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Journal of Biotechnology 127 (2007) 736–744
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Kinetic model of 1,3-specific triacylglycerols alcoholysis catalyzed by lipases

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Received 29 August 2005; received in revised form 28 July 2006; accepted 15 August 2006

Abstract

A new model of enzymatic 1,3-specific alcoholysis of triacylglycerols has been developed. The irreversibility of the acyl bounds cleavage in glycerides, a reversible monoglycerides isomerization and an irreversible enzyme deactivation have been assumed. The Ping Pong Bi Bi mechanism with competitive inhibition by alcohol has been applied to describe rates of acyl bonds cleavage.

The enzymatic propanolysis and *iso*-propanolysis of triacetin and tricaprylin catalyzed by immobilized lipase B from *Candida antarctica* (Novozym 435®) have been investigated to verify the model. Good agreement between experimental data and calculations has been obtained. It was shown that the rate of tricaprylin alcoholysis is higher than the triacetin alcoholysis and that the rate of *iso*-propanolysis reactions are higher than propanolysis. The irreversible enzyme deactivation affects the conversion of glycerides whereas the competitive alcohol inhibition may be neglected. Empirical correlations of rates for monoglycerides isomerization and enzyme deactivation have been proposed.

Keywords: Alcoholysis; Kinetic model; Lipase

1. Introduction

The ability of lipases (triacylglycerols acyl esters hydrolases; EC 3.1.1.3) to catalyze many various enzymatic reactions has attracted increasing interest throughout the world (Jaeger and Eggert, 2002). In aqueous systems lipases catalyze hydrolysis of triacylglycerols to free fatty acids, di-, monoacylglycerols and glycerol. In non-aqueous media lipases catalyze a wide spectrum of reactions such as: alcoholysis, transestrification, and esters synthesis or regiospecificity acylation. The high stereospecificity of lipases is used to produce enantiomeric pure compounds (Vulfson, 1994).

Alcoholysis of triacylglycerols is a system of sequence reactions. Three acyl bounds in triacylglycerol

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doi:10.1016/j.jbiotec.2006.08.012

Nomenclature

$C_{(i)}$	molar concentration of component (*i*) (mol/dm^3)
$D_{1,2}$	1,2-diglyceride
G	glycerol
iprOH	*iso*-propanol
k_+	parameter describing the acyl group migration from a central to extreme position in monoglycerides molecule (1/h)
k_-	parameter of acyl group migration from 1- to 2-position in monoglyceride molecule (1/h)
k_d	deactivation constant (1/h)
k'_d	modified deactivation constant (cm^3/g h)
k_i	constant describing competitive inhibition of lipase by alcohol (mol/dm^3)
k_R	kinetic constant of the maximal rate of reaction (1/h)
K_A	parameter describing lipase affinity to an alcohol (mol/dm^3)
K_{iso}	monoglycerides isomerization constant
K_T	parameter describing lipase affinity to kind of triacylglycerol (mol/dm^3)
M_1	1-monoglyceride
M_2	2-monoglyceride
prOH	1-propanol
r_{max}	maximal rate of acyl bound cleavage (mol/(dm^3 h))
R_T	rate of a single acyl bond cleavage in a triglyceride molecule (mol/(dm^3 h))
R_D	rate of diglyceride alcoholysis (mol/(dm^3 h))
R_M	rate of 1-monoacylglycerols alcoholysis (mol/(dm^3 h))
R_m	rate of monoglycerides isomerization (mol/(dm^3 h))
triAc	triacetin
triC	tricaprylin
T	triacylglycerol
e	parameter sensitivity

Subscripts

A	alcohol
$D_{1,2}$	1,2-diglyceride
E	enzyme
E_0	total enzyme
G	glycerol
M_1	1-monoglyceride
M_2	2-monoglyceride
P	fatty acid ester
T	triacylglycerol

molecule are cleavaged by an alcohol. As a result two intermediate products are formed: di- and monoacylglycerol.

