Modeling of Kinetics for the Enzymatic Hydrolysis of Sunflower Oil in a High-Pressure Reactor

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ABSTRACT: This study presents a mathematical model for characterizing the Michaelis–Menten type of sunflower oil hydrolysis, catalyzed by the lipase preparation Lipolase 100T(Novozymes Ays, Bagsvaerd, Denmark). Supercritical carbon dioxide was used as a solvent for this reaction. Computer modeling of the kinetics was done for the enzymatic hydrolysis of sunflower oil in a high-pressure reactor using the MicroMath Scientist® program (MicroMath Research, St. Louis, MO). On the basis of the experimental results, we presumed the reaction was reversible. The suitability of the model was confirmed statistically with Student's *t*-test. Good agreement was found between the experimental and calculated values for the concentrations of oleic and linoleic acids.

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KEY WORDS: Hydrolysis, lipase, Michaelis–Menten kinetics, modeling of enzyme kinetics, sunflower oil, supercritical, supercritical carbon dioxide.

Lipases constitute the most important group of biocatalysts for biotechnological applications (1). They are fat-digesting enzymes of particular interest in the food, cosmetics, and pharmaceutical industries (2–5) because of their stereo- and regiospecificity. Lipases are typical representatives of enzymes for the hydrolysis of glycerides in FA.

In the last decade, supercritical fluids have increasingly proven to be environmentally benign media for use in chemical and related processes. Supercritical media retain good enzymatic activity and stability (6) and increase the solubility of hydrophobic compounds such as FA.

The use of supercritical fluids is one possibility for carrying out chemistry and chemical processes in a sustainable manner ("green chemistry"). The suitability of supercritical fluids as solvents for enzymatic reactions has been well documented (7,8).

As a reaction engineering principle, modeling of enzymatic reactions has been playing a role (9,10). Theoretical modeling of the enzyme reactor can be used to determine optimal operation points and increase our knowledge of the process (11).

The kinetics of soluble enzymes is generally described by the Michaelis–Menten relationship, as presented in Equation 1:

$$v = \frac{v_{\max} \cdot S}{K_m + S} \qquad [1]$$

where v is the rate of an enzyme-catalyzed reaction, S is the substrate concentration, $v_{\max}$ is the maximal reaction rate of an enzyme-catalyzed reaction, and K_m is the Michaelis constant.

The hydrolysis of sunflower oil, catalyzed by a nonimmobilized lipase from *Aspergillus niger*, was performed in supercritical carbon dioxide (SC CO_2). The effects of temperature and pressure on the concentrations of oleic and linoleic acids were investigated; these experimental results are presented in a previous paper (12). The rate of enzymatic hydrolysis of sunflower oil in SC CO_2 was followed by measuring the production of FFA (products). The standard methods used to determine the Michaelis constant (K_m) and the maximal reaction rate of an enzyme-catalyzed reaction ($v_{\max}$) are typically a Lineweaver–Burk plot or an Eadie–Hofstee plot. In our case, K_m and $v_{\max}$ were determined with the help of the computer program MicroMath Scientist® (13).

EXPERIMENTAL PROCEDURES

Materials. The lipase preparation Lipolase 100T, which contains a nonimmobilized lipase from *A. niger*, was kindly donated by Novozymes A/S (Bagsvaerd, Denmark). Sunflower oil was purchased from the Oljarica Oil factory (Kranj, Slovenia) (linoleic acid, 64.6%; oleic acid, 21.1%; analyzed by GC), and all other chemicals were from Merck (Darmstadt, Germany). Carbon dioxide (99.95% pure) was supplied by Messer MG (Ruše, Slovenia).

Hydrolysis in a high-pressure batch stirred-tank reactor (HP BSTR). Sunflower oil was hydrolyzed in SC CO_2 in an HP BSTR, with the lipase preparation from *A. niger* catalyzing the reaction. The design of the batch-operated system was shown previously (14). Experiments focused on the formation of oleic and linoleic acids. Each data point represented the average of at least two measurements or, when problems with operation at high pressure appeared, the average of three measurements.

Model validation. Kinetic parameters were estimated using nonlinear regressions and a simplex optimization algorithm. Verification was done on the basis of batch reactor experiments by comparing calculated and experimental concentrations vs. time curves.

Student's t-test. For a smaller number of measurements ($n < 30$), the lawfulness of Student's distribution has been validated (15). Student's distribution, which represents 95% of all measurements, is called the confidence interval of measurements at a 95% reliability (significance level $\alpha = 0.05$). The calculated value, t_{cal}, was compared with the tabulated value, t_{theor}. The value t represents the ratio between the difference in

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the averages of two measurements and the SD of a particular group of measurements. The results are statistically equal at a 95% confidence level if $t_{\text{cal}} < t_{\text{theor}}$ (i.e., there are no significant differences).

