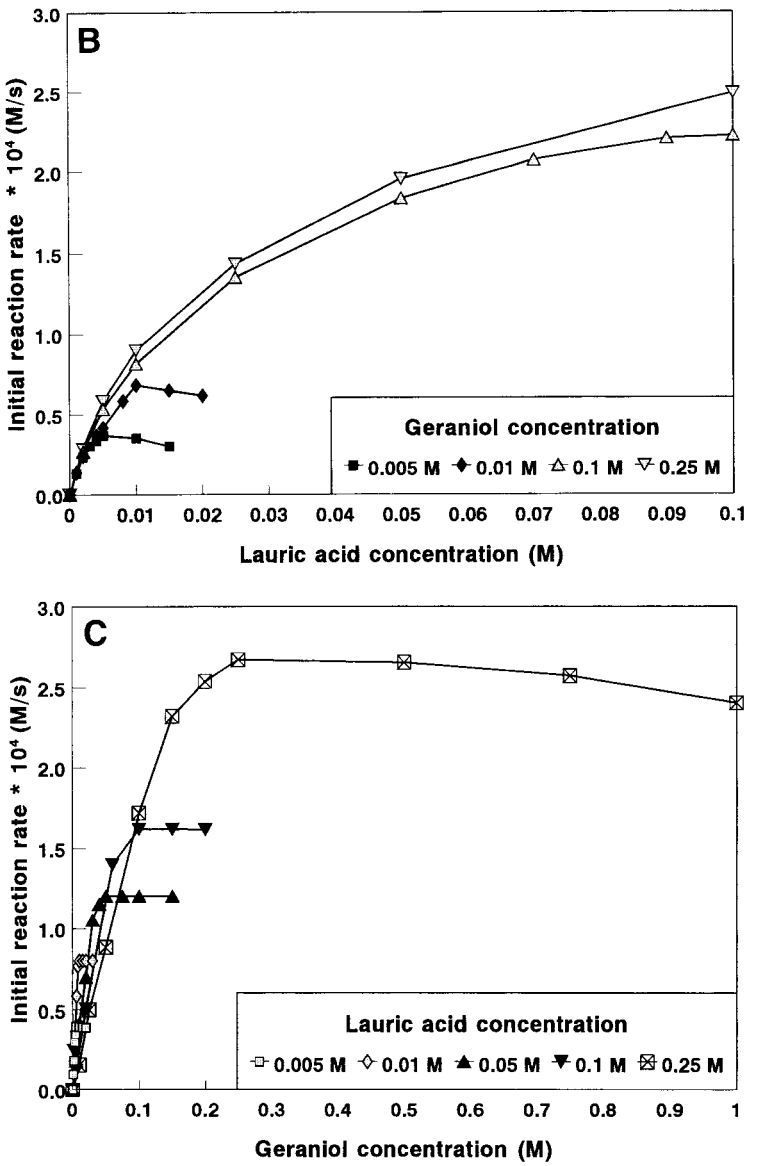
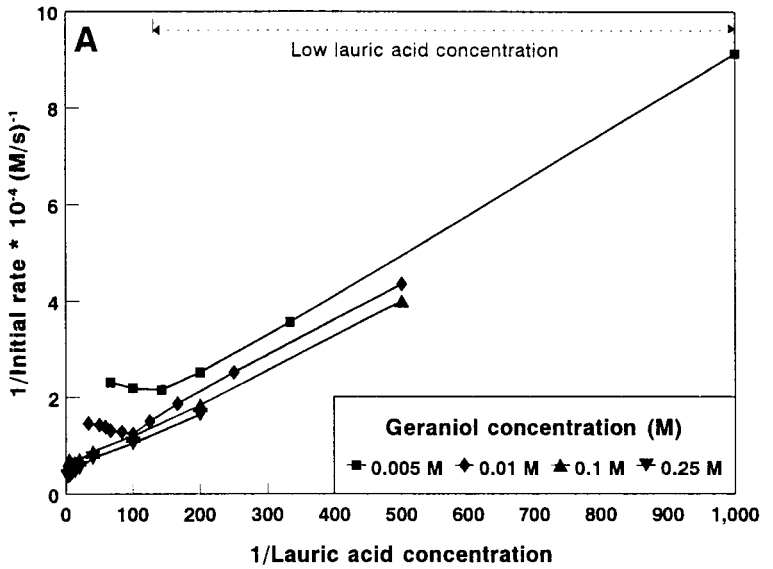


