Transesterification Kinetics of Triglycerides for a Modified Lipase in *n*-Hexane

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ABSTRACT: A simple kinetic model for the transesterification of triglycerides catalyzed by a modified lipase in *n*-hexane has been proposed. The model assumes that the enzyme has 1,3-positional specificity and does not distinguish among the different fatty acid residues considered in this study. The model is based on material balances of consecutive second-order reversible reactions and requires only one parameter that can easily be determined experimentally. The differential rate equations have been solved analytically to give explicit equations that link the concentrations of all possible triglycerides to the initial conditions and the reaction time. The model was in good agreement with experimental data for different biocatalyst concentrations with the same value of the specific rate constant. The same value of specific rate constant also gave a good fit with experimental data for an acidolysis reaction between a triglyceride and a fatty acid, implying that the modified lipase did not distinguish between free fatty acids and fatty acid residues attached to the 1 and 3 positions of a glycerol backbone.

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KEY WORDS: Acidolysis, interesterification, kinetic model, modified lipase, transesterification, triglycerides.

Lipases are able to catalyze the hydrolysis, esterification, acidolysis, alcoholysis and transesterification of a wide range of substrates and have therefore received much attention as biocatalysts for a variety of different processes (1). Examples include the production of high-value specialty fats such as cocoa butter substitute (2) and human milk fat substitutes (3), and the production of enantiomerically pure compounds (4), as well as the production of flavors and fragrances (5). One of the most promising areas of current research is the modification of fats and oils to improve their physical and nutritional properties (3,6).

Water content is crucial in determining lipase performance. The optimum water content is a compromise between minimizing hydrolysis and maximizing enzyme activity for synthesis. For this reason, lipases have been used in organic solvents with a water content between 1 and 8% (7,8). However, the activity, stability and dispersability of many lipases are poor in organic solvents as compared to that in water. Several methods have been attempted to overcome this problem, including covalent attachment of fatty acids (9), immobilization on anion exchange resins (10), coating with surfactant (11) and entrapment in reverse micelles (12–14).

In a previous study (15), 19 surfactants and 16 lipases from various fungal and microbial sources were screened for acidolysis activity. It was found that the best combination was lipase Saiken with sorbitan monostearate. Subsequent studies (16) have characterized this modified lipase and determined the optimum water content, pH and surfactant-to-enzyme weight ratio. It was also shown that this modified lipase has 1,3-positional specificity for the acidolysis reaction between a triglyceride and a fatty acid in *n*-hexane. The production of diglycerides was small (<6% of total glycerides), and monoglycerides and glycerol could not be detected at all. A kinetic model of the acidolysis of triglycerides and fatty acids has already been studied (Basheer, S., K. Mogi and M. Nakajma, unpublished data) and gave good agreement with the experimental data for the acidolysis of tripalmitin and stearic acid. However, for some important industrial applications, such as the modification of mixtures of oils (6), transesterification reactions of triglycerides are more efficient and offer an attractive alternative to direct esterification or acidolysis. For this reason it was decided to extend the original study to investigate transesterification kinetics of triglycerides. In this paper a kinetic model of the transesterification of triglycerides is proposed and compared with experimental results of the transesterification of tripalmitin and tristearin in *n*-hexane with the modified lipase described.

In the literature, several models have been proposed for the kinetics of acidolysis reactions (17,18), but little has been published on the kinetics of transesterification reactions. Zaks and Klibanov (19) studied the kinetics of the transesterification of tributyrin and heptanol and deduced that the mechanism proceeded *via* a ping-pong bi-bi mechanism with dead-end inhibition by the alcohol. Chulalaksananukul *et al.* (5) proposed a similar mechanism for the transesterification of geraniol and propyl acetate with inhibition by excess geraniol. The transesterification reaction was modeled by a modified Michaelis-Menten equation that required four parameters (two Michaelis constants, one inhibition constant and the

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maximum reaction rate). There have been no reports to date, however, on the modeling of the transesterification reaction between two triglycerides, and this paper aims to fill this gap in the literature.

Malcata *et al.* (20) have pointed out the large number of parameters necessary to model interesterification reactions, even in quite simple systems, and the prohibitive amount of experimental work and numerical analysis that would be required to determine the values of all these parameters. Two possible approaches are possible to reduce this problem to a manageable size, first, to lump together all glycerides and consider them as one component while retaining the detailed kinetic mechanism, and second, to consider all the different glyceride moieties but to simplify the kinetics. In this paper the second approach has been adopted because it is our aim to study the possible applications of these processes, in which case the concentrations of the individual glycerides are of importance; but it is not our aim to investigate the mechanisms of lipase-catalyzed reactions.

