Chapter 3

Reaction Kinetics of Interesterification Using Modified Lipase

3–1 Interesterification Kinetics of Triglycerides and Fatty Acids Using Modified Lipase in *n*-Hexane

Introduction

In Chapter 2, various surfactants and lipases were screened for interesterification activity between triglycerides and fatty acids. It was found that the best combination was lipase from *Rhizopus japonicus* with sorbitan monostearate. Subsequent studies 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 had 1.3-positional specificity for the interesterification 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 lot of quantitative experimental data dealing with lipase-catalyzed interesterification of triglycerides and fatty acids have been reported, however, many of them have not discussed on the kinetics of the reaction system. Several kinetic models based on different mechanisms have been proposed to describe the degree of conversion of substrate in the reaction system[57,58]. These models involve two to six parameters, depending on the reaction mechanism proposed. These parameters were determined by fitting the calculated results of each model with the experimental data using a trial-and-error method. Another model to analyze the kinetics of interesterification reaction of triglycerides and fatty acids that has appeared in the literature is based on a two-step reaction mechanism: initial hydrolysis and subsequent re-esterification[71]. This model is based on a proposed Ping-Pong Bi-Bi enzymatic mechanism and it consists of five parameters for the hydrolysis reaction and nine parameters for the re-esterification reaction. The lipases used in earlier kinetic studies were either crude dried cells or powdered enzymes mixed with stabilizers. Mass transfer limitations had to be considered in the first case but were neglected in the second case[66].

In this section, the interesterification kinetics of triglycerides and fatty acids using surfactant-modified lipase was studied. Tripalmitin (PPP) and stearic acid in *n*-hexane was chosen as a model system. For the sake of simplification, a simple kinetic model

with only one parameter was developed. This model is based on a material balance involving three consecutive second order reversible reactions.

Materials and methods

Materials

All chemicals and lipase used in this section were mentioned in Chapter 2-1.

Lipase modification

Lipase Saiken 100 (*R. japonicus*) and Emazol S-10 (F) (sorbitan monostearate) were used for the modification. Lipase modification was carried out according to the method described in Chapter 2-2.

Interesterification reaction

The interesterification reaction of PPP and stearic acid catalyzed by modified lipase was performed in a 60-mL glass flask with Teflon faced septa. Reactions were initiated by adding 30 mg of modified lipase into 55 mL *n*-hexane containing 0.55 g PPP (12.4 mmol/L) and 0.55 g stearic acid (35.1 mmol/L). The water content in the reaction system after the addition of the substrates and the modified lipase in *n*-hexane dried over molecular sieves was about 25 mg/L. The temperature of the reaction system was controlled at 40°C by immersion in a water bath. The reaction system was magnetically stirred at 800 rpm. Samples (0.5 mL) were periodically withdrawn from the reaction system and filtered using disposable Millipore filters (pore size 0.5 μm). All experiments, unless stated otherwise, were carried out under the above conditions.

Analytical methods

The analysis of tri-, di- and monoglycerides and fatty acids were done by GC and HPLC methods described in Chapters 2-1 and 2-2. The protein content was measured by the nitrogen analyzer (Chapter 2-1).

Water contents in the reaction system were measured with a Karl Fisher Titrator 684. After being freeze-dried, the modified lipase contained 0.041 mg H_2O/(mg biocatalyst). The water content in the *n*-hexane was reduced to 10 mg/L by drying over molecular sieves [300 g molecular sieves/(5 L *n*-hexane)]. All substrates were desiccated below 0°C.

Kinetic modeling

The interesterification reaction of tripalmitin (PPP) and stearic acid (S) catalyzed by the modified lipase with 1,3-positional specificity can be analyzed as a homogenous reaction system consisting of two consecutive interesterification reactions and also as one concurrent transesterification reaction. In the first step, stearic acid substitutes for the palmitate residue on the *sn*-1 or *sn*-3 position of the glycerol moiety to form 1,2-dipalmitoyl-3-stearoyl glycerol (PPS) (Eq. 1). In the second step, stearic acid replaces the palmitate residue in the *sn*-1 position of the PPS to produce 1,3-distearoyl-2-palmitoyl glycerol (SPS) (Eq. 2). Palmitic acid (P) is released in both steps. A transesterification reaction may also occur between PPP and SPS to produce PPS (Eq. 3).

To describe a simple mathematical model for the aforementioned interesterification reaction system, the following assumptions were adopted and later were experimentally verified:

- The lipase used has 1,3-positional specificity and therefore tristearin (SSS) is not produced in the reaction system.
- Eqs. 1, 2 and 3 are reversible reactions. The enzyme used in this study has no fatty acid specificity; it acts concurrently on any acyl group.
- There are no losses of enzyme activity during the course of the reaction. Steady state is achieved after some time and there is no further change in the compositions of the system after addition of more biocatalyst.

- Formation of diglycerides (around 6 wt% of initial concentration of triglycerides) was not taken into account for the sake of simplification of the model. Monoglycerides and glycerol were not detected under the reaction conditions employed for the kinetic study.
- Mass transfer limitations in the reaction system were ruled out because of the homogeneity of the system when the lipophilized lipase was used.

The scheme of the interesterification reaction of PPP and S can be shown as follows:

$$PPP + S \underset{k}{\overset{2k}{\rightleftharpoons}} PPS + P \qquad (1)$$

$$PPS + S \underset{2k}{\overset{k}{\rightleftharpoons}} SPS + P \qquad (2)$$

$$PPP + SPS \underset{k^0}{\overset{4k^0}{\rightleftharpoons}} 2PPS \qquad (3)$$

where k represents the interesterification reaction rate constants, and k^0 represents the transesterification reaction rate constant. If the biocatalyst used has no specificity towards either stearic or palmitic acid (P) residues, then the production rate of PPP from PPS and P in the reverse reaction of Eq. 1 is similar to that of SPS from PPS and S in Eq. 2. According to the law of probability, the disappearance rate of the symmetrical substrates, PPP and SPS, have rate constants whose values are twice of those for the nonsymmetrical substrate (PPS). A similar argument can be applied for Eq. 3.

Rate equations for S, P, PPP, SPP and SPS in the reaction system can be derived from Eqs. 1, 2 and 3 as follows:

$$\frac{d[S]}{dt} = -2k[PPP][S] + k[PPS][P] - k[PPS][S] + 2k[SPS][P] \quad (4)$$

$$\frac{d[P]}{dt} = 2k[PPP][S] - k[PPS][P] + k[PPS][S] - 2k[SPS][P] \quad (5)$$

$$\frac{d[PPP]}{dt} = -2k[PPP][S] + k[PPS][P] + k^0([PPS]^2 - 4[PPP][SPS]) \quad (6)$$

$$\frac{d[PPS]}{dt} = -k[PPS][P] + 2k[PPP][S] - k[PPS][S] + 2k[SPS][P] + k^0 (4[PPP][SPS] - [PPS]^2) \quad (7)$$

$$\frac{d[SPS]}{dt} = -2k[SPS][P] + k[PPS][S] + k^0 ([PPS]^2 - 4[PPP][SPS]) \quad (8)$$

The total concentration of triglycerides, T, is constant during the interesterification reaction because hydrolysis reactions are assumed to be negligible. Eqs. 4-8 can be solved analytically under the initial conditions (t=0) [PPP] = T, [PPS] = 0, [SPS] = 0, [S] = S_0, and [P] = 0, where S_0 is the initial concentration of stearic acid. The concentration change of each component as a function of other concentrations measured in the reaction system with time can be expressed as indicated in Eqs. 9-13 (see appendix);

$$[S] = (S_0/S^*)\{S_0 + 2T \exp(-kS^*t)\} \quad (9)$$

$$[P] = (2TS_0/S^*)\{1 - \exp(-kS^*t)\} \quad (10)$$

$$[PPP] = (T/S^{*2})\{4T^2 + 4TS_0 \exp(-kS^*t) + S_0^2 \exp(-2kS^*t)\} \quad (11)$$

$$[PPS] = (T/S^{*2})\{4TS_0 + 2S_0(S_0 - 2T)\exp(-2kS^*t) - 2S_0^2 \exp(-2kS^*t)\} \quad (12)$$

$$[SPS] = (TS_0^2/S^{*2})\{1 - 2\exp(-2kS^*t) + \exp(-2kS^*t)\} \quad (13)$$

where $S^* = S_0 + 2T$

Eq. 9 and also Eq. 10 can be rearranged for k to give Eqs. 14 and 15;

$$k = (-1/S^*t)\ln\{([S]S^* - S_0^2)/2TS_0\} \quad (14)$$

$$k = (-1/S^*t)\ln\{(2TS_0 - S^*[P])/2TS_0\} \quad (15)$$

Eqs. 14 and 15 represent the interesterification rate constant as a function of time and fatty acid concentration for a given enzyme concentration and initial concentrations of triglyceride and fatty acid. A specific interesterification reaction rate constant, k*, can be defined as

$$k^* = k/E \quad (16)$$

where E is the biocatalyst concentration.

A set of experiments where the initial concentrations of PPP and stearic acid were kept constant but the biocatalyst concentration, E, was varied, were carried out in order to determine the value of k*. k values were determined at different times before concentration of steady state was reached for each enzyme concentration. The average value of k for each experiment was plotted against the biocatalyst concentration. A straight line was fitted to the data by linear regression and the specific rate constant was calculated from the gradient.

Results and Discussion

Specificity of the modified lipase and steady state of the reaction

To check the positional specificity of the modified lipase to catalyze the substitution of fatty acids on each of the three positions of the glycerol moiety, a reaction was carried out and the components in the reaction system were monitored for 8 h. Steady state was achieved in the reaction system after around 5 h. A fresh batch of 30 mg of modified lipase was added after 10 h to the reaction mixture and the system was stirred for a further 15 h. The profile of all components in the reaction system is shown in Fig. 3-1-1. It can be seen that the modified lipase catalyzed predominantly the interesterification of PPP and stearic acid in the presence of a low water content (25 mg/L).