Fig. 1. Effect of substrate concentration on the initial reaction rate of the esterification reaction, (**A**) effect of lauric acid concentration with fixed initial concentration of geraniol. High lauric acid concentration, (**B**) effect of lauric acid concentration with fixed initial concentration of geranoil. Low lauric acid concentration, (**C**) effect of geraniol concentration with fixed initial concentration of lauric acid.

It shows that, for a given geraniol concentration, the initial rate increases when the lauric acid concentration is increased until it reaches a maximum, which occurs at the point where the concentrations of both lauric acid and geraniol are the same. A subsequent increase in the lauric acid concentration brings about a decrease in the initial rate. They also show



that the initial geraniol concentration does not seem to have a significant effect on the initial rate.

The influence of the concentration of geraniol on the initial rate at fixed initial lauric acid concentrations is shown in Fig. 1C. It indicates that, for a given lauric acid concentration, the initial rate increases as the geraniol concentration increases until it reaches a maximum at conditions corresponding to equimolar substrate concentrations. A further increase in geraniol concentration does not have any effect on the initial rate. However, as the initial lauric acid concentration is increased, the same patterns are observed, but the initial rate decreases as the fixed lauric acid concentration increases.

The results shown in Fig. 1A suggest that the Lipozyme catalyzed esterification is inhibited by lauric acid. Figure 1C suggests that the reaction obeys the Michaelis-Menten equation with respect to one substrate, providing the concentration of the other substrate is maintained fixed, however, an inhibitory effect by lauric acid needs to be considered. Another conclusion that can be drawn from these results is the fact that for all cases the apparent maximum rate is reached when the substrate concentrations are equimolar. However, these results are insufficient to determine the mechanisms of reaction and inhibition.

When the reciprocals of the initial rates obtained at constant geraniol concentrations are plotted against the reciprocals of the lauric acid concentration (Fig. 2A) this results in a series of curves showing the characteristic feature of substrate inhibition (*10–13*), with a linear section at lower lauric acid concentrations and a section where the lauric acid concentration negatively affects the initial reaction rate. When the linear sections of the curves are compared, these correspond to a series of straight lines with similar slopes but no common intercept.