The Ping Pong Bi Bi mechanism is the one most commonly used to describe reactions catalyzed by lipases (Garcia et al., 1996, 1999; Paiva et al., 2000). In many experimental studies on lipase catalyzed reactions, only initial changes of reagent concentration were used to estimate the model parameters (Zaidi et al., 2002; Bousquet-Dubouch et al., 2001; Garcia et al., 2000). Some authors assumed the reversibility of the reaction to explain the incomplete conversion of substrates (Vaysse et al., 2002; Janssen et al., 1999). In our studies on triacetin alcoholysis (Szewczyk et al., 2001), we observed total consumption of triacetin which suggests the irreversibility of the reaction. The goal of this paper is to develop a new kinetic model of enzymatic 1,3-specific triacylglycerols alcoholysis.

2. Kinetic model of enzymatic 1,3-specific triacylglycerols alcoholysis

1,3-Specyfic lipases catalyse acyl bonds cleavage only at extreme (1 and 3) positions of triacylglycerol. According to this, only one diacyloglycerol (substituted at positions 1 and 2) and only one monoglyceride (2-monoacylglycerol) are formed during the process. Fig. 1 presents a mechanism of 1,3-specific alcoholysis

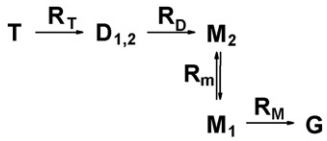

Fig. 1. The scheme of 1,3-specyfic alcoholysis according to proposed model.

assumed in this work. Monoglyceride isomerization (from position 2 to 1) may result in the complete conversion of monoglyceride. It has been assumed that the enzymatic alcoholysis of acyl bounds is an irreversible reaction following Ping Pong kinetics and the reversible monoacylglycerols isomerization is the limiting step of the process.

The rate of a single acyl bond cleavage in a triglyceride is R_T. As in a triacylglycerol, there are two bonds available and the rate of a triglyceride converting is equal to $2R_T$. The rate of diglyceride alcoholysis on 2-monoglyceride is described as R_D. The rate of 2-monoglyceride in 1-monoacyloglyceride isomerization is described as R_m. According to 1,3-regioselectivity of lipase B from *C. antarctica* isomerization of monoglycerides are necessary because the acyl bound can only be cleavaged from the external (1-) position of glyceride molecules. Glycerol, the final product of the reaction studied, is formed from 1-monoglyceride with a rate R_M.

The balances of the individual components are:

$$\frac{d}{dt}C_T = -2R_T \quad (1)$$

$$\frac{d}{dt}C_{D_{1,2}} = 2R_T - R_D \quad (2)$$

$$\frac{d}{dt}C_{M_1} = R_m - R_M \quad (3)$$

$$\frac{d}{dt}C_{M_2} = R_D - R_m \quad (4)$$

The irreversible enzyme deactivation, depending on alcohol concentration, has been assumed:

$$\frac{d}{dt}C_E = -k_d C_E C_A \quad (5)$$

The following rate equations of individual irreversible reactions are developed from the kinetics of Ping Pong Bi Bi with competitive inhibition by alcohol:

$$R_T = \frac{r_{max}}{1 + (K_T/C_T) + (K_A/C_A) + (k_i C_A/C_T)} \quad (6)$$

$$R_D = \frac{r_{max}}{1 + (K_T/C_{D_{1,2}}) + (K_A/C_A) + (k_i C_A/C_{D_{1,2}})} \quad (7)$$

$$R_M = \frac{r_{max}}{1 + (K_T/C_{M_1}) + (K_A/C_A) + (k_i C_A/C_{M_1})} \quad (8)$$

where r_{max} is the maximal rate of acyl bound cleavage and depends on total enzyme concentration:

$$r_{max} = k_R C_{E_0} \quad (9)$$

The non-enzymatic acyl group migration in monoglycerides molecules process (isomerization rate), has been described as a first order reversible reaction:

$$R_m = k_+ C_{M_2} - k_- C_{M_1} \quad (10)$$

The k_+/k_- ratio can be described as one kinetic constant K_{izo} which characterizes the isomerization process.