GC and HPLC analysis showed that the modified lipase can also catalyze the hydrolysis of triglycerides to release diglycerides (e.g. 1,2-dipalmitin, PPG). 1,2-Diglycerides are chemically unstable and therefore they undergo either spontaneously

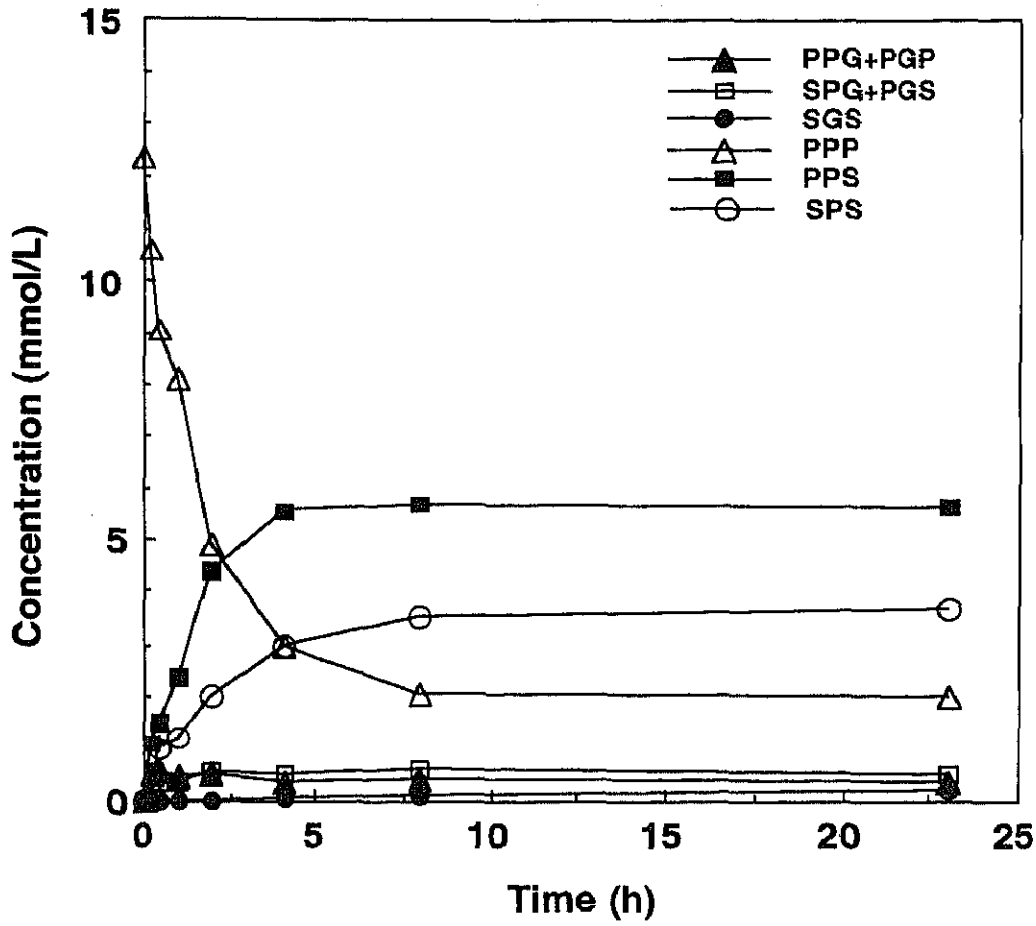

Fig. 3-1-1 Triglycerides and diglycerides concentration profiles resulting from the modified-lipase-catalyzed interesterification of tripalmitin (PPP) and stearic acid (S). PPG, 1,2-dipalmitin; PGP, 1,3-dipalmitin; SPG, 1-stearoyl 2-palmitoyl glycerol; PGS, 1-palmitoyl 3-stearoyl glycerol; SGS, 1,3-distearin; PPS, 1,2-palmitoyl 3-stearoyl glycerol; SPS, 1,3-stearoyl 2-palmitoyl glycerol; and SSS, tristearin. The reaction was carried out in 55 mL *n*-hexane containing 0.55 g PPP, 0.55 g S and 30 mg modified lipase. The reaction mixture containing 25 mg/L water was magnetically stirred at 800 rpm and thermostated at 40°C.

or enzymatically-aided acyl migration to form 1,3-diglycerides[41,61]. This isomerization of PPG explains the formation of 1-palmitoyl-3-stearoyl glycerol (PGS), which proceeds via a further lipase-catalyzed interesterification reaction with stearic acid to form 1,3-distearin (SGS) after prolonged reaction time. However, the percentage of total diglycerides produced in this system did not exceed 6 wt% of the initial PPP concentration. The degree of hydrolysis using the modified lipase to catalyze interesterification reactions of triglycerides and fatty acids is lower than the hydrolysis activities of other lipases studied to date under similar reaction conditions[64,72,73]. It can also be seen that the steady state for the interesterification of PPP and stearic acid under the conditions described was achieved after about 5 h and that of PPG, PGP and stearic acid to produce SPG, SGP and palmitic acid was rapidly established after less than 1 h. Lack of any further changes in the compositions of the reaction system after adding a fresh batch of modified lipase into the system demonstrates that the steady state corresponds to equilibrium. Furthermore, GC and HPLC analysis showed that SSS was not formed in the reaction system. This result provides further evidence of 1,3-positional specificity of the modified lipase.

The substrate specificity of lipases used to catalyze interesterification reactions is normally dependent on the type of fatty acids to be incorporated into the glycerol moiety, as well as on the types of acyl groups of the glycerol backbone. For example, it was found that the chain length of fatty acids and their degree of saturation significantly affect the extent of incorporation of fatty acids into triglycerides[39]. However, to study the substrate specificity of the modified lipase with respect to stearic and palmitic acids, the following two experiments were performed: (a) Stearic acid (35.1 mmol/L) and PPP (12.4 mmol/L), and (b) palmitic acid (35.1 mmol/L) and SSS (12.4 mmol/L) were each dissolved in 55 mL *n*-hexane and stirred magnetically with 30 mg modified lipase at 800 rpm and thermostated at 40°C. The results presented in Fig. 3-1-2 clearly show that the modified lipase does not distinguish between stearic and

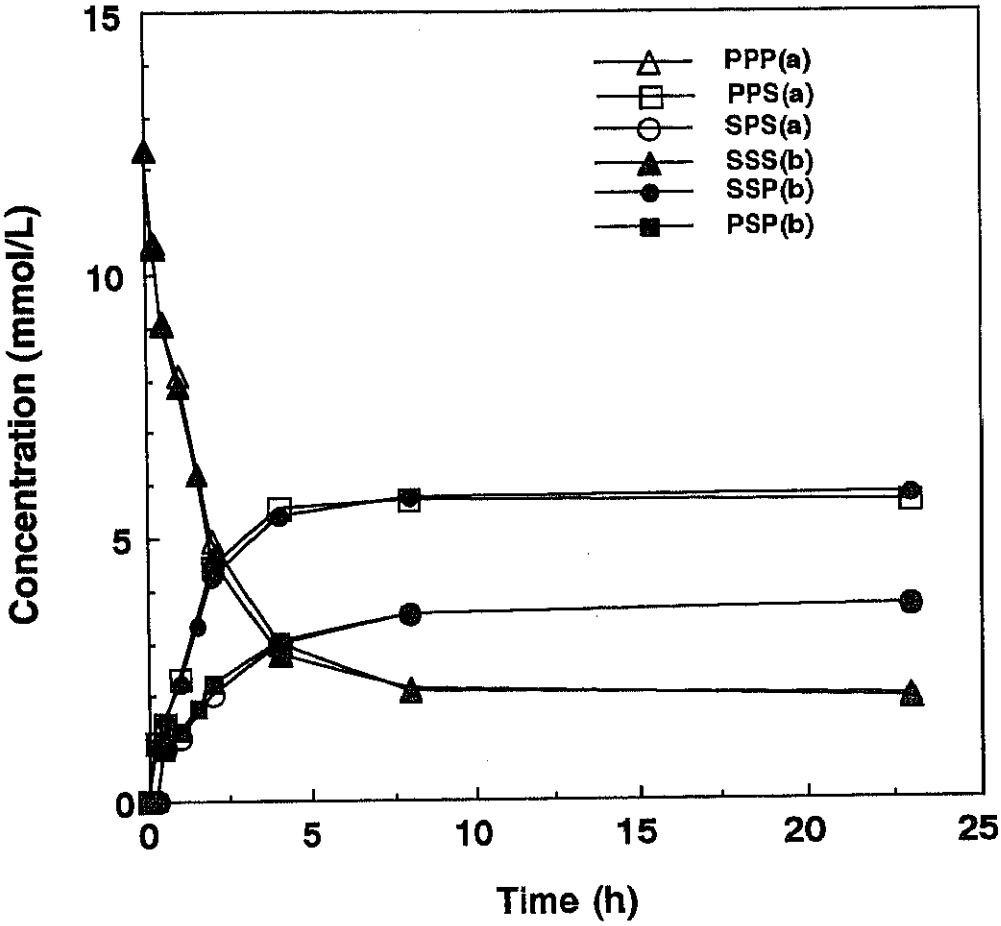

Fig. 3-1-2 Time course of the lipase-catalyzed interesterification of (a): tripalmitin (PPP) and stearic acid (S), and (b): the interesterification of tristearin (SSS) and palmitic acid (P). Initial reaction conditions of (a) were 35.1 mmol/L S and 12.4 mmol/L PPP, and of (b) were 35.1 mmol/L P and 12.4 mmol/L mmol SSS. The substrates in both systems were dissolved in 55 mL *n*-hexane and stirred magnetically with 30 mg biocatalyst at 800 rpm and thermostated at 40°C. Abbreviation as in Figure 3-1-1.

palmitic acids. Also, it was noticed that the biocatalyst has no preferential specificity towards PPP and SSS when used as substrates.

Kinetics of the interesterification reaction

It has been reported in many studies that organic solvents used for lipase-catalyzed reactions have a significant effect on the steady state position of the reaction systems. This effect is most likely to be attributed to the variable solubility of water in different organic solvents[59,98]. Therefore, in the present study, the interesterification reaction system model of PPP and stearic acid was performed in a water-dried *n*-hexane system containing constant concentrations of substrates whereas the concentration of the modified lipase was varied accordingly. Figure 3-1-3 shows the concentration profiles of PPP using varying concentrations of the biocatalyst. GC analysis showed that around 6% diglycerides of initial PPP concentration in all reaction systems were produced as byproducts due to an instantaneous hydrolysis reaction which rapidly reached steady state. Figure 3-1-3 shows that the interesterification reaction system reached steady state after 4 h when more than 1111 mg biocatalyst/L was used, and 8 h for those containing 370 and 556 mg biocatalyst/L. The values of k at different time intervals for each experiment were calculated according to Eqs. 14 and 15. The average k values of each experiment, when the concentrations of PPP and stearic acid are not limiting factors, were plotted against the biocatalyst concentration, as shown in Fig. 3-1-4. The plot is close to a straight line with slope k^* equal to 1.22×10^{-5} [L^2/(mmol•mg biocatalyst•h)]. The obtained k^* value was substituted into Eqs. 9-13 to calculate the changes of all components in the interesterification reaction system with time. It is apparent that the obtained k^* value gave a satisfactory fit with all of the experimental data using constant substrate concentrations of PPP and stearic acid and varied modified lipase concentrations (Figures 3-1-5 to 3-1-9). Therefore, this method demonstrates a simple model to describe the interesterification reaction of triglycerides and fatty acids using a constant concentration of substrates and water so that the steady