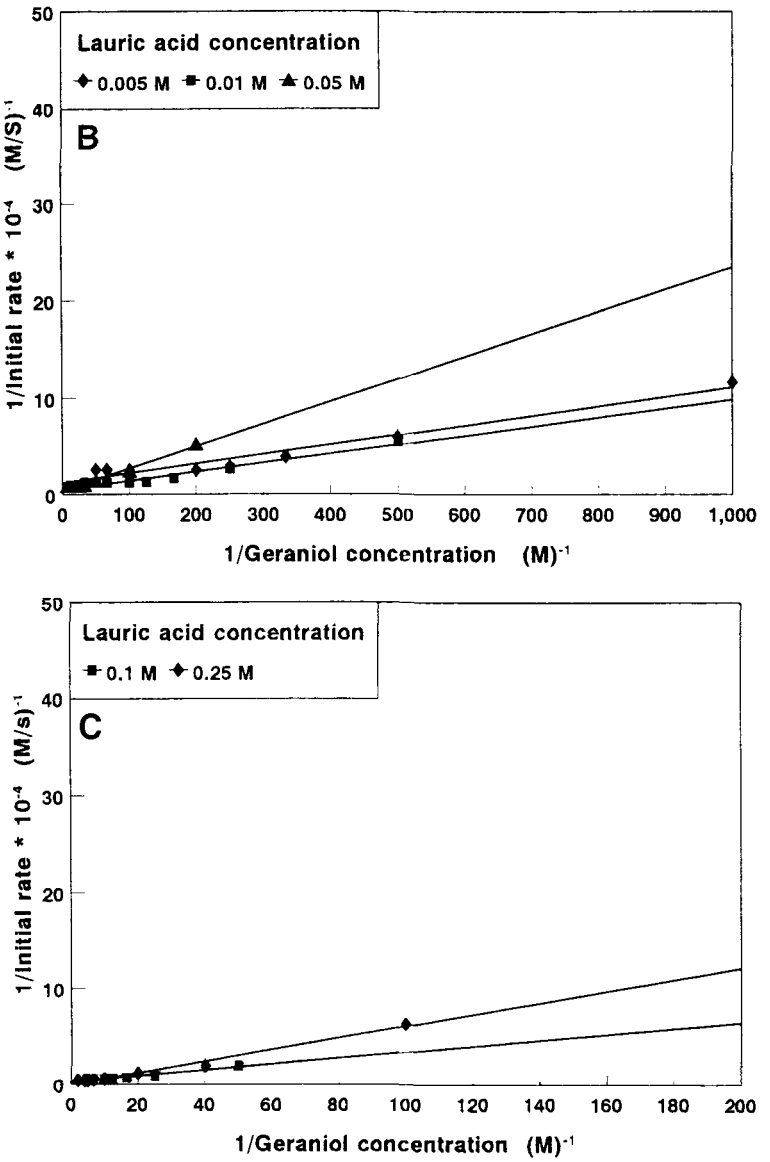


Fig. 2. Reciprocal of initial rate of reaction versus reciprocal of reactant concentration, (**A**) variation of lauric acid concentration at fixed geraniol concentrations; (**B**) and (**C**) variation of geraniol concentration at fixed lauric acid concentration, low and high concentrations of geraniol, respectively.

Figures 2B and C show the double-reciprocal plot of the initial rate of reaction as a function of geraniol concentration at fixed lauric acid concentrations (2B at low and 2C at high lauric acid concentrations). From this figure it can be seen that as the lauric acid concentration increases, the slope increases and there is a common 1/v intercept for all the lines.

When the sections of the plots presented in Fig. 2A corresponding to **low** lauric acid concentrations are compared to those for the double-

reciprocal plots of reactions following the ping-pong bi-bi mechanism, it is observed that they follow the same pattern, although it needs to be emphasized that this does not apply for **high** lauric acid concentrations. These findings can be taken as providing some evidence for the esterification reaction conforming to such a mechanism. However, the plots obtained with fixed initial lauric acid concentrations follow a different trend (Figs. 2B and C) as lauric acid is also acting as an inhibitor of the reaction. On the basis of enzyme kinetics (*5,12*) a ping-pong bi-bi mechanism with dead-end inhibition may therefore be proposed for the esterification reaction with lauric acid behaving as both substrate and inhibitor.

It has been demonstrated that lipase-catalyzed ester hydrolysis follows an acyl-enzyme intermediate mechanism (*14–16*). As the lipase-catalyzed esterification is the reverse reaction of hydrolysis it can be assumed that the acyl-enzyme mechanism also applies in the reverse direction, so that the esterification reaction between lauric acid and geraniol should proceed as follows:

$$E + LA \rightleftharpoons E{\bullet}LA \rightleftharpoons C + H_2O$$
$$C + G \rightleftharpoons C{\bullet}G \rightleftharpoons E + GL$$

where: E, enzyme; LA, lauric acid; E•LA, enzyme-lauric acid complex; C, acyl enzyme complex; G, geraniol; C•G, geraniol acyl enzyme complex; GL, geranyl laureate.

