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Research Article

Kinetic Model for the Hydrolysis of Olive Oil by Lipase in Absence of Emulsifier

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Abstract

Lipase-catalyzed hydrolysis of olive oil has been studied in the absence of emulsifier. A simple mathematical model to predict the hydrolysis rate is derived from a proposed kinetic mechanism of the reaction. The hydrolytic reaction obeys Michaelis-Menten kinetics with a simultaneous second-order enzyme deactivation. The model predictions closely agree with the experimental results.

Keywords

Lipase; hydrolysis; olive oil; kinetics

1. Introduction

Enzymatic modification of fats and oils (triglycerides) provides ambient reaction conditions unmatched in many chemical processes for producing high-value products from cheap and plentiful raw materials [1]. The use of the enzymes is advantageous because it requires less energy and

temperature and still presents more excellent selectivity [2, 3]. Lipases are the enzymes that catalyze the hydrolysis of oils and fats [4]. Lipase-catalyzed hydrolysis reactions can produce commodity chemicals with immense scope of application in food, cosmetic, detergent, explosive, and pharmaceutical industries [1, 5, 6]. Enzymic splitting of fats has gained increasing attention, as lipase is now available at a reasonable cost [7]. The industrial use of lipase for splitting lipids as an energy-saving process has been addressed in the literature, especially for producing high-value-added products or heat-sensitive fatty acids [8].

Fats and oils hydrolysis by lipases is a process to convert triglycerides (triglycerides are the main components in natural oils and fats, [9]) into fatty acids and glycerol, which are essential for food, pharmaceutical, cosmetic, and oleochemical industries [10, 11].

Most of the kinetics studies involving triglyceride hydrolysis by lipases were conducted in the presence of an emulsifier [5, 12-15], in a biphasic organic-aqueous system [5, 16-19], and a reversed micelle [20-24]. From an industrial viewpoint, these processes increase the cost of product separation and are energy-intensive, in addition to their adverse effect on the reaction rate [25]. To solve these issues, methods for lipase assay, in which the reaction mixtures containing water and oil were emulsified by magnetic stirring, have been developed; these methods showed that the rate of oil hydrolysis could be determined reproducibly in the absence of any added emulsifier [26-28].

Some efforts have been expended on developing kinetic models to explain the phenomenon of the enzymatic hydrolysis of oils by lipases without added emulsifiers. Still, these studies have been conducted using complex equations that, unfortunately, do not hold for all operating conditions [29-32].

It is apparent from the preceding discussion that a reliable kinetic model to predict the rate of oil hydrolysis by lipases in the absence of additional emulsifiers is still lacking.

In these circumstances, the purpose of the present study is to investigate the kinetics of lipase-catalyzed hydrolysis of edible oil in the absence of any added emulsifier and to develop a model with a simple kinetic equation to predict the hydrolysis rate of lipids at different reaction time, and enzyme and substrate concentrations. For this research, olive oil was chosen and used due to its well-known properties in the food industry.

2. Materials and Methods

Lipozyme TL 100L, a 1,3 specific lipase from *Thermomyces lanuginosus* [33], was a Novo Nordisk A/S, Denmark gift. The declared activity of Lipozyme TL 100 L was 100 KLU/g (1KLU is the amount of enzyme activity that liberates 1000 µmol of titratable butyric acid from the substrate glycerol tributyrate per minute under defined standard conditions [33]), and its density was 1050 g/l [34]. This lipase is optimally active in the pH range from 7 to 10 and at temperatures from 20 to 50°C [33, 34].

Refined olive oil from Carbonell (Córdoba, Spain) was purchased at a local market.

Experimental work was performed in a 0.5 l laboratory jacketed reactor operated in batch mode. The operating conditions were as follows: temperature 35°C, pH controlled at 7.0 by adding 0.2 M potassium hydroxide (KOH), stirring speed 300 rpm, working volume 0.4 l at the beginning of each run, consisting of olive oil and distilled water. Temperature and pH were selected according to the optimal Lipozyme TL 100 L values. Stirring speed was chosen to maintain the homogeneity of the

reaction mixture and prevent vortices. After the desired conditions were reached in the bioreactor, the required amount of enzyme was added to initiate the reaction.