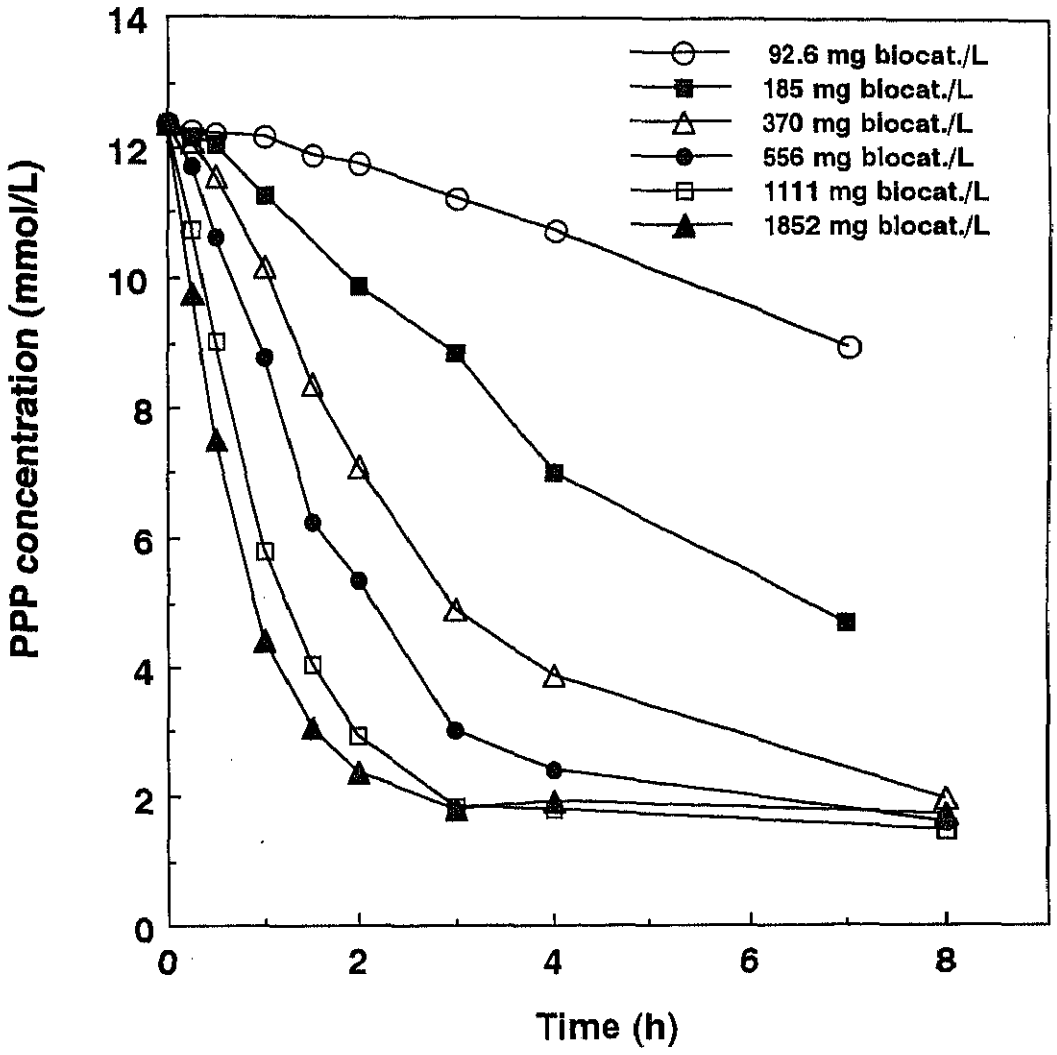

Fig. 3-1-3 The concentration of tripalmitin (PPP) with time in the reaction system using different concentrations of biocatalyst. Reaction conditions: 0.55 g S and 0.55 g PPP dissolved in 55 mL *n*-hexane and stirred at 800 rpm and 40°C. The concentrations of the biocatalyst were 92.6, 185, 370, 556, 1111, and 1852 mg/L.

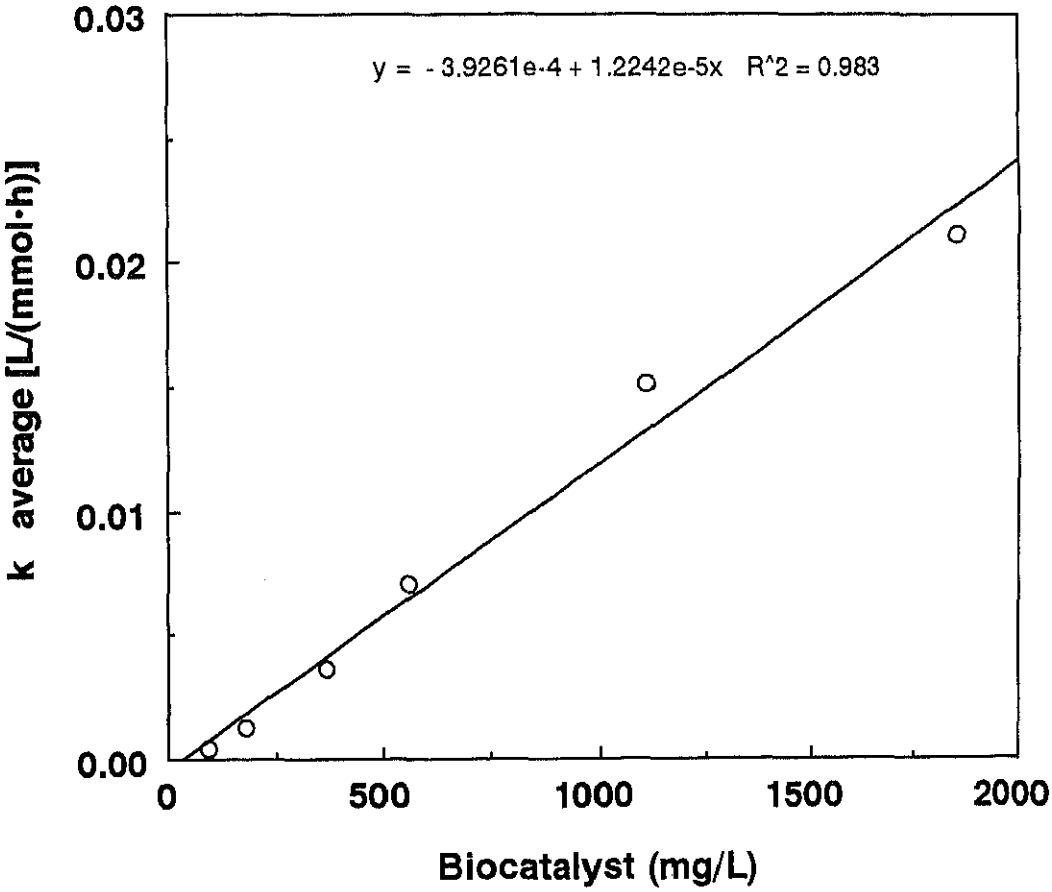

Fig. 3-1-4 The average of k values calculated according to Eq. 14 for each experiment at different time intervals against the biocatalyst concentration.

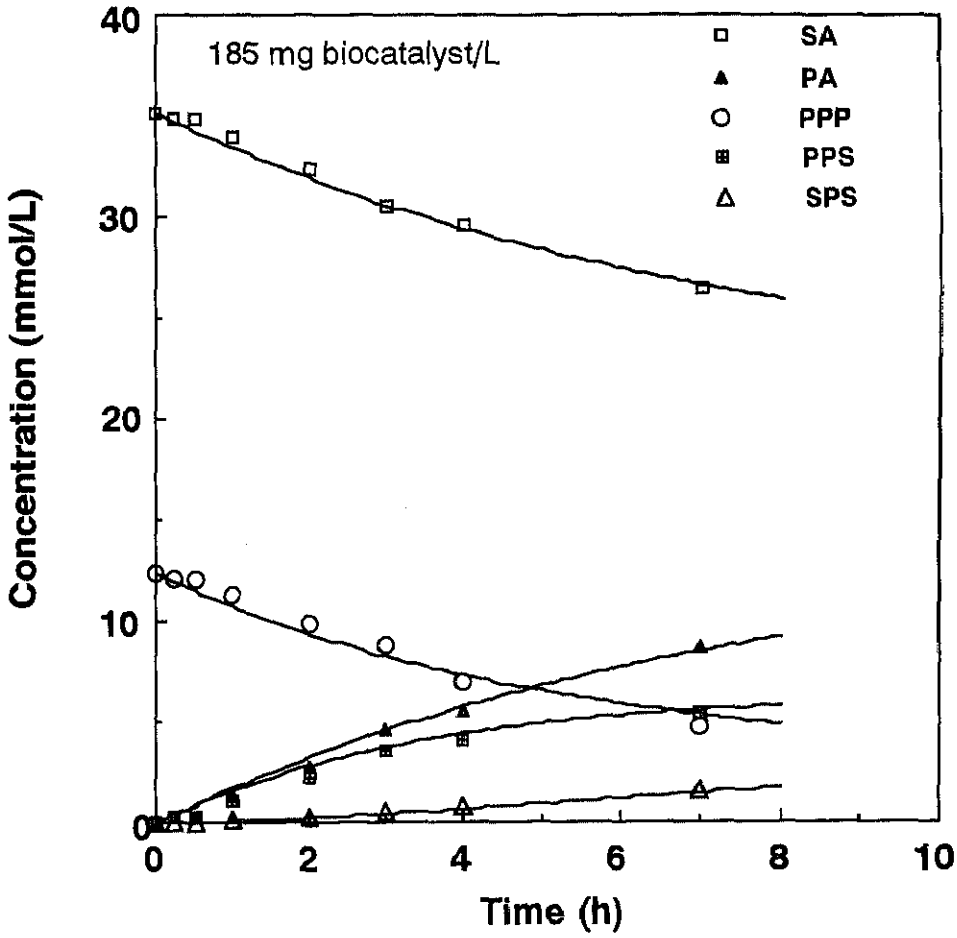

Fig. 3-1-5 Concentration profiles of fatty acids and triglycerides in the reaction system using 185 mg biocatalyst/L. Reaction conditions as in Figure 3-1-3. The points in the graph represent the experimental data, and the lines represent the analytical solutions of Eqs. 9-13 for each component in the reaction mixture using $k^*=1.22 \times 10^{-5}$ [L^2/(mmol·mg biocatalyst·h)]. SA, stearic acid; PA, palmitic acid; PPP, tripalmitin; PPS, 1,2-palmitoyl 3-stearoly glycerol; SPS, 1,3-stearoly 2-palmitoyl glycerol.