According to the proposed ping-pong mechanism, lipase first binds lauric acid; then the acyl-enzyme intermediate is formed and water is released; the geraniol then attacks this intermediate and the geranyl laureate is formed. However, if the synthesis of the ester is under dead-end inhibition by substrate, this would imply that lauric acid is also able to bind the acyl-enzyme intermediate to form a dead-end acyl-enzyme-lauric acid complex that cannot participate in the reaction:

$$C + LA \rightarrow CLA$$

where: CLA, acyl enzyme lauric acid complex.

A schematic representation of this reaction mechanism using the Cleland (*5,10*) notation is given in Fig. 3.

Moreover, since the model is tested based on initial rates, it is assumed that the esterification reaction proceeds only in the forward direction; and since lauric acid is also an inhibitor, the expression for the reaction rate becomes:

$$v / v_{max} = ([LA]\,[G]) / \{ K_{m_G}\,[LA]\,(1 + [I] / K_i) + K_{m_{LA}}\,[G] + [LA]\,[G]\} \quad (7)$$

where $K_{m_{LA}}$ is the Michaelis constant for lauric acid; K_{m_G} is the Michaelis constant for geraniol: [LA], [G], and [I] are the lauric acid, geraniol, and inhibitor concentrations respectively and, K_i is the inhibition constant.

When the lauric acid concentration is varied and considering that [LA] = [I], the equation is:

E: free enzyme
LA: lauric acid
ELA: enzyme-lauric acid complex
C: acyl enzyme complex
CG: geraniol-acyl enzyme complex
CAL: acyl enzyme-lauric acid complex
GL: geranyl laurate

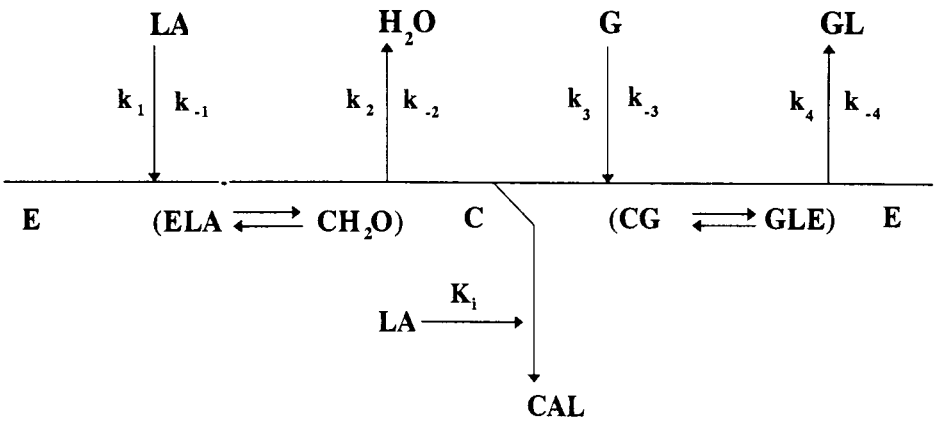


Fig. 3. Schematic representation using the Cleland notation (*14,17*) of the esterification reaction following the ping pong bi-bi mechanism with dead-end inhibition.

$$v / v_{max} = ([LA] / \{K_{m_{LA}} + [LA] (1 + K_{m_G} / [G] (1 + [LA] / K_i))\} \quad (8)$$

With a double-reciprocal form:

$$1 / v = 1 / v_{max} (1 + K_{m_G} / [G] + K_{m_G} / K_i) + K_{m_{LA}} / v_{max} (1 / [LA]) \quad (9)$$

When the geraniol concentration is varied, the equation is:

$$v / v_{max} = [G] / \{ K_{m_G} (1 + [LA] / K_i) + [G] (1 + K_{m_{LA}} / [LA])\} \quad (10)$$

and its corresponding double-reciprocal form:

$$1 / v = 1 / v_{max} (1 + K_{m_{LA}} / [LA]) + K_{m_G} / v_{max} (1 + [LA] / K_i) (1 / [G]) \quad (11)$$

Equation 9 indicates that the slopes of the double-reciprocal curves obtained for a fixed geraniol concentration depend on only two constants ($K_{m_{LA}}$ and v_{max}) and, therefore, that it has the same value regardless of the geraniol concentration. This behavior is observed in Fig. 2A.