The following balances of reagents create additional constraints:

- Balance of glycerol frame:

$$C_G = C_{T_0} - C_T - C_{D_{1,2}} - C_M; \quad (11)$$

- Balance of fatty acid residues:

$$C_P = 3C_{T_0} - 3C_T - 2C_{D_{1,2}} - C_M; \quad (12)$$

- Alcohol balance:

$$C_A = C_{A_0} - 3C_{T_0} + 3C_T + 2C_{D_{1,2}} + C_M; \quad (13)$$

where C_M describes the total molar concentration of monoglycerides ($M = M_1 + M_2$) and C_{T_0} is the initial molar concentration of triglycerides in the reaction mixture.

The model includes six parameters: k_R, K_T, K_A, k_i, k_d and K_{izo} which should be estimated from experimental data.

3. Materials and methods

3.1. Enzyme and chemicals

The 1,3-specific lipase B from *Candida antarctica* immobilized on a macroporous acrylic resin (commercial name: Novozym 435®; producer: Novo-Nordisk A/S, Denmark), with a water content of 2%, was used as a catalyst. The nominal activity of the ester synthesis was 7000 Propyl Laurate Units (PLU) per gram of Novozyme (Novozymes, 2002). The initial enzyme concentration was 7% of the initial mass of triglyceride substrate (0.153 g of immobilized lipase for triacetin as substrate and 0.330 g of enzyme when tricaprylin was used).

All the chemicals used (triacetin, tricaprylin, 1-propanol, *iso*-propanol) exceeded 99% purity and were supplied by Sigma–Aldrich (Germany). The chemicals appointed as standards by Sigma–Aldrich were used as chromatography standards.

3.2. Experimental procedure

The reaction was carried out in Erlenmeyer flasks (25 cm^3) in a water bath shaker (250 rpm) at 50 °C. Alcohols were dried for 24 h over molecular sieves (4 Å) before use. Only triglyceride (triacetin or tricaprylin) and alcohol (1- or 2-propanol) were included in initial reaction mixtures; no additional aqueous or organic buffer systems were used. Reaction volume ranged from 6.4 to 15.9 cm^3, depended on molar mass and density of substrates. The processes with the highest alcohol:triglyceride ratio (10:1) were carried out in 50 cm^3 flasks to ensure the proper mixing of components.

The reaction mixtures were pre-incubated (15 min) in the water bath shaker to set the reaction conditions. Enzymatic alcoholysis of triacylglycerols was initiated by the addition of Novozym 435®. The samples (20 μl) of the pure reaction mixture, free of immobilized enzyme, were collected and analyzed.

3.3. Analytical methods

The reaction mixtures were analyzed by a Hewlett-Packard 5890 Series II gas chromatograph equipped with a split–splitless injection system, flame ionization detector and a capillary column. Chromatography grade nitrogen was used as a carrier gas. Two different capillary columns were used for qualitative GC analyses.

The CP-Wax 52CB column (Chrompack, The Netherlands) was used to analyse products of the triacetin alcoholysis. The following oven temperature program was used: 75 °C → 85 °C (2 °C/min) → 220 °C (25 °C/min) → 220 °C (12.5 min). The temperature of the injector and detector was set at 300 °C.

The HP 1 capillary column (Agilent Technologies) was used for the analysis of products of the tricaprylin alcoholysis. The oven temperature program had three steps: 70 °C (1 min) → 180 °C (35 °C/min) → 310 °C (15 °C/min) → 310 °C (7 min). The temperature of the injector and detector was set at 320 °C.

HP 3 365 ChemStation application (Hewlett-Packard, USA) was used to collect and integrate the detected signals. The quantitative analysis of tri-, di-, monoglycerides, glycerol, alcohols and fatty acid esters was performed by the internal normalization method. The external standard method has been used to analyze ester concentration and to verify the accuracy of the results. Propyl and *iso*-propyl acetate (GC purity) have been used as an external standard.

3.4. Numerical methods

A Visual Basic programme has been developed for simulation and sensitive analysis. The standard Runge-Kutta method and minimizing procedure has been used.