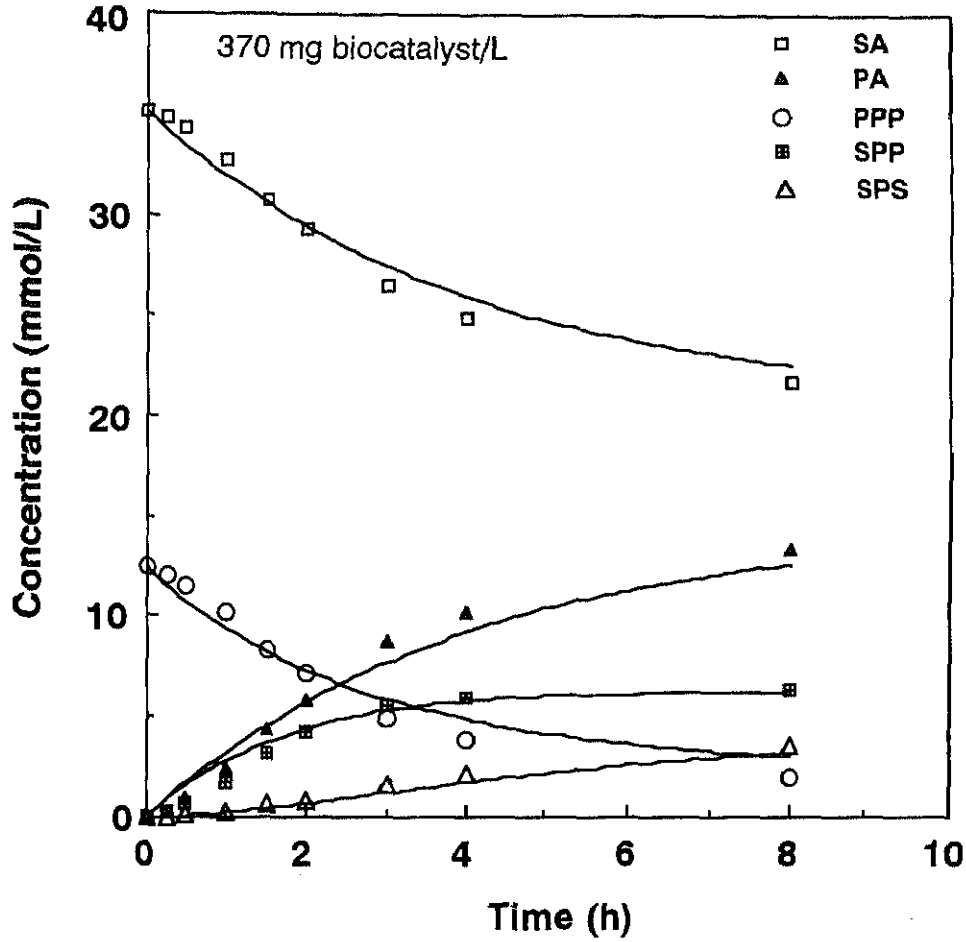

Fig. 3-1-6 Concentration profiles of fatty acids and triglycerides. Reaction conditions as in Figure 3-1-5 but using 370 mg biocatalyst/L. Abbreviation as in Figure 3-1-5

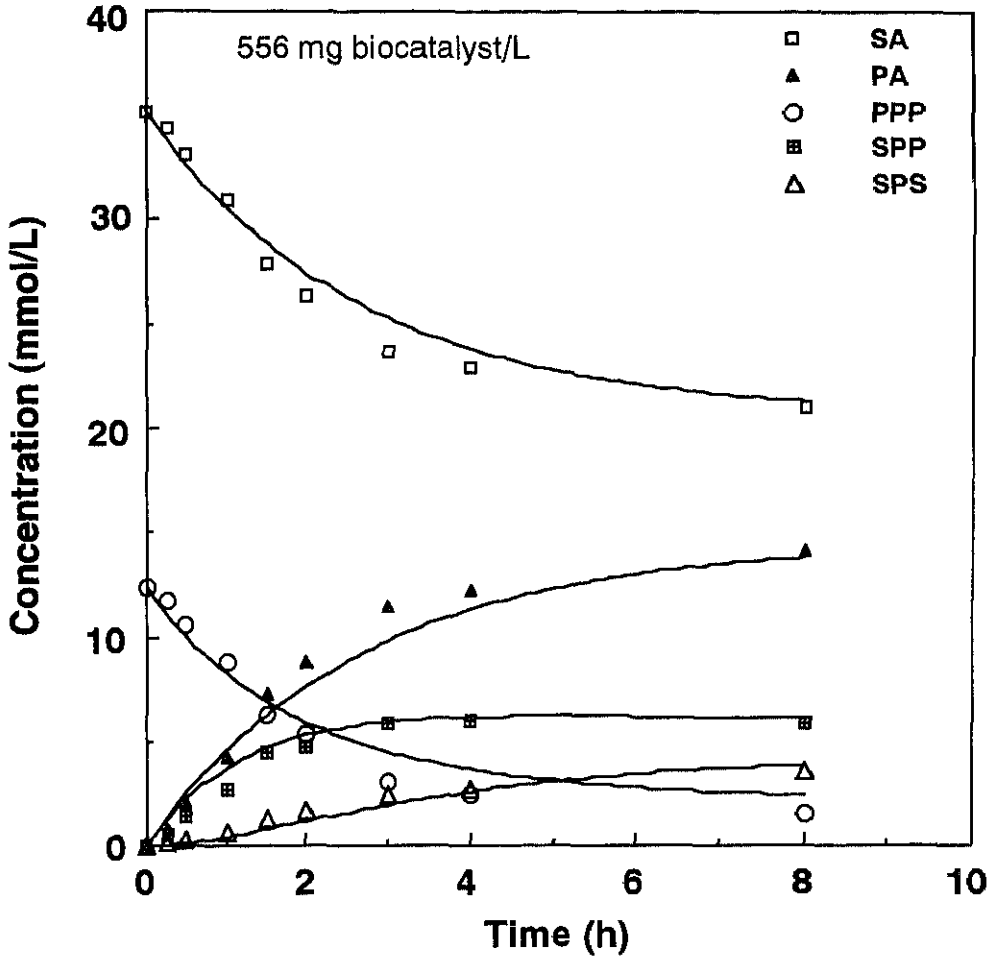

Fig. 3-1-7 Concentration profiles of fatty acids and triglycerides. Reaction conditions as in Figure 3-1-5 but using 556 mg biocatalyst/L. Abbreviation as in Figure 3-1-5.

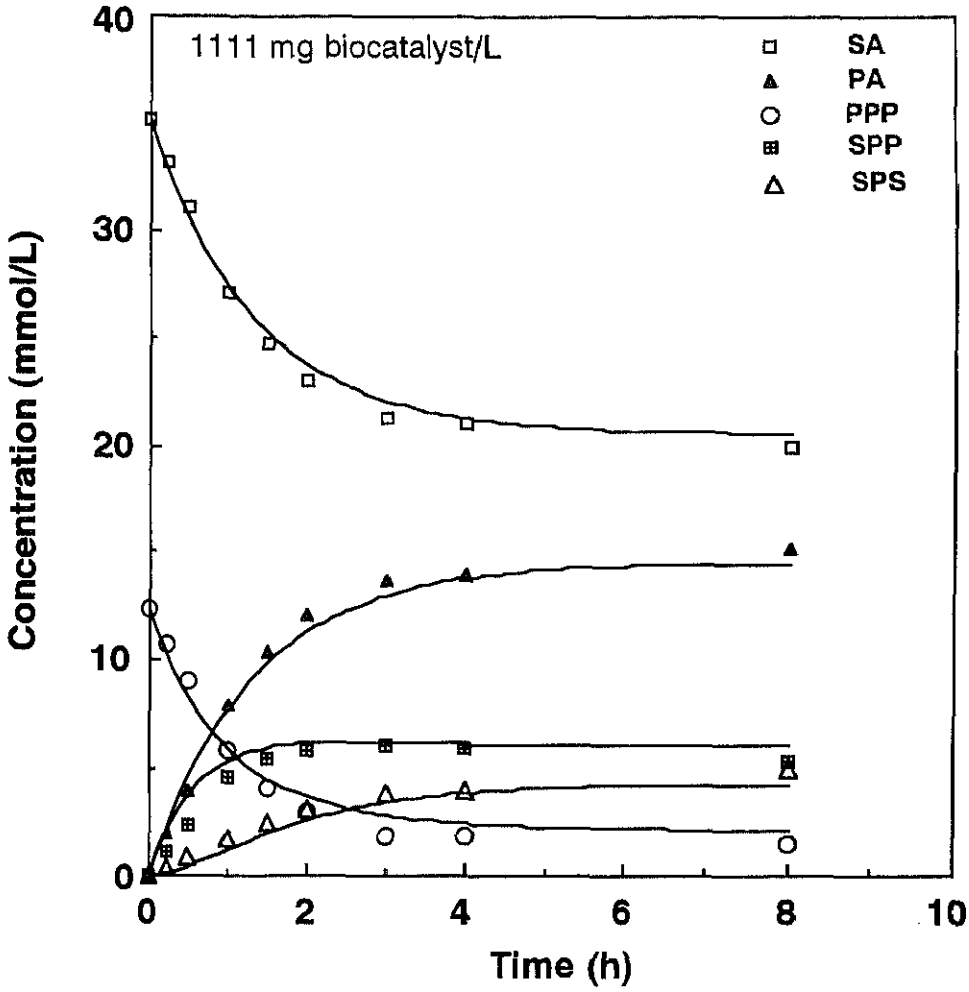

Fig. 3-1-8 Concentration profiles of fatty acids and triglycerides. Reaction conditions as in Figure 3-1-5 but using 1111 mg biocatalyst/L. Abbreviation as in Figure 3-1-5.

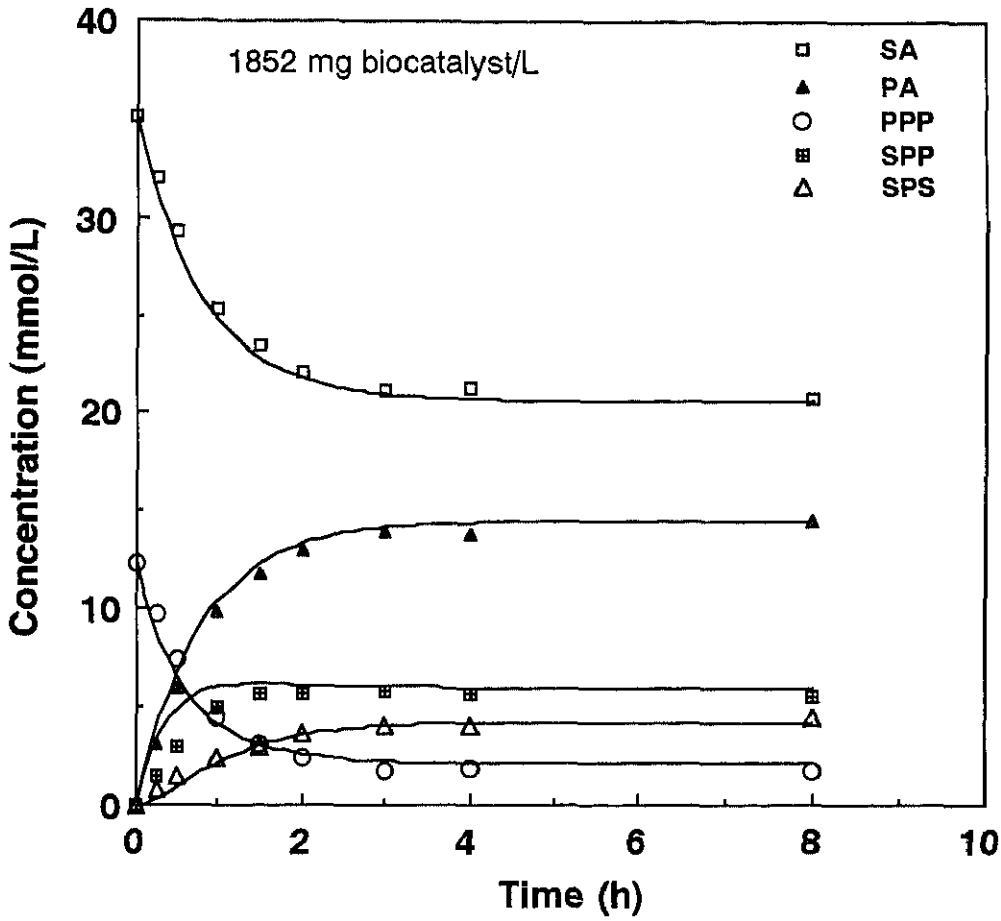

Fig. 3-1-9 Concentration profiles of fatty acids and triglycerides. Reaction conditions as in Figure 3-1-5 but using 1852 mg biocatalyst/L. Abbreviation as in Figure 3-1-5.

state of the interesterification reaction is not shifted. The proposed model has the advantage that it has only one parameter that can be determined very 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 to be very effective in screening of different lipase and surfactant combinations for interesterification activity whereas the concentrations of substrates are kept constant in micro-aqueous media.