For fixed lauric acid concentrations the expression representing the slope (Eq. 11) includes the concentration of lauric acid, hence an increase in the slope is expected when the lauric acid concentration is increased. This pattern is found in Fig. 2B and C.

Moreover, when the slopes of the lines obtained in Fig. 2B and C are plotted against the lauric acid concentration, a straight line is obtained with a horizontal intercept equal to $-K_i$.

The values of K_i, v_{max}, $K_{m_{LA}}$, and K_{m_G} have been calculated according to the following procedure: the K_i value was obtained by linear regression

Table 1
Kinetic Constants for the Esterification of Lauric Acid with Geraniol Obtained from Initial Rate Studies

v_{max}	$2.74 \cdot 10^{-4}$ M/s
$K_{m_{LA}}$	0.024M
K_{m_G}	0.042M
K_i	0.125M

plotting the slope of Eq. 11 vs lauric acid concentration, and is shown in Table 1. Then, it is introduced in Eq. 9 to determine the values of K_{m_G} and v_{max} from the experimental data for variable geraniol and fixed lauric acid concentrations, using the 'Enzfitter' data analysis program (*17*). Then, the value of v_{max} is substituted in the expression for the slope in Eq. 11 to determine the value of $K_{m_{LA}}$. The values obtained are presented in Table 1 and comparisons between the experimental results and model predictions are given in Figs. 4A and B. They show that in general, the proposed model predicts reasonably well the behavior of the system for both fixed lauric acid and geraniol concentrations.

Several authors have postulated the ping-pong mechanism to describe reactions catalyzed by lipase subjected to inhibition; Chulalaksananukul et al. (*18*) in transesterification reactions with lipase from *Mucor miehei* found that the reaction was inhibited by high geraniol concentrations, Chulalaksananukul et al. (*19*) found a similar effect on the same lipase in the esterification of oleic acid by ethanol, Rizzi et al. (*20*) have suggested an inhibitory effect by both substrates (ethyl acetate and isoamyl alcohol) in transesterification reactions with lipase from *Mucor miehei.*

With respect to the possible reasons for lauric acid inhibition, it has been established that some free fatty acids inhibit the activity of lipases (*21,22*), possibly because of the tendency of fatty acids to accumulate at the lipid/water interface blocking the access of the enzyme to unreacted molecules (*4,23*). However, such an effect was not found with lauric acid when working at concentrations up to 1 μmol/mL (*21*). It could also be hypothesized that, when working with lauric acid concentrations above the 1:1 ratio with respect to geraniol, the excess in lauric acid could accumulate at the interface causing a reduction in the activity of the enzyme, especially considering that the working concentrations used for this study were far above 1 μmol/mL. Nevertheless, as interfacial phenomena involved in lipase catalyzed reactions are still not well understood, it is difficult to propose an explanation for the inhibition.

CONCLUSIONS

The kinetics of the enzymatic esterification of lauric acid with geraniol catalyzed by the commercial lipase preparation Lipozyme in iso-octane