MATERIALS AND METHODS

The lipase used was lipase Saiken 100, *Rhizopus japonicus*, obtained from Nagase Biochemicals Ltd. (Osaka, Japan), and the surfactant used was sorbitan monostearate (Kao Chemicals, Wakayama, Japan). The triglycerides were purchased from Sigma (St. Louis, MO) and had better than 99% purity. All other chemicals were purchased from Wako Pure Chemical Ind. (Tokyo, Japan).

Triglycerides were analyzed by high-performance liquid chromatography (HPLC) (Waters 600E; Waters Associates, Milford, MA) equipped with a light-scattering evaporative mass analyzer (ACS Ltd., Macclesfield, England) by following the protocol studied by Basheer *et al.* (Basheer, S., K. Mogi and M. Nakajima, unpublished data). The column used was LiChrospher 100, RP-18 (Cica Merck, Tokyo, Japan), and the mobile phase was a gradient mixture of acetone and acetonitrile. Tripentadecanoin was used as an internal standard. This technique made it possible to separate and accurately quantitate triglycerides that contained different numbers of palmitic acid and stearic acid residues (see Fig. 1), but it was not, however, possible to separate triglyceride isomers, i.e., 1,3-dipalmitoyl-2-stearoyl glycerol (PSP) and 1,3-dipalmitoyl-3-stearoyl glycerol (PPS), and 1,2-distearoyl-3-palmitoyl glycerol (PSS) and 1,3-distearoyl-2-palmitoyl glycerol (SPS).

Gas-chromatographic (GC) analysis was used to detect mono- and diglycerides. The glycerides were first silylated and then analyzed by GC (Shimadzu, Kyoto, Japan) in a capillary column (Shinwa Chemical Industries Ltd., Tokyo, Japan) with a flame-ionization detector (FID). Hexadecane was used as an internal standard.

Transesterification reactions of tripalmitin and tristearin, catalyzed by the lipase surfactant complex, were initiated by adding modified lipase (5–60 mg) to 55 mL of *n*-hexane that contained 266.5 mg (6 mmol/L) tripalmitin and 294.2 mg (6 mmol/L) tristearin. The temperature was maintained at 40°C by immersion in a water bath, and the contents were stirred magnetically at 800 rpm. Samples (0.5 mL) were taken periodically and filtered with Millipore filters (0.5 μm pore diameter) before analysis.

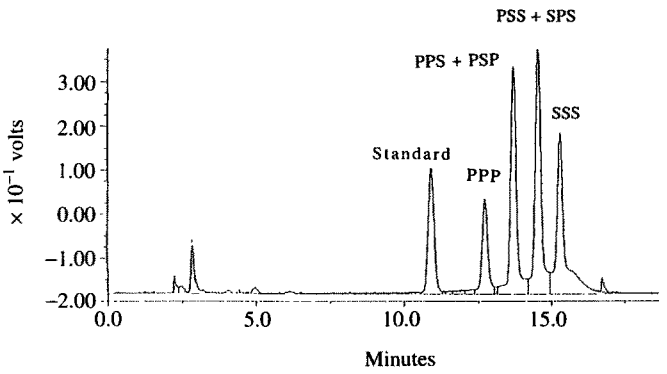

FIG. 1. High-performance liquid chromatography analysis of triglycerides. Internal standard, tripentadecanoin; PPS, 1,3-dipalmitoyl-3-stearoyl glycerol; PSP, 1,3-dipalmitoyl-2-stearoyl glycerol; PPP, tripalmitin; PSS, 1,2-distearoyl-3-palmitoyl glycerol; SPS, 1,3-distearoyl-2-palmitoyl glycerol; SSS, tristearin.

The concentration of water in the reaction system was measured by a Karl Fischer Titrator (684 KF Coulometer; Metrohm, Herisau, Switzerland) and was determined to be 25 mg/L.