Hydrolysis was followed by the base consumption (0.2 M KOH). The base required to keep pH constant is proportional to the hydrolysis rate:

$$\begin{array}{l} H_2COOR_1 \\ | \\ HCOOR_2 \\ | \\ H_2COOR_3 \end{array} + 3H_2O \xrightarrow{\text{Lipase}} \begin{array}{l} H_2COH \\ | \\ HCOH \\ | \\ H_2COH \end{array} + \begin{array}{l} R_1OOH \\ R_2OOH \\ R_3OOH \end{array} \quad (1)$$

$$\begin{array}{l} R_1OOH \\ R_2OOH \\ R_3OOH \end{array} + 3KOH \xrightarrow{\text{pH=constant}} \begin{array}{l} R_1OOK \\ R_2OOK \\ R_3OOK \end{array} + 3H_2O \quad (2)$$

The degree of hydrolysis was calculated from the acid value of the oil in the reactor during hydrolysis and the saponification value of the used oil [35]:

$$X = \frac{AV}{SV} \cdot 100 \quad (3)$$

$$AV = \frac{56.1 \cdot B \cdot M_B}{W} \quad (4)$$

where *X* is the degree of hydrolysis (%), *SV* is the saponification value (mg KOH/g oil), *AV* is the acid value (mg KOH/g oil), *B* is the consumption of KOH (ml), M_B is the concentration of KOH (M), and *W* is the weight of oil in the reactor (g).

All the tests were triplicated, and a Student's t-test was performed (significant level $p \leq 0.05$) to evaluate the statistical confidence of the data. The saponification value was determined from the number of milligrams of potassium hydroxide required to neutralize the free fatty acid in the oil according to the analytical method 920.160 of the Association of Official Analytical Chemists [36]. The result is expressed as a mean (±SD).

Nonlinear regression analysis of the data was performed using the statistical software Statgraphics Centurion 18 program. To examine the goodness of fit of the model, the coefficient of the determination (R^2) was calculated [37]:

$$R^2 = 1 - \frac{SS_{res}}{SS_{tot}} \quad (5)$$

SS_{res} and SS_{tot} are the sum of squares of residuals and the total sum of squares, respectively:

$$SS_{res} = \sum_{i=1}^{n} (X_i - \ddot{X}_i)^2 \quad (6)$$

$$SS_{tot} = \sum_{i=1}^{n} \left(X_i - \overline{X}\right)^2 \quad (7)$$

where n is the number of data points, X_i are the experimental data, $\overline{X}$ is the mean of the observed data and $\ddot{X}_i$ are the values predicted from the model.

3. Results and Discussion

3.1 Kinetic Model

Batch reactions were carried out at several initial substrate and enzyme concentrations. To calculate the hydrolysis degree, a saponification value for the commercial olive oil of 190 ± 1.84 mg KOH/g oil (determined as indicated in Section 2 as the average of four replicates) was used. This value agrees with those found in the literature [38], where saponification values for olive oil between 187 and 196 are reported.

In Figure 1, the value of the hydrolysis degree, X, is plotted against the reaction time, t, for initial concentrations of olive oil (S_0) from 1.1 to 4.6 g/l at an initial enzyme concentration (E_0) of 7.7 KLU/l (0.077 g/l).

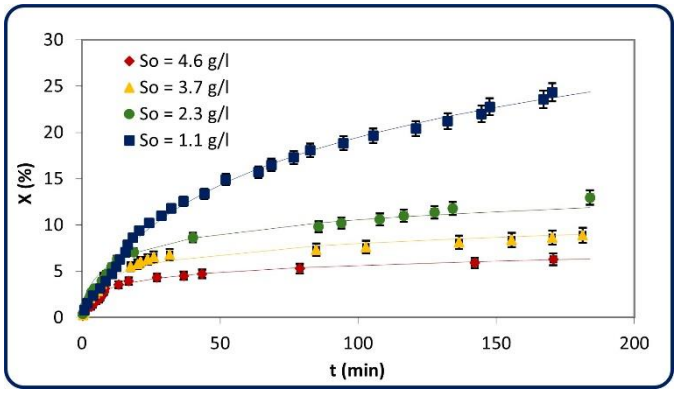

Figure 1 Effect of substrate concentration on hydrolysis curves for E_0 = 7.7 KLU/l (dots) and adjustment of the kinetic model (solid lines).

In Figure 2, the value of the hydrolysis degree, X, is plotted versus the reaction time, t, for initial concentrations of lipase (E_0) from 2.6 to 10.3 KLU/l (0.026-0.103 g/l) at an initial olive oil concentration (S_0) of 4.6 g/l.