4. Results and discussion

The enzymatic alcoholysis of two triacyloglycerols: triacetin (glycerol triacetate) and tricaprylin (glycerol tricaprylate) was studied. In these triglyceride molecules, glycerol is substituted by different fatty acid rests: acetic or caprylic.

The reaction conditions have been established in preliminary experiments (Szewczyk et al., 2001). All experiments were carried out at 50 °C. The initial enzyme concentration was 7% of the initial mass of triglyceride substrate.

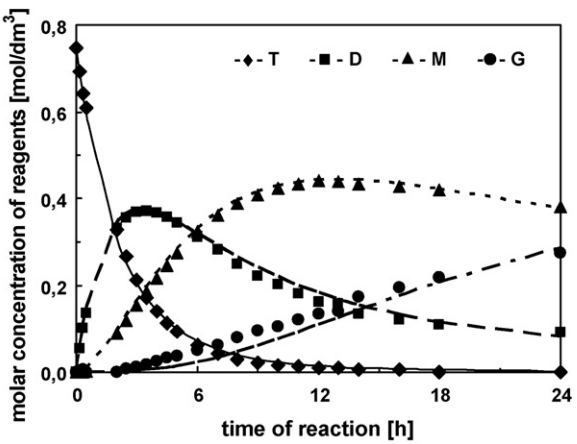

Fig. 2. Comparison of triacetin *iso*-propanolysis (T = 50 °C; 7% of Novozym 435®; A:T = 5:1) simulation and experimental data.

Fig. 2 shows an example of the results of enzymatic 1,3-specific triacylglycerol alcoholysis. Dots present experimental results, whereas lines present the model simulation. Good agreement between the model and experiments has been achieved. The concentration of tricaprylin drops to zero. It confirms the assumption that the alcoholysis reaction is irreversible. Peaks of intermediate products concentration (di- and monoglycerides) occur. This is typical for a sequential reaction as well as considering the fact that a noticeable concentration of glycerol is observed some time after the beginning of the reaction.

The concentration of monoglycerides does not decrease to zero. According to the model, this is the result of the enzyme deactivation. To prove this some amounts of triglycerides were added to the mixture after 5 and 24 h of the reaction. Fig. 3a and b present results of the experiment. The concentration of tricaprylin added after 5 h of the process decreased more slowly than in a standard reaction, while the concentration of triglyceride added after 24 h almost did not decrease. This is the evident effect of enzyme deactivation.

The problem of estimation of the mathematical model parameters was taken into consideration to minimize total error. Total relative error was given by the following formula:

$$\text{estimation error} = \sum_i \left(\frac{c_i^{\text{exp}} - c_i^{\text{m}}}{c_i^{\text{m}}} \right)^2 \tag{14}$$

where c_i^{m} is reagents concentration calculated from the model and c_i^{exp} is the concentration measured during the experiments. The error defined as above is a function of all the model parameters and its value was not higher than 6%.

Sensitivity analysis of the kinetic parameters of the presented model of triacylglycerols alcoholysis has revealed that not every one of them has an essential influence on the summary estimation error. In these calculations parameter sensitivity was given as follows:

$$\varepsilon = \frac{\Delta\text{estimation error values/estimation error value}}{\Delta\text{parameters values/parameter value}} \tag{15}$$

These calculations show that only three parameters: the kinetic constant of the maximal rate of reaction (k_{R}) with parameter sensitivity equal to 1.2, the parameter describing the acyl group migration from the central to the extreme position in monoglyceride molecules (k_{+}) with $\varepsilon = 0.46$ and the deactivation constant (k_{d}) with $\varepsilon = 0.74$ have a significant influence on the estimation error. The analysis of parameter sensitivity shows that the constant, which describes competitive inhibition of lipase by the alcohol used (k_{i}) and the acyl group migration in monoglycerides molecules from 1- to 2-position (k_{-}), can be neglected and therefore will not be discussed further. All of them were characterized by parameter sensitivity less than 0.05.