Kinetic modeling. In a previous study (Basheer, S., K. Mogi and M. Nakajima, submitted for publication), it was shown that the lipase surfactant complex has 1,3-positional specificity, and therefore, the *sn*-2 position of the glycerol backbone is inaccessible to any species at or near the active site of the enzyme. It was also shown that the modified lipase acted on any acyl group and showed no specificity toward the kind of fatty acid moiety used in the study. Equilibrium was always reached, and addition of further enzyme did not result in a change in the concentrations of the products. No loss of enzyme activity was detected in the time scale of the experiments. The formation of diglycerides was detected, but the concentration was always less than 6% of the initial triglyceride concentration and was not taken into account in the kinetic model. Monoglycerides and glycerol were not detected under these conditions. Mass transfer limitations were not included in the model because of the homogeneity of the system. Based on these experimental findings, the following kinetic model was derived. Assuming no mono- or diglycerides are produced, there are six potential products of the transesterification reactions between tripalmitin (PPP) and tristearin (SSS) as:

$$\mathrm{PPP} + \mathrm{SSS} \underset{k}{\overset{4k}{\rightleftharpoons}} \mathrm{PPS} + \mathrm{PSS} \qquad [1]$$

$$\mathrm{PPP} + \mathrm{PSS} \underset{2k}{\overset{2k}{\rightleftharpoons}} \mathrm{PPS} + \mathrm{PSP} \qquad [2]$$

$$\mathrm{PPS} + \mathrm{SSS} \underset{2k}{\overset{2k}{\rightleftharpoons}} \mathrm{PSS} + \mathrm{SPS} \qquad [3]$$

$$PPS + PSS \underset{4k}{\overset{k}{\rightleftharpoons}} PSP + SPS \quad [4]$$

$$PPP + SPS \underset{k}{\overset{4k}{\rightleftharpoons}} 2PPS \quad [5]$$

$$SSS + PSP \underset{k}{\overset{4k}{\rightleftharpoons}} 2PSS \quad [6]$$

where k represents the transesterification reaction constant and PPS, SPS, PSP and PSS represent the mixed triglycerides as defined previously. Equation 1 represents the exchange of a palmitic acid residue from PPP with a stearic acid residue from SSS. There are four possible transfers, a palmitic acid residue from position 1 or 3 of the PPP can be exchanged with a stearic acid residue from positions 1 or 3 of the tristearin. The reverse reaction, however, has only one possible transfer. By the law of probability, it can therefore be assumed that the forward rate constant is four times greater than the reverse rate constant. Because the lipase shows no specificity toward the kind of fatty acid residue and by symmetry, the above argument can be applied to the other transesterification reactions. In other words, when a symmetrical triglyceride reacts with another symmetrical triglyceride, two asymmetrical triglycerides are formed with a rate constant that is four times greater than the reverse reaction (Eqs. 1, 4, 5 and 6). When a symmetrical triglyceride reacts with an asymmetrical triglyceride, a symmetrical and an asymmetrical triglyceride are produced, and, in this case, the forward reaction has the same rate constant as the reverse reaction (Eqs. 2 and 3).

Differential rate equations for SSS, PSP, PPP, PPS, PSS and SPS in the reaction system can be derived from Equations 1–6 as follows:

$$\frac{1}{k}\frac{d[SSS]}{dt} = [PPS][PSS] + 2[PSS][SPS] + [PSS]^2 - 4[PPP][SSS] - 2[PPS][SSS] - 4[SSS][PSP] \quad [7]$$

$$\frac{1}{k}\frac{d[PPP]}{dt} = [PPS][PSS] + 2[PPS][PSP] + [PPS]^2 - 4[PPP][SSS] - 2[PPP][PSS] - 4[PPP][SPS] \quad [8]$$

$$\frac{1}{k}\frac{d[SPS]}{dt} = 2[PPS][SSS] + [PPS][PSS] - [PPS]^2 - 2[PSS][SPS] - 4[SPS][PSP] + 4[SSS][PSP] \quad [9]$$

$$\frac{1}{k}\frac{d[PSP]}{dt} = [PPS][PSS] + 2[PPP][PSS] + [PSS]^2 - 4[PSP][SPS] - 2[PPS][PSP] - 4[SSS][PSP] \quad [10]$$

$$\frac{1}{k}\frac{d[PPS]}{dt} = -[PPS][PSS] + 2[PPP][PSS] + 4[PPP][SSS] - 2[PPS][PSP] - [PPS][PSS] - 2[PSS][SPS] + 2[PSS][SPS] + 4[PSP][SPS] + 4[PPP][SPS] - [PPS]^2 \quad [11]$$

$$\frac{1}{k}\frac{d[PSS]}{dt} = -PPS][PSS] - 2[PPP][PSS] + 4[PPP][SSS] + 2[PPS][PSP] - 2[PSS][SPS] + 2[PPS][SSS] + 4[PSP][SPS] - [PPS][PSS] + 4[SSS][PSP] - [PSS]^2 \quad [12]$$