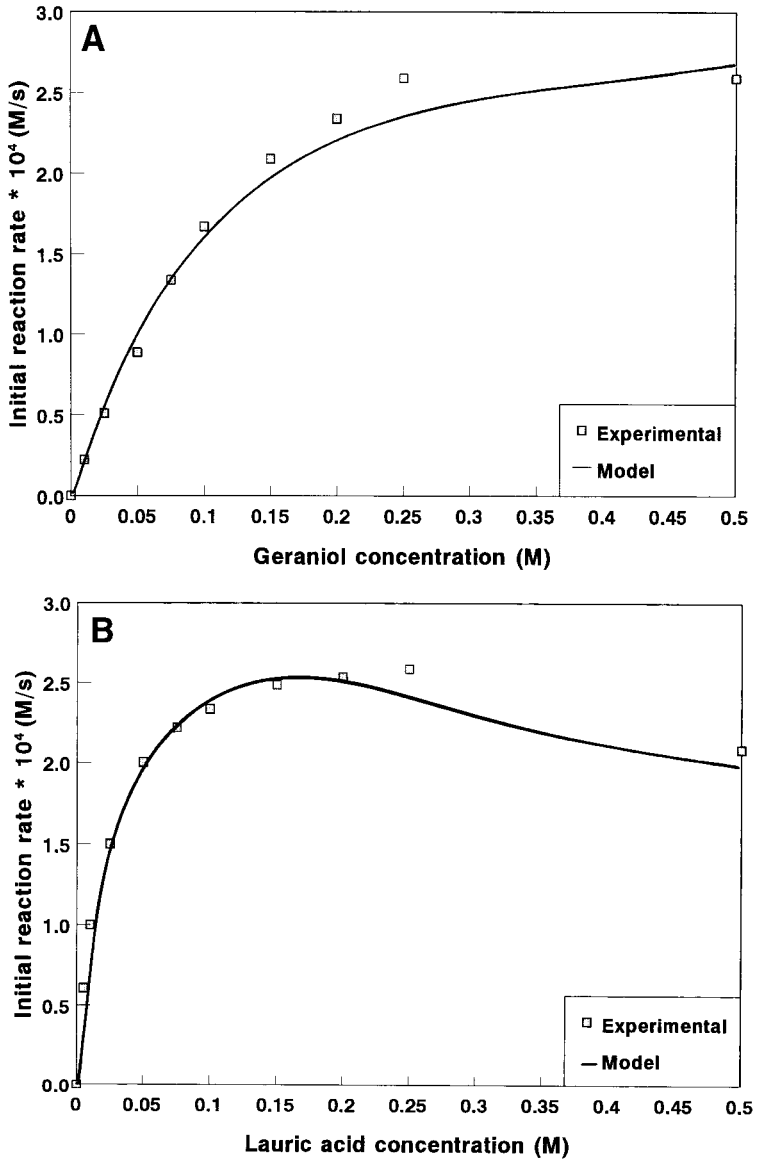


Fig. 4. Comparison of model predictions and experimental results. (**A**) 0.25*M* initial lauric acid concentration; (**B**) 0.25*M* geraniol concentration.

have been analyzed. On the basis of the results reported here, it can be concluded that the reaction mechanism can be described as Ping-Pong Bi-Bi, and also that the reaction is under dead-end inhibition by lauric acid with no negative effect exerted by geraniol.

ACKNOWLEDGMENTS

Financial support provided by CONACYT and CINVESTAV (Mexico) to FVL is gratefully acknowledged.