Appendix

This appendix contains the derivation of the kinetic model employed to describe the interesterification reaction of triglycerides and fatty acids in the presence of a modified lipase.

For simplicity;

A = [PPP], B = [PPS], C = [SPS], P = [P], and S = [S]

The rate equation for each component described in Eqs. 1-3 is as follows;

$$\frac{dS}{dt} = k[-2AS + BP - BS + 2CP] \quad (A1)$$

$$\frac{dP}{dt} = k[2AS - BP + BS - 2CP] \quad (A2)$$

$$\frac{dA}{dt} = k[-2AS + BP + (\frac{k^o}{k})(B^2 - 4AC)] \quad (A3)$$

$$\frac{dB}{dt} = k[2AS - BP - BS + 2CP + (\frac{k^o}{k})(4AC - B^2)] \quad (A4)$$

$$\frac{dC}{dt} = k[-2CP + BS + (\frac{k^o}{k})(B^2 - 4AC)] \quad (A5)$$

Initial conditions: A = T, B = 0, C = 0, S = S_0, P = 0

Mass balances on the triglycerides (T) and fatty acids give:

$$A + B + C = T \quad (A6)$$

$$S + P = S_0 \quad (A7)$$

A mass balance on stearic acid yields:

$$S + B + 2C = S_0 \quad (A8)$$

where the subscript zero denotes the initial value. Symbols without subscripts denote concentrations at any reaction time t.

From Eqs. A3, A4, A5, and A6;

$$\left(\frac{1}{k}\right)\frac{d(A+B+C)}{dt} = \left(\frac{k^0}{k}\right)(B^2 - 4AC) = 0 \qquad (A9)$$

Substituting Eqs. A6-A8 in Eqs. A1, A2, A3, A4, A5, and A9 produces;

$$(1/k)\frac{dS}{dt} = -(S_0 + 2T)S + S_0^2 \qquad (A10)$$

$$(1/k)\frac{dP}{dt} = -(S_0 + 2T)P + TS_0 \qquad (A11)$$

$$(1/k)\frac{dA}{dt} = -2S_0A + 2TS_0 - 2TS - P^2 \qquad (A12)$$

$$(1/k)\frac{dB}{dt} = -2S_0B + 2TS + P^2 - SP \qquad (A13)$$

$$(1/k)\frac{dC}{dt} = -2S_0C + SP \qquad (A14)$$

Solving Eqs. A10 and A11 for the initial conditions of the interesterification reaction at t = 0 gives the concentrations of S and P measured as a function with time;

$$S = (S_0/S^*)\{S_0 + 2T\exp(-kS^*t)\} \qquad (A15)$$

$$P = (2TS_0/S^*)\{1 - \exp(-kS^*t)\} \qquad (A16)$$

where, $S^* = S_0 + 2T$

Substituting Eqs. A15 and A16 in Eqs. A12-A14 gives;

$$(1/kS_0)dA/dt = -2(A - 4T^3/S^{*2}) - \{4T^2(2T-S_0)/S^{*2}\}\exp(-kS^*t) - (4T^2S_0/S^{*2})\exp(-2kS^*t) \qquad (A17)$$

$$(1/kS_0)dB/dt = -2(B - 4T^2S_0/S^{*2}) + \{2T(2T-S_0)^2/S^{*2}\}\exp(-kS^*t) + \{8T^2S_0/S^{*2}\}\exp(-2kS^*t) \qquad (A18)$$

$$(1/kS_0)dC/dt = -2(C - TS_0^2/S^{*2}) + \{2T\,S_0\,(2T-S_0)/S^{*2}\}\exp(-kS^*t) - \{4T^2S_0/S^{*2}\}\exp(-2kS^*t) \qquad (A19)$$

Eqs. A17-A19 can be solved to give;

$$\frac{A}{T} = 4T^2/S^{*2} + A_1\exp(-2kS_0t) + 4TS_0/S^{*2}\exp(-kS^*t)$$

$$+ S_0^2/S^{*2}\exp(-2kS^*t) \qquad \text{(A20)}$$

$$\frac{B}{T} = 4TS_0/S^* + B_1\exp(-2kS_0t) + 2S_0(S_0-2T)/S^{*2}\exp(-2kS^*t)$$

$$- 2S_0^2/S^{*2}\exp(-2kS^*t) \qquad \text{(A21)}$$

$$\frac{C}{T} = S_0^2/S^{*2} + C_1\exp(-2kS_0t) - 2S_0^2/S^{*2}\exp(-kS^*t)$$

$$+ S_0^2/S^{*2}\exp(-2kS^*t) \qquad \text{(A22)}$$

Substituting the initial concentrations A = T, B = 0, C = 0 at t = 0 in Eqs. A20-A22 gives the change of triglycerides concentrations with time;

$$A = (T/S^{*2})\{4T^2 + 4TS_0\exp(-kS^*t) + S_0^2\exp(-2S^*t)\} \qquad \text{(A23)}$$

$$B = (T/S^{*2})\{4TS_0 + (2S_0^2 - 4TS_0)\exp(-kS^*t) - 2S_0^2\exp(-2kS^*t)\} \qquad \text{(A24)}$$

$$C = (TS_0^2/S^{*2})\{1 - 2\exp(-kS^*t) + \exp(-2kS^*t)\} \qquad \text{(A25)}$$

The reaction rate constant k as a function of either stearic or palmitic acid concentration with time can be obtained by rearranging Eq. A15 or A16;

$$k = (-1/S^*t)\ln\{(S^*S/S_0 - S_0)/2T\} \qquad \text{(A26)}$$

or;

$$k = (-1/S^*t)\ln\{1-(S^*P/2T\,S_0\} \qquad \text{(A27)}$$

3–2 Interesterification Kinetics of Triglycerides Using Modified Lipase in *n*-Hexane

Introduction

A kinetic model of the interesterification of triglycerides and fatty acids was proposed in Chapter 3-1 and gave good agreement with the experimental data for the interesterification of tripalmitin and stearic acid. However, for some important industrial applications such as oil modification[56], interesterification of triglycerides offer an attractive alternative. For this reason it was decided to extend the original study to investigate interesterification kinetics of triglycerides. In the literature, several models have been proposed for the kinetics of interesterification between triglycerides and fatty acids[58,71] but little has been published on the kinetics of interesterification between triglycerides. Zaks and Klibanov[107] studied the kinetics of the interesterification 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.*[15] proposed a similar mechanism for the interesterification of geraniol and propyl acetate with inhibition by excess geraniol. The interesterification reaction was modeled by a modified Michaelis-Menten equation that required four parameters (two Michaelis constants, one inhibition constant and the maximum reaction rate). Malcata *et al.*[66] have pointed out the large number of parameters necessary to model interesterification between triglycerides, 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.

In this section, similar approach as the interesterification model described in Chapter 3-1 was applied to the interesterification between triglycerides.

Materials and Methods

Materials

All chemicals and lipase used in this section were mentioned in Chapter 2-1.

Lipase modification

Lipase Saiken 100 (*R. japonicus*) and Emazol S-10 (F) (sorbitan monostearate) were used for the modification. Lipase modification was carried out according to the method described in Chapter 2-2.

Analysis method

Triglycerides were analyzed by HPLC (Chapter 2-2). Tripentadecanoin was used as an internal standard. This technique made it possible to separate and accurately quantify triglycerides that contained different numbers of palmitic acid and stearic acid residues (Fig. 3-2-1), but it was not, however, possible to separate triglyceride isomers i.e., 1,3-dipalmitoyl-2-stearoyl glycerol (PSP) and 1,2-dipalmitoyl-3-stearoyl glycerol (PPS), and 1,2-distearoyl-3-palmitoyl glycerol (PSS) and 1,3-distearoyl-2-palmitoyl glycerol (SPS). GC analysis was used to detect mono- and diglycerides. The glycerides were first silylated and then analyzed by in a capillary column with FID (Chapter 2-1).

The concentration of water in the reaction system was measured by a Karl Fischer Titrator and was determined to be 25mg/L.