Equations 7–12 can be solved analytically for the special case when the initial concentrations of the triglycerides are equal. Therefore, the concentration change of each component as a function of other concentrations measured in the reaction system with time can be expressed as (see appendix for derivation):

$$[PPP] = \left(\frac{T}{4}\right)\{1 + \exp(-4kTt)\}^2 \quad [13]$$

$$[PPS] = \left(\frac{T}{2}\right)\{1 - \exp(-8kTt)\} \quad [14]$$

$$[SPS] = \left(\frac{T}{4}\right)\{1 - \exp(-4kTt)\}^2 \quad [15]$$

where T is the initial concentration of each triglyceride. By symmetry, the equations for SSS, PSS and PSP will be the same as Equations 13, 14 and 15, respectively. Equations 13, 14 or 15 can be rearranged to give the following expressions for the rate constant:

$$k = -\left(\frac{1}{4Tt}\right)\ln\left\{\left(\frac{4[PPP]}{T}\right)^{0.5} - 1\right\} \quad [16]$$

$$k = -\left(\frac{1}{8Tt}\right)\ln\left\{1 - \left(\frac{2[PPS]}{T}\right)\right\} \quad [17]$$

$$k = -\left(\frac{1}{4Tt}\right)\ln\left\{1 - \left(\frac{4[SPS]}{T}\right)^{0.5}\right\} \quad [18]$$

This model can therefore predict the kinetics of all six triglycerides from only one parameter, the reaction rate constant k, and the initial concentrations of the triglycerides.

RESULTS AND DISCUSSION

To compare the model with experimental data, it was first necessary to obtain a value for the transesterification rate constant, k. The following experiments were done to determine the value of k for a given initial substrate concentration: Equal molar concentrations of tripalmitin and tristearin were added to *n*-hexane containing variable amounts of modified lipase (92.6–1111 mg/L). Figures 2–6 show the time course of the changes in concentration of PPP, SSS, PPS plus PSP, and SPS plus PSS. It was assumed in the model that by symmetry the concentration time courses of PPP and SSS, PSP and SPS, and also of PSS and PPS, would be identical, and it can be seen that this was a reasonable assumption. The time taken for the system to reach equilibrium depended on the enzyme concentration and was about 1 h with 1111 mg/L biocatalyst concentration and about 10 h with 92.6 mg/L biocatalyst concentration. The values of k at different time intervals before equilibrium was reached were calculated according to Equation 16 for each enzyme concentration. The average value of k for each experiment was calculated and plotted against the enzyme concentration (Fig. 7). The data could be fitted by linear regression ($r^2 = 0.98$), and the resulting straight line had a gradient, the specific rate constant (k^*), of $2.52 \cdot 10^{-4}$ [L^2/(mmol • h • mg biocatalyst)]. This value was substituted

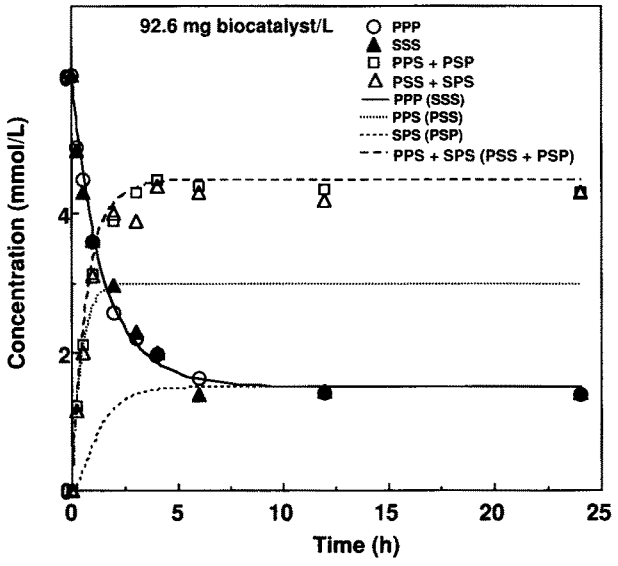

FIG. 2. Concentration profiles of triglycerides with 92.6 mg modified lipase/L. The points represent the experimental data, and the lines represent the solutions of Equations 9–13 for a value of 2.5×10^{-4} [L^2/(mmol • h • mg cat.)]. Reaction conditions: 6 mmol/L SSS and 6 mmol/L PPP dissolved in 55 mL *n*-hexane stirred at 800 rpm at 40°C. Abbreviations as in Figure 1.