REFERENCES

1. Lombardo, D. and Guy, O. (1981), Effect of alcohols on the hydrolysis catalyzed by human pancreatic carboxylic-ester hydrolase. *Biochem. Biophys. Acta.* **657,** 425–437.
2. Lombardo, D. (1982), Catalytic properties of modified human pancreatic carboxylic-ester hydrolase. *Biochem. Biophys. Acta.* **700,** 75–80.
3. Miller, D. A., Prausnitz, J. M., and Blanch, H. W. (1991), Kinetics of lipase-catalyzed interesterification of triglycerides in cyclohexane. *Enzyme Microb. Technol.* **13,** 98–103.
4. Malcata, F. J., Reyes, H. H., Garcia, H. S., Hill, Jr., C. G., and Amundson, C. H. (1992), Kinetics and mechanism of reactions catalyzed by immobilized lipases. *Enzyme Microb. Technol.* **14,** 426–446.
5. Segel, I. H. (1975), *Enzyme kinetics. Behavior and analysis of rapid equilibrium and steady-state enzyme systems.* Wiley Interscience, New York.
6. Sugiura, M. and Isobe, M. (1976), Studies on the mechanism of lipase reaction. III. Adsorption of *Chromobacterium* lipase on hydrophobic glass beads. *Chem. Pharm. Bull.* **24,** 72–78.
7. Wang, C. S. (1981), Human milk bile salt-activated lipase. Further characterization and kinetic studies. *J. Biol. Chem.* **256,** 10,198–10,202.
8. Vázquez Lima, F. (1993), *Studies on the modelling of esterification reactions with immobilized biocatalysts in organic solvents, use of a fluidized bed reactor.* Ph.D. thesis. University of Reading.
9. Vázquez Lima, F., Pyle, D. L., and Asenjo, J. A. (1995), Factors affecting the esterification of lauric acid using an immobilized biocatalyst, enzyme characterization and studies in a well-mixed reactor. *Biotechnol. Bioeng.* **46,** 69–79.
10. Palmer, T. (1985), *Understanding enzymes.* 2nd Ed. Ellis Horwood Ltd., Chichester.
11. Novo Industri (1986), *A/S Lipozyme.* Novo Enzymes preliminary product information sheet B 347b/GB 440. Novo Industri A/S, Bagsvaerd.
12. Cleland, W. W. (1979), Substrate Inhibition, in *Methods in Enzymology, vol. 63* (Colowick, S. O. and Kaplan, N. O., eds.), Academic, New York.
13. Fersht, A. (1985), *Enzyme structure and mechanism.* W. H. Freeman and Co., New York.
14. Burdette, R. A. and Quinn, D. M. (1986), Interfacial reaction dynamics and acyl-mechanism for lipoprotein lipase-catalyzed hydrolysis of lipid *p*-nitrophenyl esters. *J. Biol. Chem.* **261,** 12,016–12,021.
15. Chapus, C., Sémériva, M., Bovier-Lapierre, C., and Desnuelle, P. (1976), Mechanism of pancreatic lipase. 1. Interfacial activation of pancreatic lipase. *Biochemistry* **15,** 4980–4987.
16. Sémériva, M., Chapus, C., Bovier-Lapierre, C., and Desnuelle, P. (1974), On the transient formation of an acetyl enzyme intermediate during the hydrolysis of p-nitrophenyl acetate by pancreatic lipase. *Biochem. Biophys. Res. Commun.* **58,** 808–813.
17. Leatherbarrow, R. J. (1987), *Enzfitter.* Elsevier-Biosoft, Cambridge.
18. Chulalaksananukul, W., Condoret, J. S., Delorme, P., and Willemot, R. M. (1990), Kinetic study of esterification by immobilized lipase in n-hexane. *FEBS Lett.* **276,** 181–184.
19. Chulalaksananukul, W., Condoret, J. S., and Combes, D. (1992), Kinetics of geranyl acetate synthesis by lipase-catalysed transesterification in n-hexane. *Enzyme Microb. Technol.* **14,** 293–298.
20. Rizzi, M., Stylos, P., Riek, A., and Reuss, M. (1992), A kinetic study of immobilized lipase catalysing the synthesis of iso-amyl acetate by transesterification in n-hexane. *Enzyme Microb. Technol.* **14,** 709–714.
21. Smith, J. L. and Alford, J. A. (1966), Inhibition of microbial lipases by fatty acids. *Appl. Microbiol.* **14,** 699–705.
22. Rostrup-Nielsen, T., Pedersen, L. S., and Villadsen, J. (1990), Thermodynamics and kinetics of lipase catalysed hydrolysis of oleyl oleate. *J. Chem. Tech. Biotechnol.* **48,** 467–482.
23. Benzonana, G. and Desnuelle, P. (1968), Action of some effectors on the hydrolysis of long-chain triglycerides by pancreatic lipase. *Biochem. Biophys. Acta.* **164,** 47–58.