RESULTS AND DISCUSSION

Enzyme-catalyzed hydrolysis of sunflower oil in SC CO_2. The enzyme-catalyzed reaction was performed in an HP BSTR. The reaction mixture, with an optimal ratio of sunflower oil/phosphate buffer of 1:1, was stirred with a magnetic stirrer and heated to the operational temperature in an oil bath. An optimal enzyme concentration of 0.0714 g enzyme/mL of CO_2-free reaction mixture was added. The dry CO_2 was then pumped into the reactor up to the desired pressure. The reaction medium was monophasic. During the reaction, samples were taken from the reactor and the concentration of FFA was determined.

The experimental data and methods of enzymatic hydrolysis of sunflower oil in an HP BSTR were presented in detail in a previous work (12). Optimal reaction parameters, which are essential for a determination of kinetics, were also set up experimentally in the previous work: optimal lipase concentration, 0.0714 g/mL of CO_2-free reaction mixture; optimal buffer/oil ratio, 1:1 (w/w); optimal pH, 7; optimal pressure and temperature, 200 bar and 50°C; and optimal rotation speed, 600 min^{-1}.

To confirm the presumed kinetic model, the kinetics was studied on the basis of experiments performed at different pressures and temperatures. Temperature is one of the most important reaction parameters; it influences enzymatic activity much more than pressure. During an increase in temperature, the reaction rate increases with higher temperatures, and enzyme activation/deactivation occurs. In supercritical fluids, the optimal temperature is also connected with the optimal pressure. With changes in pressure and temperature, the transport properties of supercritical fluids may change significantly, resulting in changes in the reaction rate. Although an increase in temperature enhances the rate of hydrolysis, the biocatalyst becomes more susceptible to inactivation.

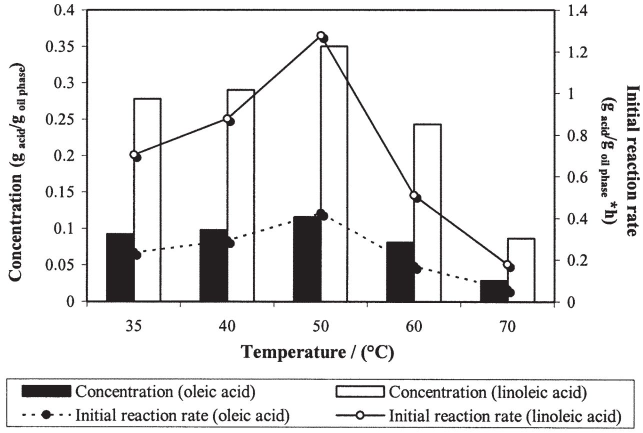

FIG. 1. Influence of temperature on the enzyme-catalyzed hydrolysis of sunflower oil in SC CO_2 at 200 bar after 5 h of reaction. The reaction mixture was stirred at a rotational speed of 600 rpm.

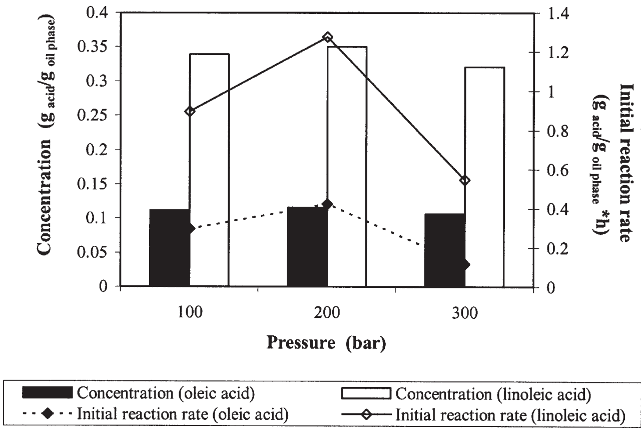

FIG. 2. Concentration of oleic and linoleic acids and initial rates of enzyme-catalyzed hydrolysis of sunflower oil in a high-pressure batch stirred-tank reactor (HP BSTR) after 5 h at 50°C, different pressures, and an enzyme concentration of 0.0714 g/mL of CO_2-free reaction mixture.

The FFA concentration increased after 5 h at 200 bar with temperatures between 35 and 50°C (Fig. 1), whereas with further temperature increases it decreased. The highest degree and highest initial rate of hydrolysis in SC CO_2 at 200 bar were observed at 50°C. As one can see from Figure 2, the optimal pressure at 50°C for this reaction was 200 bar. With higher and lower pressures, the concentrations of both acids decreased slightly.

The experimental results obtained showed that the reaction followed Michaelis–Menten kinetics.

Kinetic constants for the enzymatic hydrolysis of sunflower oil in SC CO_2. Experimental results showed that the first 5 h of the reaction followed Michaelis–Menten kinetics, after which a linear dependence between the concentration of product and time was observed. Therefore, we decided to study the kinetics for only the first 5 h of the reaction.