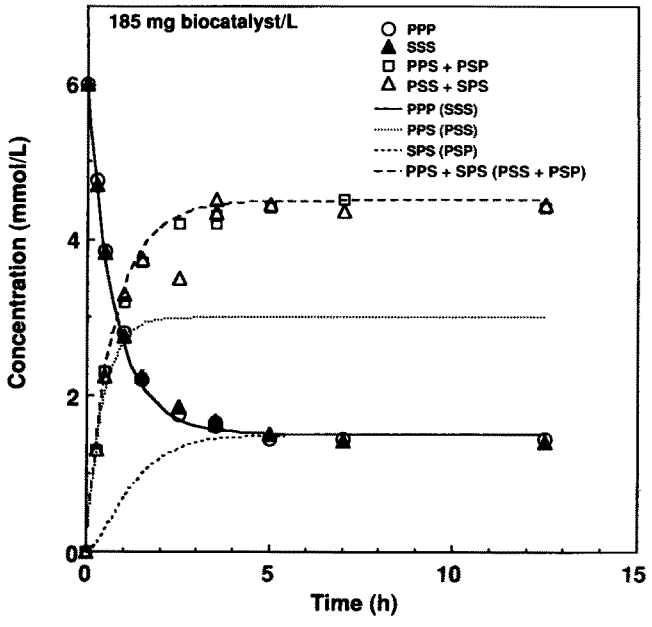

FIG. 3. Triglyceride concentration time profiles for 185 mg modified lipase/L. Abbreviations as in Figure 1.

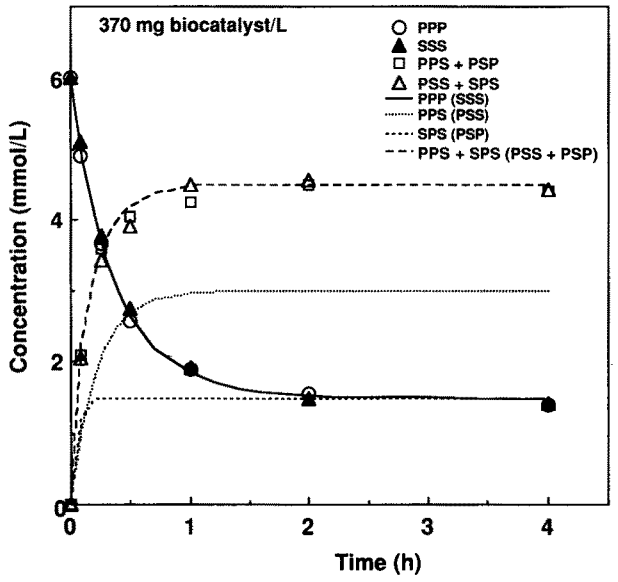

FIG. 4. Triglyceride concentration time profiles for 370 mg modified lipase/L. Abbreviations as in Figure 1.

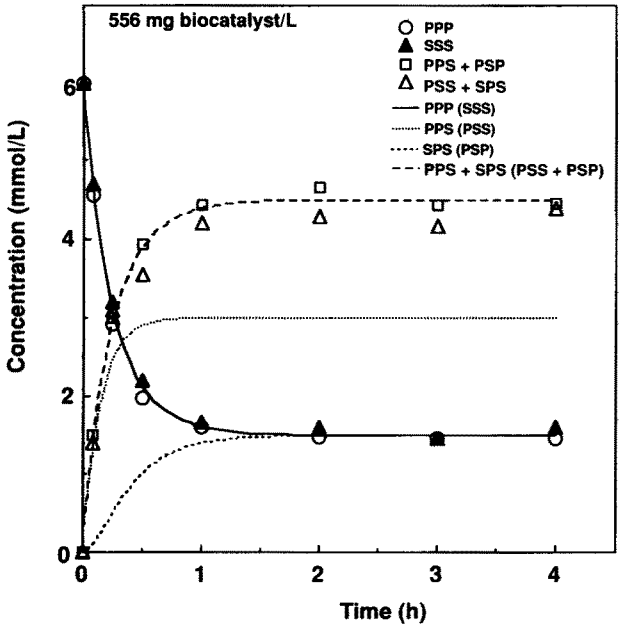

FIG. 5. Triglyceride concentration time profiles for 556 mg modified lipase/L. Abbreviations as in Figure 1.