Table 1 shows the values of the parameters of the discussed kinetic model of enzymatic triacylglyc-

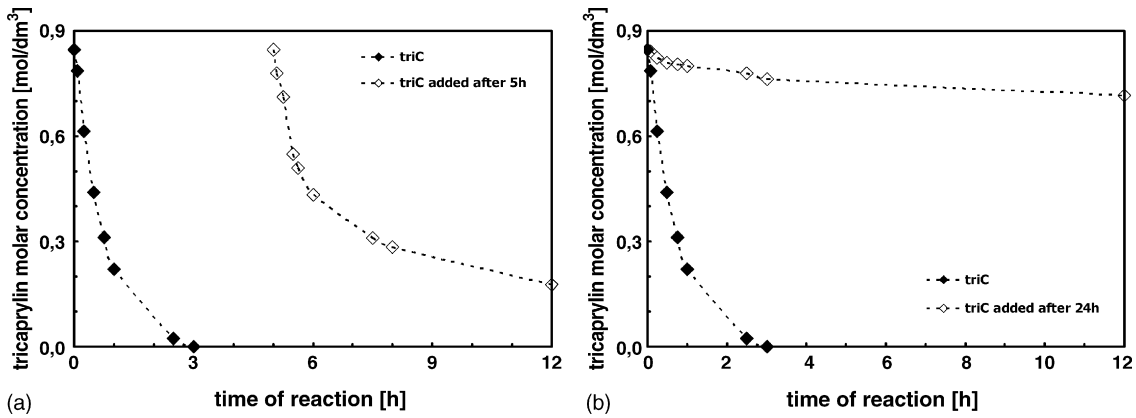

Fig. 3. (a) Concentration changes of tricaprylin added after 5 h of *iso*-propanolysis (A:T = 3:1). (b) Concentration changes of tricaprylin added after 24 h of *iso*-propanolysis (A:T = 3:1).

Table 1
The values of estimated parameters of the proposed 1,3-enzymatic alcoholysis kinetic model

A:T ratios	k_R (1/h)	K_T (mol/dm^3)	K_A (mol/dm^3)	k_i (1/h)	$K_{izo} = k_+/k_-$	k_d (1/h)	k'_d (cm^3/g h)
Triacetin propanolysis							
2:1	328	23.6	2.3	0.29	1.12	0.00941	6.6
3:1					0.58	0.000841	
5:1					0.46	0.000453	
10:1					0.21	0.000159	
Triacetin *iso*-proapanolysis							
2:1	539	24.4	2.3	0.242	2.64	0.0237	45.5
3:1					1.70	0.0128	
5:1					1.01	0.00672	
10:1					0.68	0.00173	
Tricaprylin propanolysis							
2:1	791	23.4	1.1	0.167	3.28	0.0889	38.5
3:1					1.91	0.0343	
5:1					1.20	0.0155	
10:1					0.34	0.000169	
Tricaprylin *iso*-propanolysis							
2:1	1158	24.2	1.1	0.146	4.18	0.118	85.9
3:1					3.48	0.0652	
5:1					2.74	0.0337	
10:1					0.68	0.0108	

erols alcoholysis which were calculated by numerical estimation.

The maximal rate of alcoholysis is characterized by k_R kinetic constant. The values of k_R for individual reagents systems showed in Table 1 confirm the experimental results. Higher values were noted for alcoholysis of triacylglycerol with longer acyl rests (tricaprylin) and in triacylglycerols alcoholysis with secondary alcohol—*iso*-propanol. According to these, the highest k_R value was observed in *iso*-propanolysis of tricaprylin.

The parameter K_T describing the lipase affinity for the kind of triacylglycerols used in the reaction achieves almost identical values (see Table 1). According to Ping Pong kinetics, this parameter characterizes the enzyme-substrate active complex formation and its disintegration. The independence of K_T from the alcohol type as well as from the length of fatty acid rest confirms the reaction model.

The data show that K_A depends only on the kind of triglyceride. The same values have been estimated for propanolysis and *iso*-propanolysis of a particular triglyceride. The K_A is about two times higher for alcoholysis of tricaprylin than the alcoholysis of triacetin. It is a result of the molecular mechanism of alcoholysis (Pleiss et al., 1998; Sainz-Diaz et al., 1997). According to the mechanism, the alcohol reacts with an enzyme-acyl complex. The results of the presented experiments suggest that the rate of alcoholysis is mainly affected by the length of acyl moiety.