A mathematical model was developed for the enzymatic hydrolysis of sunflower oil in SC CO_2. The scheme for hydrolysis or esterification may be written as

$$A + B \underset{k_2}{\overset{k_1}{\leftrightarrow}} C + D \qquad [2]$$

where *A* is glyceride, *B* is water, *C* is FFA, and *D* is glycerol.

The reversibility of the reaction is dependent not only on the regiospecificity of the lipase but also on other factors such as the amount of water in the reaction mixture. On the basis of our experimental results, we presumed the reaction to be reversible.

We assumed that the hydrolysis was 0th order and could be presented as in the following equation:

$$-\frac{dc_A}{dt} = v_{\max} \qquad [3]$$

where $v_{\max}$ is the maximal reaction rate of hydrolysis and c_A is the concentration of TAG. When the reaction is 0th order, the

TABLE 1
Calculated Kinetic Constants[a] for the Production of Linoleic Acid (LA) at Different Temperatures

Temperature (°C)	$v_{\max}$ (g LA/$g_{\text{oil phase}}$ · h)	$v_{\max_1}$ (g LA/$g_{\text{oil phase}}$ · h)	K_{m_1} (g LA/$g_{\text{oil phase}}$)
35	28.709	35.920	0.93786
40	35.879	44.027	0.93786
50	85.430	102.660	0.93786
60	33.344	42.556	0.93786
70	11.404	20.059	0.93786

[a] Abbreviations: $v_{\max}$, maximum reaction rate of hydrolysis; $v_{\max_1}$, maximum reaction rate of esterification; K_{m_1}, Michaelis constant.

TABLE 2
SD and Regression Coefficients (R^2) for the Production of LA at Different Temperatures[a]

Temperature (°C)	R^2	SD for $v_{\max}$ (%)	SD for $v_{\max_1}$ (%)
35	0.9993	1.866	2.395
40	0.9994	2.157	2.712
50	0.9991	7.907	9.670
60	0.9989	2.784	3.643
70	0.9991	1.308	2.357

[a] For abbreviations see Table 1.

reaction rate is independent of both the concentration of glycerol and the concentration of FFA.

Esterification, which is a reversible reaction, obeys Michaelis–Menten kinetics and can be presented with the second part of the equation for the production of FFA (Eq. 4). The rate of product formation follows Michaelis–Menten kinetics and can be written using the following expression:

$$\frac{dc_C}{dt} = v_{\max} - \frac{v_{\max_1} \cdot c_C}{K_{m_1} + c_C} \qquad [4]$$

where $v_{\max}$ represents the maximal reaction rate of hydrolysis, $v_{\max_1}$ represents the maximal reaction rate of esterification, K_{m_1} is the Michaelis constant for the reaction, and c_C indicates the concentration of FFA.

With the support of the Scientist computer program, we simulated the model described in the aforementioned equations. The results were then compared with experimental results.

The enzyme-catalyzed hydrolysis of sunflower oil was simulated when temperature and pressure were optimized. The Michaelis constant would be the same for each reaction, whereas the maximal reaction rates could be dependent on the course of the reaction, the reaction parameters, and the enzymatic activity.

Reaction kinetics at different temperatures. Calculated values of $v_{\max}$, $v_{\max_1}$, and K_{m_1} for different temperatures are presented in Table 1. Table 2 presents SD and regression coefficient (R^2) values. Figure 3 shows good agreement between the experimental and calculated values for linoleic acid concentrations vs. time for the hydrolysis of sunflower oil in SC CO_2 in the presence of Lipolase 100T at different temperatures.

Reaction kinetics at different pressures. The same calculation was also performed for pressure optimization. The calculated values for $v_{\max}$ and $v_{\max_1}$ in K_{m_1} are presented in Table 3, and SD and R^2 values are given in Table 4. The comparison of calculated and experimental linoleic acid concentrations vs. time curves for the enzymatic hydrolysis of sunflower oil in SC CO_2 at different pressures also showed good prediction accuracy (Fig. 4). The suitability of our model was confirmed statistically by using Student's *t*-test (15).