into Equations 13–15 to calculate the time course of the concentrations of all six triglycerides. Figures 2–6 compare the experimental data with the model prediction for five different enzyme concentrations by using the same value of k^*. There is good agreement between the experimental data and the model prediction in all cases. It was not possible to separate triglyceride isomers, but if the predicted concentrations of the two triglycerides are added together, good agreement with the experimental data is obtained.

Figure 8 shows the time course of the acidolysis reaction between PPP (6 mmol/L) and stearic acid (12 mmol/L) with 92.6 mg of modified lipase/L. The molar ratio of stearic acid to PPP was 2 to 1, which allowed a comparison between the acidolysis reaction and the transesterification reaction between PPP and SSS. Because the enzyme has 1,3-positional specificity, the molar ratio of stearic acid residues to palmitic

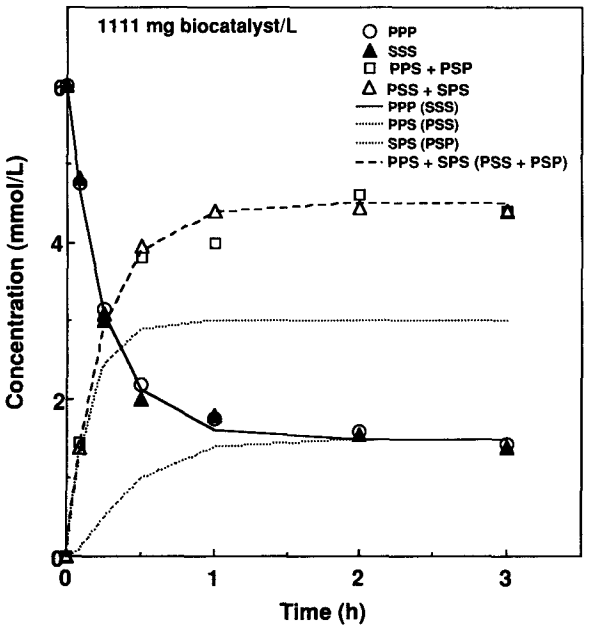

FIG. 6. Triglyceride concentration time profiles for 1111 mg modified lipase/L. Abbreviations as in Figure 1.

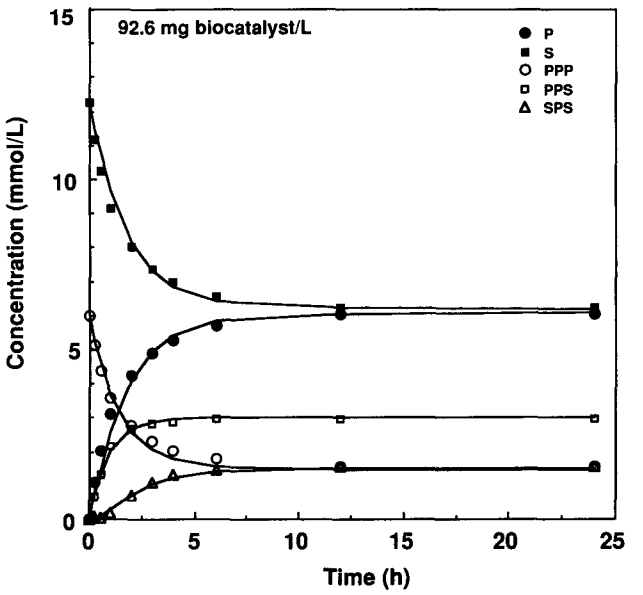

FIG. 8. Time course of the acidolysis of PPP (6 mmol/L) and stearic acid (12 mmol/L) with 92.6 mg of modified lipase/L. Abbreviations as in Figure 1. P, palmitic acid; S, stearic acid.