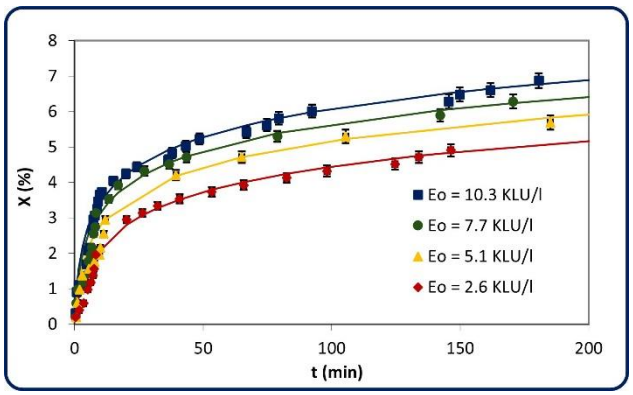

Figure 2 Effect of enzyme concentration on hydrolysis curves for S_0 = 4.6 g/l (dots) and adjustment of the kinetic model (solid lines).

Initial lipase and olive oil concentrations were chosen to obtain the E_0/S_0 rate typically used in oil hydrolysis with lipases. In this study, E_0/S_0 rate ranges from $5 \cdot 10^{-3}$ to $70 \cdot 10^{-3}$ g/g which agree with studies of Lares et al. ($2.5 \cdot 10^{-3}$-$7.5 \cdot 10^{-3}$ g/g) [39], Hermansyah et al. ($20 \cdot 10^{-3}$-$100 \cdot 10^{-3}$ g/g) [19], Yao et al., ($30 \cdot 10^{-3}$-$120 \cdot 10^{-3}$) [22] and Raspe et al. ($10 \cdot 10^{-3}$-$200 \cdot 10^{-3}$ g/g) [6].

The following kinetic equation can fit the experimental data (Figure 1 and Figure 2):

$$\frac{dX}{dt} = a\, e^{-bX} \tag{8}$$

This suggests a simple Elovich *equation* may describe the *kinetics* of olive oil hydrolysis by lipases.

A kinetic model of Michaelis-Menten can explain this equation with simultaneous second-order enzyme deactivation.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P \tag{9}$$

As the starting point, it is assumed that the enzyme *E* combines reversibly with the substrate *S* to form an enzyme-substrate complex *ES*, which then breaks down to form product P and regenerate the enzyme *E*. k_1 and k_{-1} are the forward and backward reaction constants of the first reaction, respectively; and k_2 represent the rate constant of the second reaction.

The irreversible decomposition of the complex to product and free enzyme determines the reaction rate:

$$r = \frac{d[P]}{dt} = S_0 \frac{dX}{dt} = k_2[ES] \tag{10}$$

For reaction in a well-mixed closed vessel at a steady state, the following mass balance for *ES* complex can be written:

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] \approx 0 \tag{11}$$

$$[ES] = \frac{k_1}{k_{-1} + k_2}[E][S] = \frac{[E][S]}{K_M} \tag{12}$$

where K_M is the Michaelis-Menten constant.

Making use of the previous equation (12), the reaction rate can be expressed in terms of enzyme and substrate concentrations:

$$S_0 \frac{dX}{dt} = k_2[ES] = k_2 \frac{[E][S]}{K_M} = \frac{k_2}{K_M}[E][S] \tag{13}$$

Since all enzyme present (*e*) is either free or complexed,

$$e = [E] + [ES] = [E]\left(1 + \frac{[S]}{K_M}\right) \tag{14}$$

and

$$[E] = \frac{e}{1 + \frac{[S]}{K_M}} \tag{15}$$

Substituting *[E]* into eq. (13)

$$S_0 \frac{dX}{dt} = \frac{k_2}{K_M} \frac{e}{1 + \frac{[S]}{K_M}} [S] \tag{16}$$

rearranging this equation

$$S_0 \frac{dX}{dt} = k_2 \frac{e}{K_M + [S]} [S] \tag{17}$$

and assuming

$$[S] >>> K_M$$

kinetic equation reduces to:

$$S_0 \frac{dX}{dt} = k_2 \, e \tag{18}$$

If thermal deactivation of the enzyme follows second-order kinetics, the time course of active enzyme concentration is described by:

$$r_e = -\frac{de}{dt} = k_d \, e^2 \tag{19}$$

Combining this deactivation equation with the equation (18), the following equation is obtained:

$$\frac{S_0 dX}{de} = -\frac{k_2 \, e}{k_d \, e^2} \tag{20}$$

Separation of variables and integration between the limits of the initial and final reaction conditions provides:

$$S_0 \int_0^X dX = -\frac{k_2}{k_d} \int_{E_0}^{e} \frac{de}{e} \tag{21}$$

and

$$e = E_0 e^{\left(-\frac{k_d}{k_2} S_0 \, X\right)} \tag{22}$$

Then, the reaction rate can be derived as:

$$\frac{dX}{dt} = k_2 \, \frac{E_0}{S_0} \, e^{\left(-\frac{k_d}{k_2} S_0 \, X\right)} \tag{23}$$

And substituting

$$k_2 \frac{E_0}{S_0} = a \tag{24}$$

$$\frac{k_d}{k_2} S_0 = b \tag{25}$$

equation (8) is obtained.

3.2 Model Verification

Values of *a* and *b* obtained from nonlinear regression analysis of the hydrolysis curves show the dependence of *a* on the enzyme and substrate concentrations (Figure 3A) and the dependence of *b* on the substrate concentration (Figure 3B).

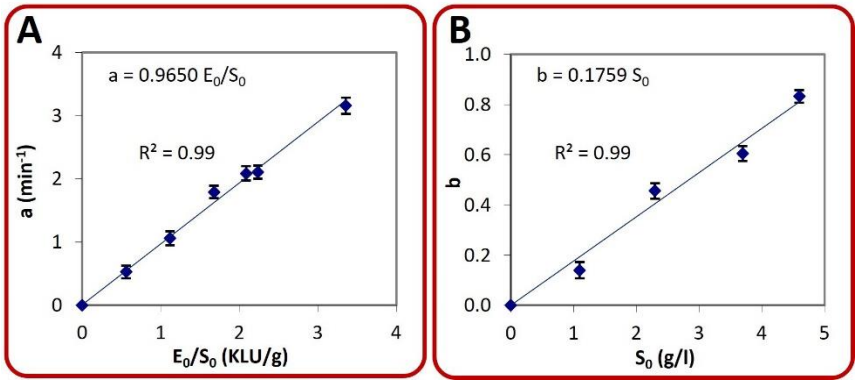

Figure 3 Variation of kinetic parameters: A) Parameter *a* for different E_0/S_0 values, B) Parameter *b* for different S_0 values.

From Figure 3A and Figure 3B, kinetic parameters have been deduced:

$$k_2 = 96.50 \cdot 10^{-2} \text{ g.KLU}^{-1}\text{.min}^{-1}$$

$$k_d = 16.97 \cdot 10^{-2} \text{ l.KLU}^{-1}\text{.min}^{-1}$$

The theoretical curves calculated through the model equation (8) are shown as solid lines in Figure 1 and Figure 2. The fit of the proposed model is expressed by the coefficients of determination R^2 whose values greater than 0.95 (Table 1) imply that more than 95% of experimental data is compatible with the data predicted by the model. Values of R^2 higher than 0.75 indicate the aptness of the model, and the closer R^2 is to unity, the better the empirical model fits the actual data [40]. This proves that equation (8) is a suitable model to describe the response of the experiments about the enzymatic hydrolysis of olive oil.

Table 1 Coefficients of determination for fit between experimental and calculated data.

S_0 (g/l)	E_0 (KLU/l)	R^2
1.1	7.7	$99.63 \cdot 10^{-2}$

2.3	7.7	$97.27 \cdot 10^{-2}$
3.7	7.7	$96.62 \cdot 10^{-2}$
4.6	7.7	$95.44 \cdot 10^{-2}$
4.6	2.6	$96.79 \cdot 10^{-2}$
4.6	5.1	$95.63 \cdot 10^{-2}$
4.6	7.7	$95.44 \cdot 10^{-2}$
4.6	10.3	$96.42 \cdot 10^{-2}$

The formation of the *ES* complex suggested for the mechanism of the hydrolysis of olive oil agrees with previous studies [16-18, 41], where authors propose that first, the enzyme (*E*) forms an activated or penetrated enzyme (*E**) at the interface, then forms a complex (*E*S*) with the substrate and finally this complex breaks down to yield the product, *P*. Hence, the rate-limiting step could be any of the three steps mentioned earlier in the hydrolysis of oils by lipase.