Values of the k_i parameter, which describes the competitive inhibition of the lipase show that the aforementioned kinetic constant depends more on the kind of triglyceride used as substrate than on the kind of alcohol. The k_i values are somewhat higher for alcoholysis with *iso*-propanolysis in both of the studied reactions alcoholysis of triacetin and tricaprylin.

During the experiments the initial ratio of Novozyme 435® to triacylglycerol was constant. So, along with the increase of alcohol concentration in the reaction mixture (increase the A:T ratio), the enzyme concentration was decreased. The values of the deactivation constant (k_d) and the parameter describing the acyl group migration from the center to the extreme position (k_+) are dependent on the A:T ratio. As shown in Table 1, the k_d values decrease when the A:T ratio increases. Fig. 4 shows that the ratio $k_d/C_{E_0}^2$ is independent of the A:T ratio. It suggests that the lipase

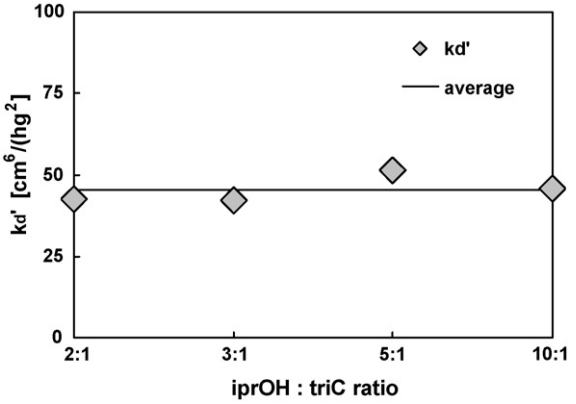

Fig. 4. After division of deactivation constant (k_d) by $C_{E_0}^2$ the modified constant value can be achieved (triacetin *iso*-propanolysis data).

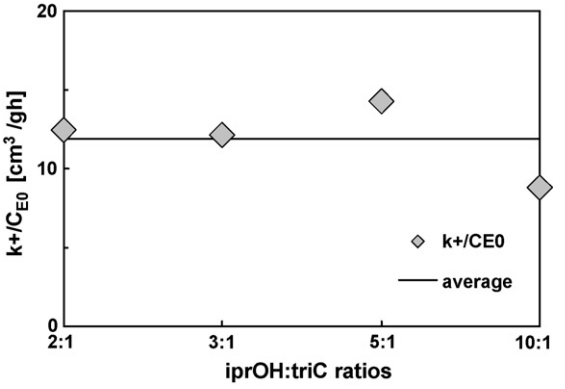

Fig. 5. Relationship between kinetic constant of acyl group migration from central to extreme position (k_+) and enzyme initial concentration in tricaprylin *iso*-propanolysis reaction mixtures.

deactivation rate should be described by the following modified equation:

$$R'_d = -k'_d C_A C_{E_0}^2 \quad (16)$$

where k'_d is modified deactivation constant.

The dependence of the deactivation rate (see Eq. (16)) on the total enzyme concentration (C_{E_0}) is probably an effect of polarity of reaction mixtures. Some authors (Halling, 1994; Bhandarkar and Neau, 2000) report that in non-aqueous systems the polarity of the reaction mixture is the main factor which determines the activity of an enzyme. Even small changes of system polarity cause significant changes in enzyme activity.

Lipase B from *Candida antarctica* reveals 1,3-regioselectivity and catalyzes cleavages of bonds located in triglyceride extreme positions but not in the central one (Novozymes, 2002). So it is impossible to obtain glycerol (the final product) in the reaction mixture this way. The presence of glycerol in the reaction mixture must be the result of an acyl group migration occurring in monoglyceride molecules. It can be described as the migration of an acyl group from the center to the extreme position (from 2- to 1-position) of glycerol's frame in a monoglyceride molecule. In the presented mathematical model of enzymatic triglycerides alcoholysis, this phenomenon is characterized by k_+ kinetic constant.