Interesterification reaction

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

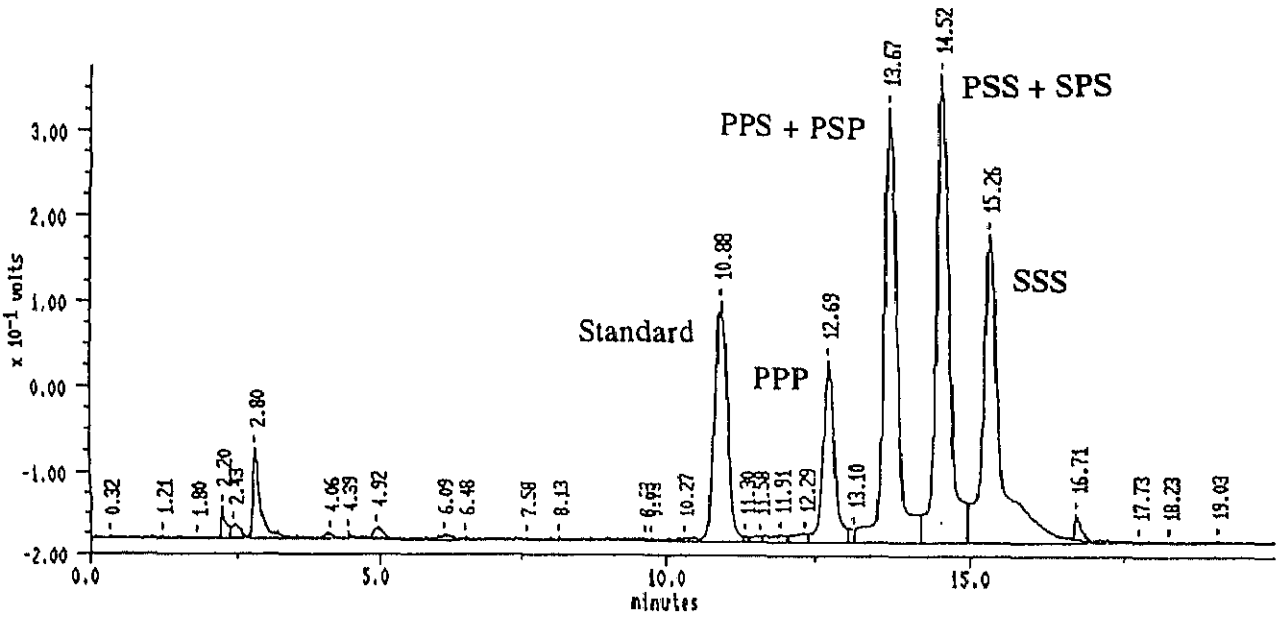

Fig. 3-2-1 HPLC analysis of triglycerides. Internal standard, tripentadecanoin; PPS, 1,2-dipalmitoyl-3-stearoyl glycerol; PSP, 1,3-dipalmitoyl-2-stearoyl glycerol; PSS, 1,2-distearoyl-3-palmitoyl glycerol; PPP, tripalmitin; PSS, 1,2-distearoyl-3-palmitoyl glycerol; SPS, 1,3-distearoyl-2-palmitoyl glycerol; SSS, tristearin.

Kinetic modeling

In a previous study (Chapter 2-1), 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 towards the kind of fatty acid moiety used in this 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 interesterification reactions between tripalmitin (PPP) and tristearin (SSS) as summarized below:

$$\text{PPP} + \text{SSS} \underset{k}{\overset{4k}{\rightleftharpoons}} \text{PPS} + \text{PSS} \qquad (1)$$

$$\text{PPP} + \text{PSS} \underset{2k}{\overset{2k}{\rightleftharpoons}} \text{PPS} + \text{PSP} \qquad (2)$$

$$\text{PPS} + \text{SSS} \underset{2k}{\overset{2k}{\rightleftharpoons}} \text{PSS} + \text{SPS} \qquad (3)$$

$$PPS + PSS \underset{4k}{\overset{k}{\rightleftharpoons}} PSP + SPS \qquad (4)$$

$$PPP + SPS \underset{k}{\overset{4k}{\rightleftharpoons}} 2PPS \qquad (5)$$

$$SSS + PSP \underset{k}{\overset{4k}{\rightleftharpoons}} 2PSS \qquad (6)$$

where k represents the interesterification 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 4 times greater than the reverse rate constant. Because the lipase shows no specificity towards the kind of fatty acid residue and by symmetry, the above argument can be applied to the other interesterification reactions. In other words, when a symmetrical triglyceride reacts with another symmetrical triglyceride, two asymmetrical triglycerides are formed with a rate constant that is 4 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 to 6 as follows:

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

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

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

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

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

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

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 (appendix for derivation):

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

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

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

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:

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

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

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

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 interesterification 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 to 1111mg/L). Figures 3-2-2 to 3-2-6 show the time course of the changes in concentration of PPP, SSS, PPS plus PSP and SPS plus SSP. It was assumed in the model that by symmetry the concentration time courses of PPP and SSS, PSP and SPS and also SSP 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 1111mg/L biocatalyst concentration and about 10 h with 92.6mg/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. 3-2-7). 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 x 10^{-4} [L^2/(mmol•h•mg biocatalyst)]. This value was substituted into Equations 13 to 15 to calculate the time course of the concentrations of all six triglycerides. Figures 3-2-2 to 3-2-6 compare the experimental data with the model prediction for five different enzyme concentrations 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

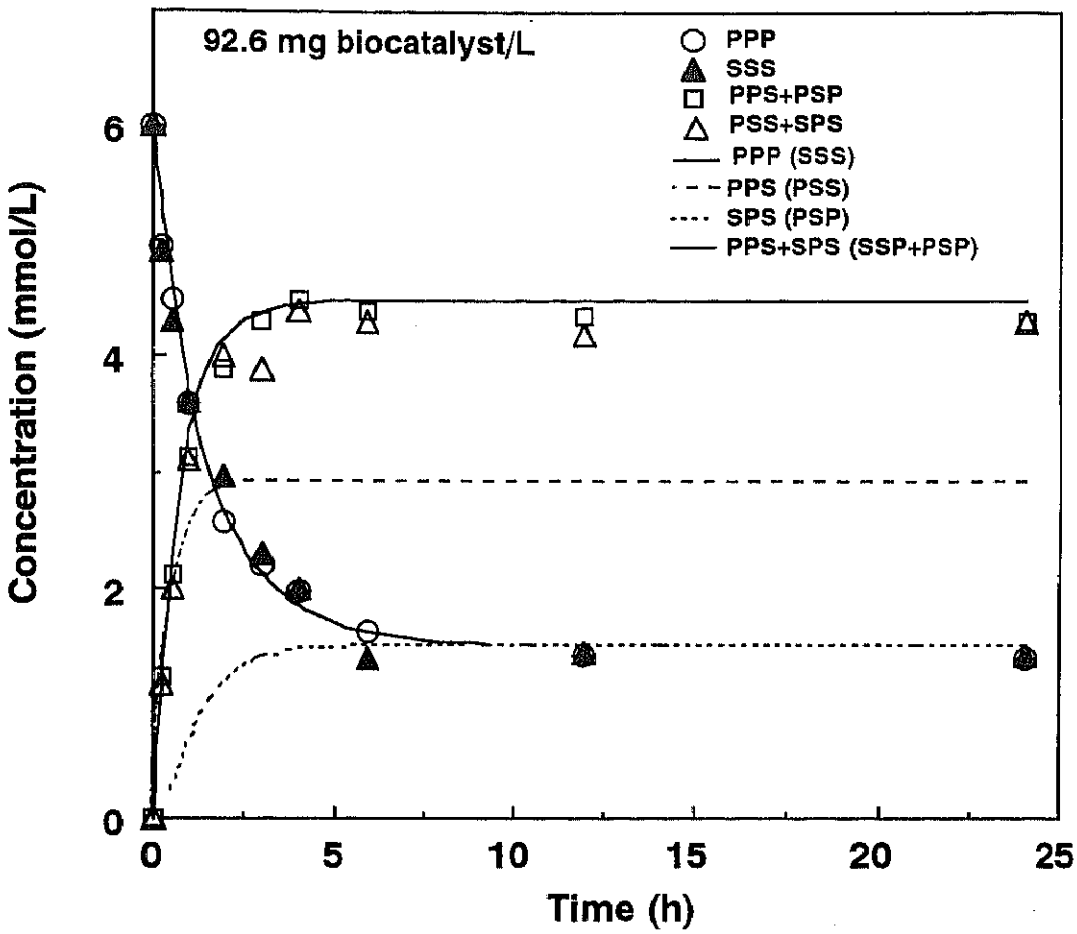

Fig. 3-2-2 Concentration profiles of triglycerides with 92.6 mg modified lipase/L. The points represent the experimental data and the lines represent the solutions of Equations 9-13 for a value of 2.5 x 10^{-4} [L^2/(mmol·h·mg cat.)]. Reaction conditions: 6 mmol/L SSS and 6 mmol/L PPP dissolved in 55 mL *n*-hexane stirred at 800 rpm at 40°C. Abbreviations as in Figure 3-2-1.

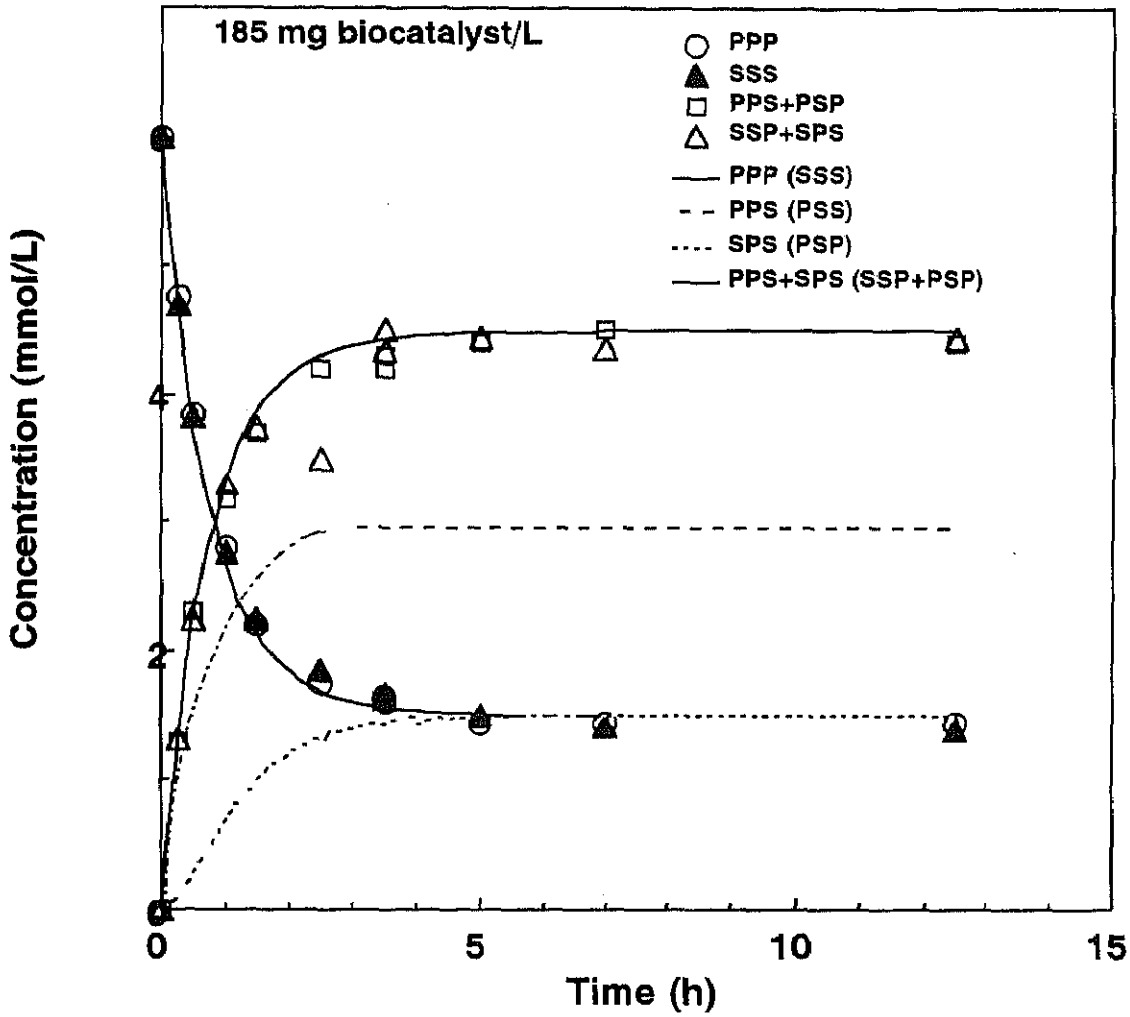

Fig. 3-2-3 Triglyceride concentration time profiles for 185 mg modified lipase/L. Abbreviations as in Figure 3-2-1.