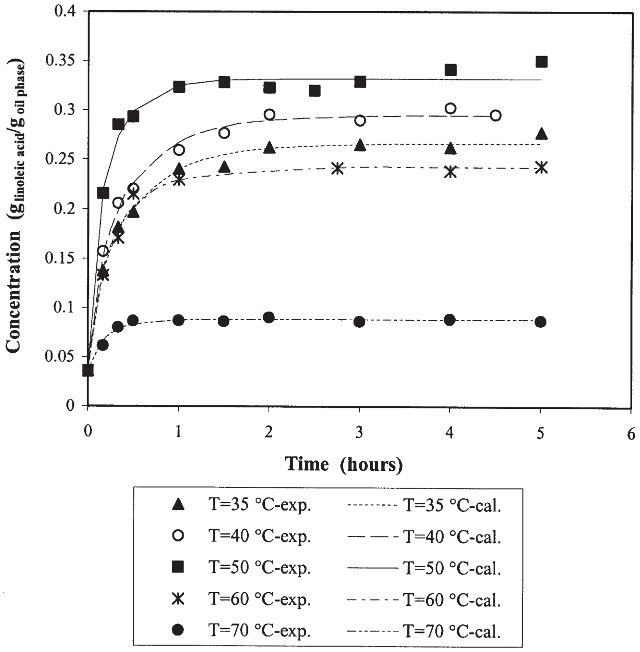

FIG. 3. Fits of experimental data to the kinetic model for the enzyme-catalyzed hydrolysis of sunflower oil at 200 bar and different temperatures. The reaction was performed in SC CO_2. Abbreviations: exp., experimental value for the concentration of linoleic acid; cal., calculated value for the concentration of linoleic acid obtained using the software package Scientist® (MicroMath Research, St. Louis, MO).

TABLE 3
Calculated Kinetic Constants[a] for the Production of LA at Different Pressures

Pressure (bar)	v_{max} (g LA/$g_{oil\ phase}$ · h)	v_{max_1} (g LA/$g_{oil\ phase}$ · h)	K_{m_1} (g LA/$g_{oil\ phase}$)
100	23.384	28.279	0.93786
200	85.430	102.660	0.93786
300	14.084	16.846	0.93786

[a]For abbreviations see Table 1.

TABLE 4
SD and Regression Coefficients (R^2) for the Production of LA at Different Pressures

Pressure (bar)	R^2	SD for v_{max} (%)	SD for v_{max_1} (%)
100	0.9985	2.046	2.055
200	0.9991	7.907	9.670
300	0.9984	1.116	1.410

[a]For abbreviation see Table 1.

TABLE 5
t_{theor} and t_{cal} for the Kinetic Model at Different Temperatures[a]

t	35°C	40°C	50°C	60°C	70°C
t_{cal}	0.0069	0.0084	–0.0481	–0.0066	–0.0123
t_{theor}	2.0860	2.0860	2.0739	2.1448	2.1009

[a]The values t_{theor} and t_{cal} refer to the theoretical and calculated values obtained with Student's *t*-test.

TABLE 6
t_{theor} and t_{cal} for the Kinetic Model at Different Pressures[a]

t	100 bar	200 bar	300 bar
t_{cal}	0.0163	–0.0481	–0.0003
t_{theor}	2.0860	2.0739	2.0739

[a]For abbreviations see Table 5.

Student's t-test. The experimental and calculated data were compared on the basis of the predicted kinetic model. Tables 5 and 6 present the calculated and tabulated values for *t* when temperature and pressure were optimized. From these tables, one can see that $t_{cal} < t_{theor}$. Hence, it follows that our predicted kinetic model was at a 95% confidence level, which is appropriate for all the given measurements.

One can conclude that mathematical models, especially when coupled with modern computer techniques, prove to be effective in searching for optimal conditions and creating an optimal environment for the biocatalyst in order to optimize productivity.

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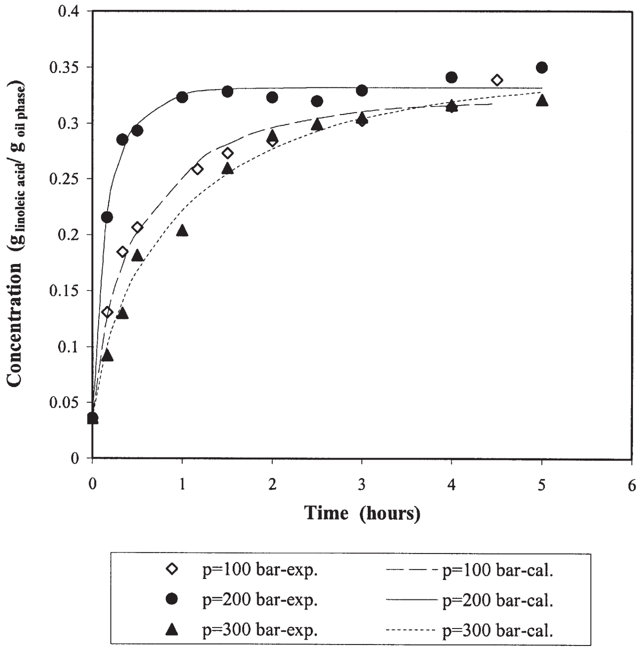

FIG. 4. Fits of experimental data to the kinetic model for enzyme-catalyzed hydrolysis of sunflower oil at 50°C and different pressures. The reaction was performed in SC CO_2. For abbreviations see Figure 3.

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