Values of k_+ decrease as the amount of alcohol added to the mixture increases. Fig. 5 shows the values of k_+/C_{E_0} ratio for different A:T ratios. It seems that the rate of acyl group migration from the center to extreme position in the monoglyceride molecule is proportional to the total enzyme concentration. The reason for this dependence is not clear. The value of parameter k_+ depends on the reaction system and the values for *iso*-propanolysis are higher than for propanolysis. The triacylglycerols used in the reaction have a smaller influence on k_+ than the alcohol.

The significantly higher estimated value of kR for tricaprylin alcoholysis, shown in Table 1, indicates that this reaction is faster than the triacetin *iso*- and 1-propanolysis. It means that *Candida antarctica* lipase B has a higher affinity for triglycerides with longer fatty acid chains in their molecules. Fig. 6a shows a comparison of triacetin and tricaprylin conversion rates. The tricaprylin alcoholysis is faster than triacetin alcoholysis. Similar effects in alcoholysis and hydrolysis reactions were reported by some authors (Vaysse et al., 2002; Janssen et al., 1996) for fatty acids estrification. Fig. 6b presents a comparison of tricaprylin 1-proponolysis and *iso*-propanolysis. The results show that enzymatic *iso*-propanolysis of triacyloglycerols is only a little faster in comparison with propanolysis. A similar effect of alcohol structure on estrification in organic media was also reported (Chang et al., 1999).

Triglycerides alcoholysis by tertiary alcohol was also studied on *tert*-butanolysis of triacetin. In no reaction mixture (from A:T equimolar to tenfold alcohol excess) did the reaction occur. No monoacylglycerols or glycerol were detected in the reaction mixture during 24 h of the reaction. Only trace quantities (3–5%

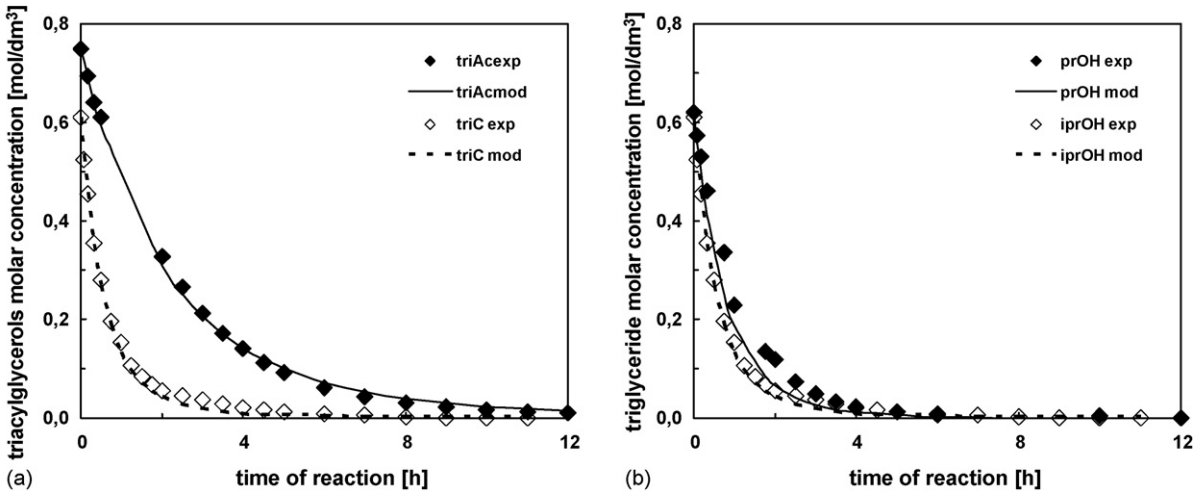

Fig. 6. (a) Changes in triglyceride concentration during triacylglycerols *iso*-propanolysis (A:T = 5:1)—model and experimental data comparison. (b) Changes in triglyceride concentration during the tricaprylin 1- and *iso*-propanolysis (A:T = 5:1)—model and experimental data comparison.

of total reaction mixture volume) of diglycerides were noticed after this time. These results are similar to those mentioned earlier (Rangheard et al., 1992).