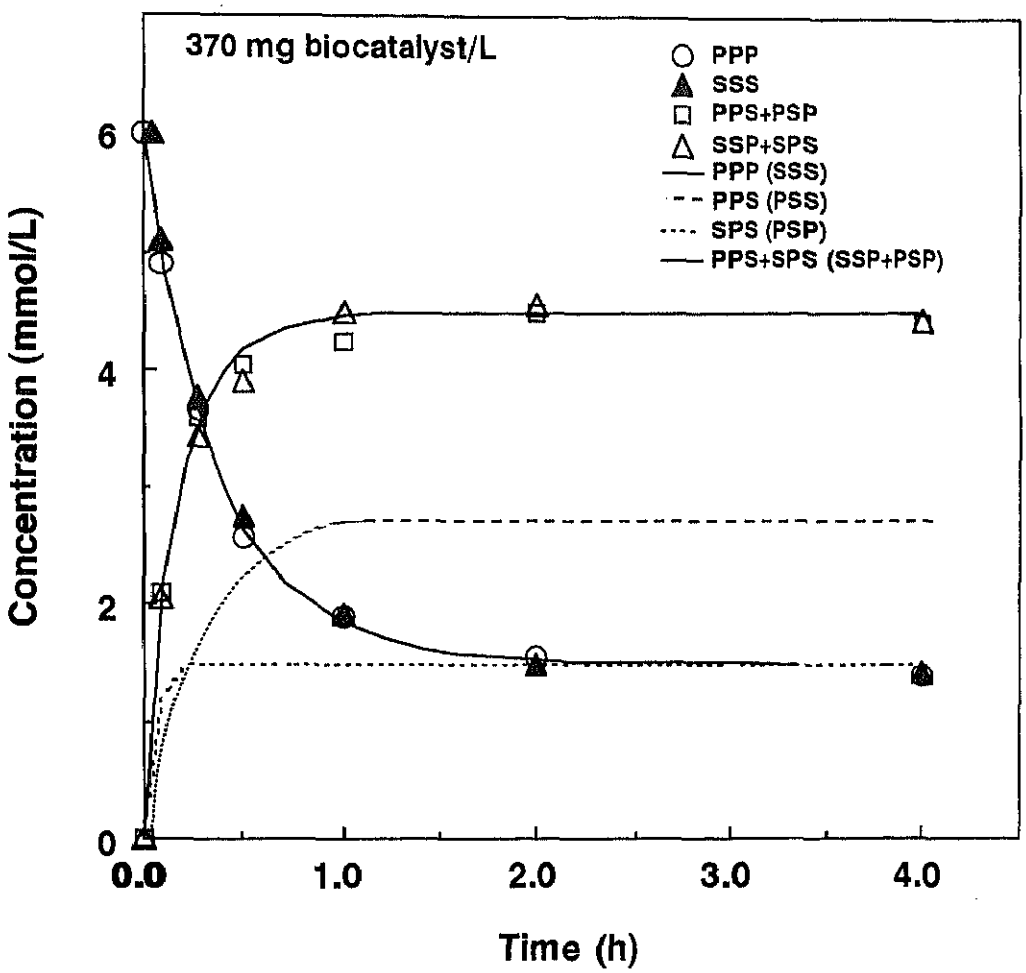

Fig. 3-2-4 Triglyceride concentration time profiles for 370 mg modified lipase/L. Abbreviations as in Figure 3-2-1.

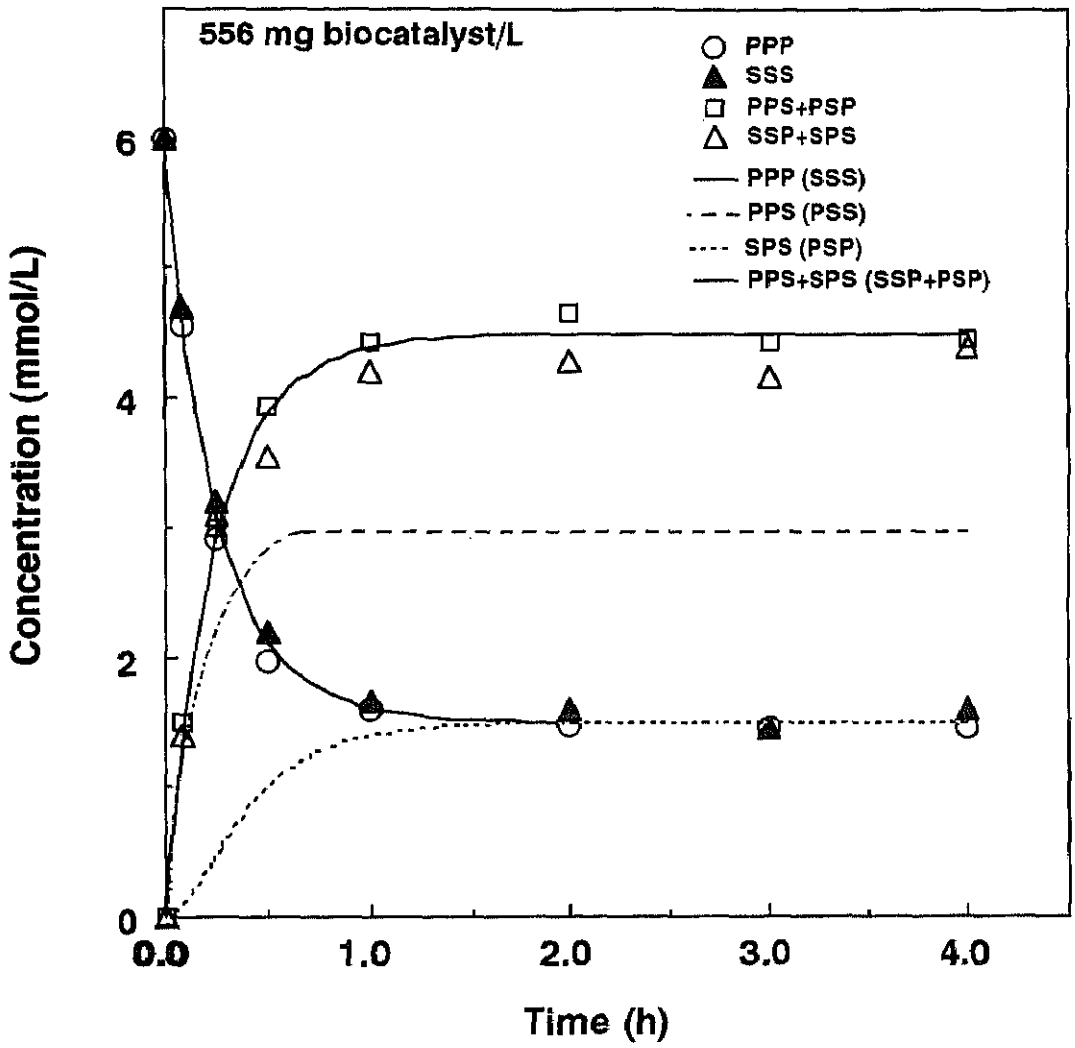

Fig. 3-2-5 Triglyceride concentration time profiles for 556 mg modified lipase/L. Abbreviations as in Figure 3-2-1.

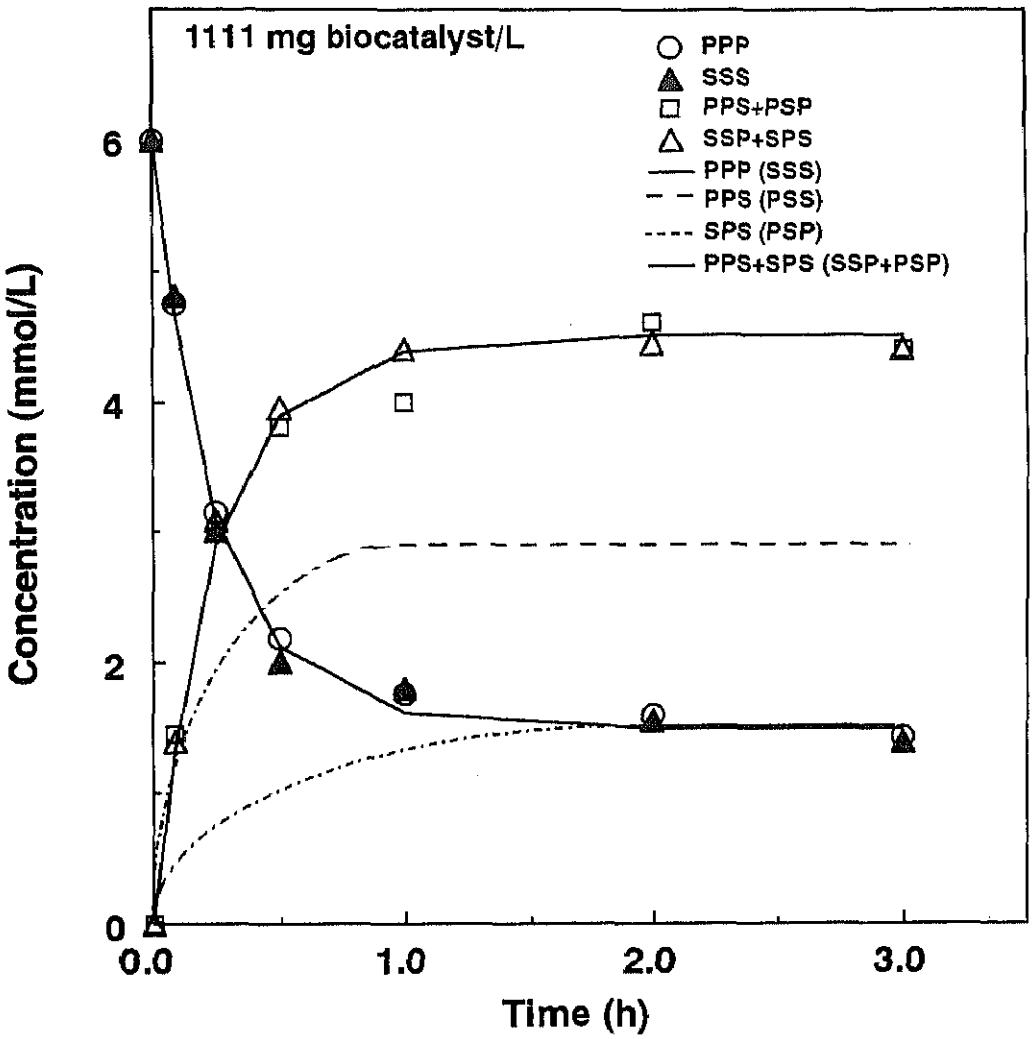

Fig. 3-2-6 Triglyceride concentration time profiles for 1111 mg modified lipase/L. Abbreviations as in Figure 3-2-1.