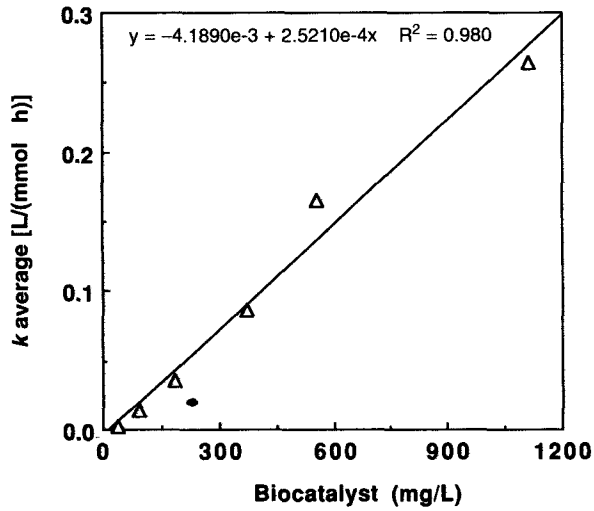

FIG. 7. Average k values calculated according to Equation 14 for different concentrations of modified lipase.

acid residues was the same in both experiments. In a previous study, a kinetic model was proposed for the acidolysis of PPP by stearic acid with the same modified lipase in *n*-hexane. The value of k^* obtained above for the transesterification reaction [$2.52 \cdot 10^{-4}$ L^2/(mmol • h • mg biocatalyst)] was substituted into this acidolysis model. In Figure 8, the points show the experimental data, and the lines show the model prediction. There is excellent agreement between the experimental data and the model. The fact that the same value of k^* could be used to model both transesterification and acidolysis reactions implies that the enzyme does not distinguish between a free fatty acid and a fatty acid attached to positions 1 and 3 of a glycerol backbone.

The proposed model accurately predicted the transesterification kinetics of the lipase-catalyzed reaction between SSS and PPP in microaqueous *n*-hexane. The advantages of this model are that it requires only one parameter, which can be easily determined experimentally, and that an analytical solution is possible when the initial concentrations of the substrates are equal. When the initial substrate concentrations are not equal, the model can easily be solved by simulation software. The limitations of the model are that it ignores the production of the diglycerides detected, albeit at low concentrations. The water content is not taken into account in the model, although it is known that it significantly affects enzyme performance. The justification for this is that all experiments were performed at constant water content and, in practice, this is likely to be the case. Also, the precise mechanism by which the water content influences the behavior of lipases is not well understood. The model is based on material balances for second-order reversible reactions and does not take into account the formation of enzyme substrate complexes. Although it has been demonstrated that transesterification reactions proceed *via* a ping-pong bi-bi mechanism (19), many experiments are required to determine the kinetic constants. The proposed model has the advantage of having only one parameter that can be determined simply and allows a comparison of different lipases and different substrates, as well as different reactions with a minimum of experimentation. This approach has proved effective in the screening of different lipase and surfactant combinations for acidolysis activity (15).

The transesterification rate constant is expected to be a function of the initial substrate concentration, and further

work is in progress to characterize the effect of substrate concentration on the kinetics of transesterification reactions.

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APPENDIX

This appendix shows the derivation of the kinetic model employed to describe the transesterification reaction of two triglycerides using modified lipase.

For simplicity, let:

$$A = [PPP],\ B = [PPS],\ C = [SPS],\ D = [PSP],\ E = [PSS],\ F = [SSS]$$

rate equations,

$$\frac{dA}{dt} = k[-4AF + BE - 2AE + 2BD - 4AC + B^2] \quad [A1]$$

$$\frac{dB}{dt} = k[4AF - BE + 2AE - 2BD + 4AC - B^2 + 4CD - BE - 2BF + 2CE] \quad [A2]$$

$$\frac{dC}{dt} = k[-4CD + BE + 2BF - 2CE - 4AC + B^2] \quad [A3]$$

$$\frac{dD}{dt} = k[-4CD + BE + 2AE - 2BD - 4DF + E^2] \quad [A4]$$

$$\frac{dE}{dt} = k[4CD - BE - 2AE + 2BD + 4DF - E^2 + 4AF - BE + 2BF - 2CE] \quad [A5]$$

$$\frac{dF}{dt} = k[-4AF + BE - 2BF + 2CE - 4DF + E^2] \quad [A6]$$

For the special case when the initial concentrations of both triglycerides are equal, these differential equations can be solved. The initial conditions are therefore:

$$A = T,\ B = 0,\ C = 0,\ D = 0,\ E = 0,\ F = T \quad [A7]$$

Assuming 1,3 specificity:

$$A + B + C = T \quad [A8]$$

$$D + E + F = T \quad [A9]$$

Mass balances on stearic acid (S) and palmitic acid (P) yield:

$$3A + 2B + C + 2D + E = 3T \quad [A10]$$

$$B + 2C + D + 2E + 3F = 3T \quad [A11]$$

Rearranging equations A8, A9, A10 and A11 gives:

$$2D + E = 2T - 2A - B \quad [A12]$$

$$2F + E = 2T - 2C - B \quad [A13]$$

Combining equations A1, A2, A3 and A8 leads to:

$$\frac{1}{k}\frac{d(A + B + C)}{dt} = B^2 - 4AC = 0 \quad [A14]$$

Similarly, for equations A4, A5, A6 and A11:

$$\frac{1}{k}\frac{d(D + E + F)}{dt} = E^2 - 4DF = 0 \quad [A15]$$

From equations A1 to A6 and A12 to A15:

$$\frac{1}{k}\frac{dA}{dt} = -2T(2A - B) \quad [A16]$$

$$\frac{1}{k}\frac{dB}{dt} = -4T(B - A - C) \quad [A17]$$

$$\frac{1}{k}\frac{dC}{dt} = -2T(2C - B) \quad [A18]$$

$$\frac{1}{k}\frac{dD}{dt} = -2T(2D - E) \quad [A19]$$

$$\frac{1}{k}\frac{dE}{dt} = -4T(E - D - F) \quad [A20]$$

$$\frac{1}{k}\frac{dF}{dt} = -2T(2F - E) \quad [A21]$$

From equations A16 and A18:

$$\frac{1}{k}\frac{d(A - C)}{dt} = -4T(A - C) \quad [A22]$$

Solving equation A22:

$$A - C = C_1 \exp(-4kTt) \quad [A23]$$

Initial conditions:

$$A = T, C = 0, \text{ at } t = 0; \quad [A24]$$

From equations A23 and A24:

$$C = A - T\exp(-4kTt) \quad [A25]$$

From equations A8 and A25:

$$B = T - 2A + T\exp(-4kTt) \quad [A26]$$

From equations A16 and A26:

$$\frac{1}{k}\frac{dA}{dt} = -8T(A - T/4) + 2t^2 \exp(-4kTt) \quad [A27]$$

Solving equation A27:

$$A = \frac{T}{4} + C_2 \exp(-8kTt) + \left(\frac{T}{2}\right)\exp(-4kTt) \quad [A28]$$

Initial conditions:

$$A = T \text{ at } t = 0; \quad [A29]$$

From equations A28 and A29

$$A = \left(\frac{T}{4}\right)\{1 + \exp(-4kTt)\}^2 \quad [A30]$$

From equations A25, A26 and A30

$$B = \left(\frac{T}{2}\right)\{1 - \exp(-8kTt)\} \quad [A31]$$

$$C = \left(\frac{T}{4}\right)\{1 - \exp(-4kTt)\}^2 \quad [A32]$$

$$D = C, E = B, \text{ and } F = A; \quad [A33]$$

Rearranging equations A30 to A33 gives the following expressions for k:

$$k = -\left(\frac{1}{4Tt}\right)\ln\left\{\left(\frac{4A}{T}\right)^{0.5} - 1\right\} \quad [A34]$$

$$k = -\left(\frac{1}{8Tt}\right)\ln\left\{1 - \left(\frac{2B}{T}\right)\right\} \quad [A35]$$

$$k = -\left(\frac{1}{4Tt}\right)\ln\left\{1 - \left(\frac{4C}{T}\right)^{0.5}\right\} \quad [A36]$$

$$k = -\left(\frac{1}{4Tt}\right)\ln\left\{1 - \left(\frac{4D}{T}\right)^{0.5}\right\} \quad [A37]$$

$$k = -\left(\frac{1}{8Tt}\right)\ln\left\{1 - \left(\frac{2E}{T}\right)\right\} \quad [A38]$$

$$k = -\left(\frac{1}{4Tt}\right)\ln\left\{\left(\frac{4F}{T}\right)^{0.5} - 1\right\} \quad [A39]$$

Hence k can be estimated from any of the equations A34 to A39.