Fig. 7a and b show diglyceride concentrations in the reaction studied. The higher diglycerides concentrations were observed in triacetin alcoholysis. This is the result of a lower rate of the triacetin reaction, which caused higher accumulation of the intermediate product. Fig. 7b presents the effect of the kind of alcohol used on diacylglycerols concentrations. In this case the differences in diglycerides conversions were less obvious, which is a result of similar triglyceride consumption rates showed in Fig. 6. The effects of the kind of substrates on the changes of monoglycerides concentration were similar as discussed above for diglycerides.

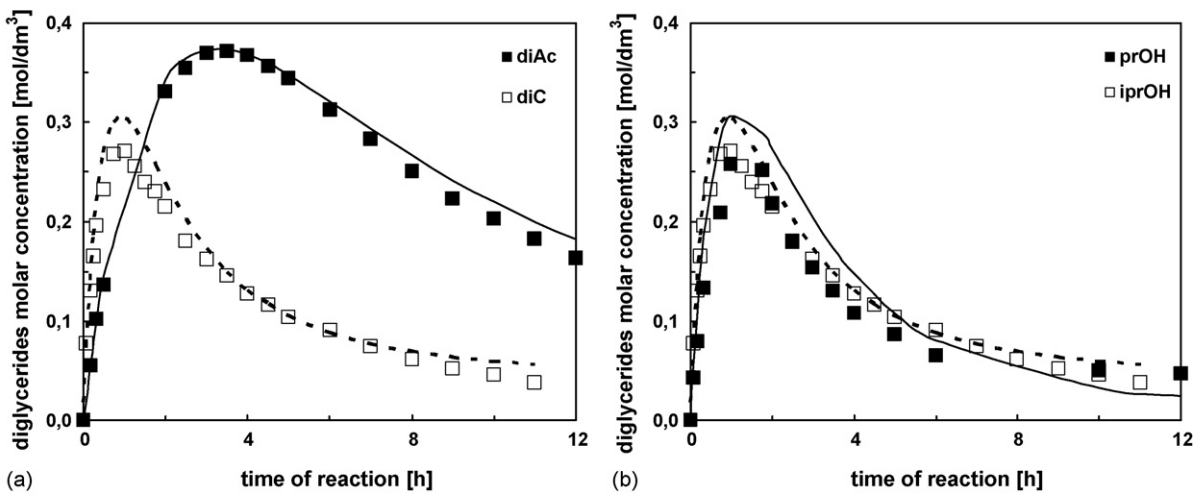

Fig. 7. (a) Time course of diglyceride concentration during triacylglycerols *iso*-propanolysis (A:T = 5:1)—model and experiment comparison. (b) Time course of diacylglycerol concentration during the tricaprylin 1- and *iso*-propanolysis (A:T = 5:1)—model and experiment comparison.

5. Conclusions

A kinetic model of enzymatic 1,3-specific alcoholysis of triacylglycerols has been developed. An irreversible acyl bond cleavage in glycerides, a reversible monoglycerides isomerization and an irreversible enzyme deactivation have been assumed. Experimental investigations on tracetin and tricaprylin 1-propanolysis and *iso*-propanolysis have been used to estimate the model parameters. Good agreement between experimental data and the model simulations has been obtained. The enzymatic 1,3-specific triacylglycerols alcoholysis by secondary alcohol (*iso*-propanol) is a little faster than that of the first order (propanol). Alcoholysis of triacetin and tricaprylin does not occur with tertiary alcohol. The rate of alcoholysis is higher for tricaprylin than for triacetin.

The kinetic model of enzymatic triacylglycerols alcoholysis can be reduced without any loss of calculation accuracy. Sensitivity analysis of its parameters has allowed a reduction of their number. Three parameters: the kinetic constant of the maximal rate of reaction (k_R), the parameter describing the acyl group migration in monoglyceride molecules (k_+) and the deactivation constant (k_d) mainly influence the estimation error.

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