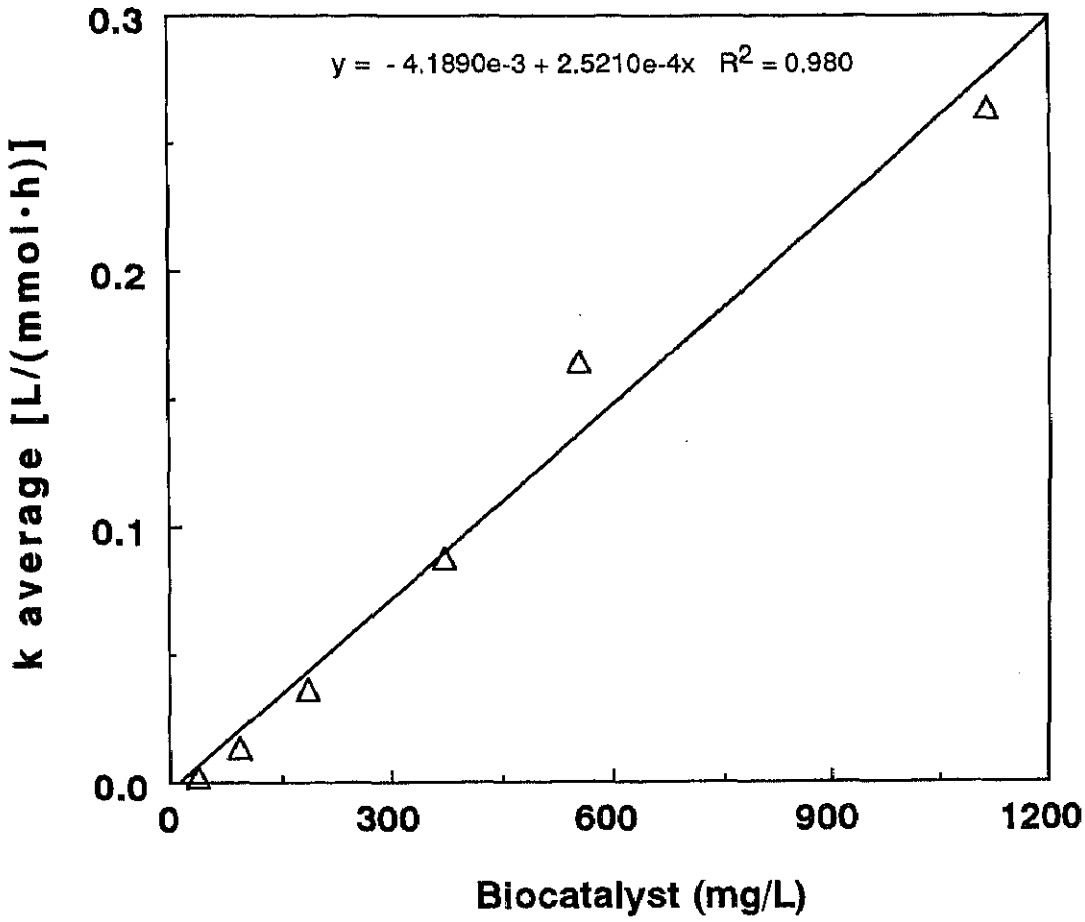

Fig. 3-2-7 Average k values calculated according to Equation 14 for different concentrations of modified lipase.

separate triglyceride isomers, but if the predicted concentration of the two triglycerides are added together, good agreement with the experimental data is obtained.

Figure 3-2-8 shows the time course of the interesterification reaction between PPP (6mmol/L) and stearic acid (12mmol/L) using 92.6mg of modified lipase/L. The molar ratio of stearic acid to PPP was 2 to 1, which allowed a comparison between the interesterification between triglycerides and fatty acids and the interesterification between PPP and SSS. Because the enzyme has 1,3-positional specificity, the molar ratio of stearic acid residues to palmitic acid residues was the same in both experiments. In a previous study (Chapter 3-1), a kinetic model was proposed for the interesterification of PPP by stearic acid with the same modified lipase in *n*-hexane. The value of k* obtained above for the interesterification reaction [2.52×10^{-4} L^2/(mmol•h•mg biocatalyst)] was substituted into this interesterification model. Figure 3-2-8, the points shows 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 the interesterification between triglycerides and interesterification between triglycerides and fatty acids 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 interesterification kinetics of the lipase-catalyzed reaction between PPP and SSS 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 for the case 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 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

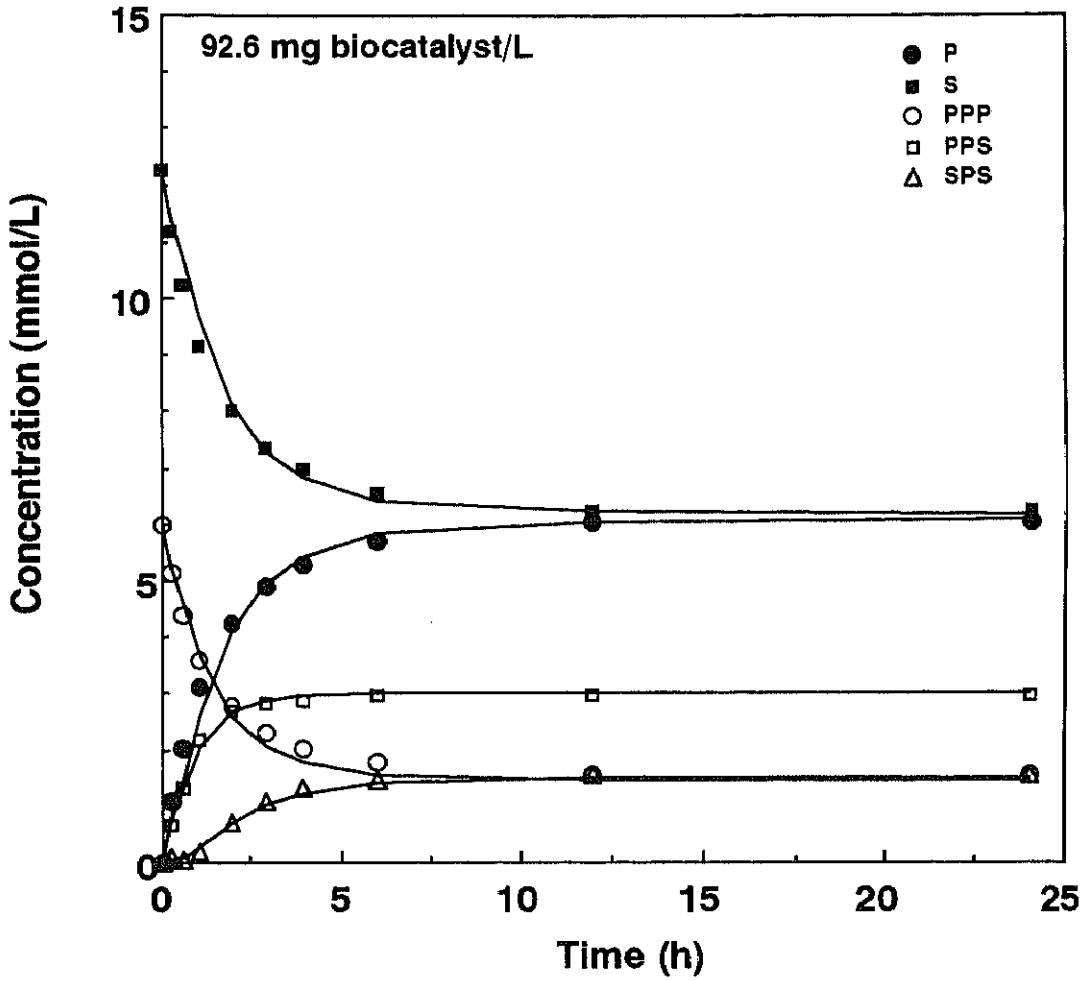

Fig. 3-2-8 Time course of the acidolysis of PPP (6 mmol/L) and stearic acid (12 mmol/L) with 92.6 mg of modified lipase/L. Abbreviations as in Figure 3-2-1. P, palmitic acid; S, stearic acid.

and, in practice, this is likely to be often 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 interesterification reactions proceed via a Ping-Pong Bi-Bi mechanism[71], may experiments are required to determine the kinetics 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 proven effective in the screening of different lipase and surfactant combinations for interesterification activity.

The interesterification 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 interesterification reactions.

Appendix

This appendix shows the derivation of the kinetic model employed to describe the interesterification 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 (A7)

Assuming 1,3 specificity,

A + B + C = T (A8)

D + E + F = T (A9)

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

3A + 2B + C + 2D + E = 3T (A10)

B + 2C + D + 2E + 3F = 3T (A11)

Rearranging equations A8, A9, A10 and A11 gives:

$$2D + E = 2T - 2A - B \tag{A12}$$

$$2F + E = 2T - 2C - B \tag{A13}$$

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

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

Similarly, for equations A4, A5, A6 and A11

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

From equations A1 to A6 and A12 to A15

$$\frac{1dA}{k\,dt} = -2T(2A - B) \tag{A16}$$

$$\frac{1dB}{k\,dt} = -4T(B - A - C) \tag{A17}$$

$$\frac{1dC}{k\,dt} = -2T(2C - B) \tag{A18}$$

$$\frac{1dD}{k\,dt} = -2T(2D - E) \tag{A19}$$

$$\frac{1dE}{k\,dt} = -4T(E - D - F) \tag{A20}$$

$$\frac{1dF}{k\,dt} = -2T(2F - E) \tag{A21}$$

From equations A16 and A18:

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

Solving equation A22:

$$A - C = C_1\exp(-4kTt) \tag{A23}$$

Initial conditions:

$A = T$, $C = 0$, at $t = 0$; (A24)

From equations A23 and A24:

$$C = A - T\exp(-4kTt) \quad \text{(A25)}$$

From equations A8 and A25:

$$B = T - 2A + T\exp(-4kTt) \quad \text{(A26)}$$

From equations A16 and A26:

$$\frac{1}{k}\frac{dA}{dt} = -8T\left(A - \frac{T}{4}\right) + 2T^2\exp(-4kTt) \quad \text{(A27)}$$

Solving equation A27:

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

Initial conditions:

$$A = T \text{ at } t = 0; \quad \text{(A29)}$$

From equations A28 and A29

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

From equations A25, A26 and A30

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

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

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

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

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

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

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

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

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

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

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