Some researchers consider that the adsorption and desorption of the enzyme at the interface are the most critical steps that affect the kinetics of the oil hydrolysis with the enzyme and elaborate complex models considering the specific interfacial area [30, 31]. However, although low enzyme concentration model predictions fit experimental data, the high enzyme concentration model curve deviates from the experimental data. Deviations observed for several operating conditions indicate this model is not satisfactory, and adsorption and desorption are not the determinant steps in the process. This theory agrees with mechanisms proposed by other authors [29] and with our mechanism where adsorption is not included and the decomposition of the complex to product is considered the rate-limiting step, obtaining a closely fitting for low and high enzyme concentrations.

Hydrolysis modeled as a Michaelis-Menten kinetics with a simultaneous second-order deactivation of enzyme agrees with previous studies that suggest Michaelis-Menten kinetics [29] but seems to disagree with other studies that indicate competitive product inhibition and a first-order deactivation mechanism for lipase [22, 39, 42].

To check a possible enzyme inhibition, the reaction rate (expressed as $S_0 \cdot \Delta X/\Delta t$ according to equation 10) was calculated for different degrees of hydrolysis and substrate concentrations. Figure 4 shows that the reaction rate increases with the substrate concentration but decreases with the hydrolysis degree.

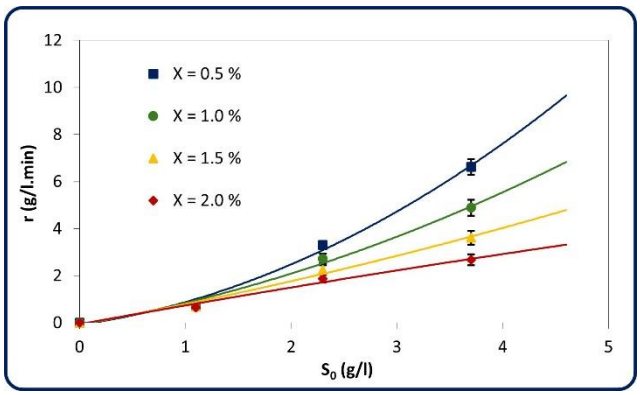

Figure 4 Variation of the reaction rate with the hydrolysis degree and the substrate concentration.

An increase of *r* with S_0 means that no inhibition by substrate exists. However, the decrease of *r* with *X* indicates the presence of inhibition by the product. This observation might explain the downward curvature of the hydrolysis curve as a case of product inhibition: the hydrolysis products formed at high *X* are inhibiting the reaction. The existence of inhibition by products in the hydrolysis of oils with lipases is a phenomenon already reflected by other authors [43]. As reported by Becker and Märkl [44], the oleic acid liberated in the degradation of the olive oil is the leading cause of this inhibition. However, product inhibition does not seem essential in the hydrolysis of olive oil with lipase because, when Michaelis-Menten kinetics is applied to experiments, calculated data agree better with experimental data than for product inhibition kinetics.

To prove the enzyme's second-order deactivation, X's variation was plotted versus E_0t. Figure 5 shows all data situated on the same curve.

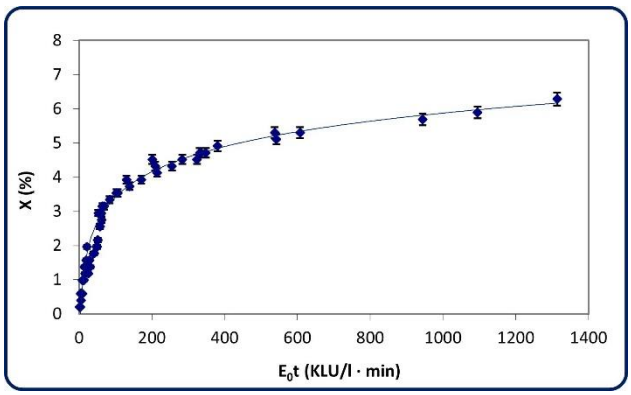

Figure 5 Influence of E_0t on the hydrolysis degree.

This result can be explained by the fact that there is no deactivation of the lipase or second-order kinetics for enzyme deactivation [45]. As the thermal deactivation of Lipozyme TL 100 L is referred to by Novozymes [46] and Moreno-Pérez et al. [47], Figure 5 confirms the second-order deactivation.

The kinetic model proposed by Yao et al. [22], including product inhibition and a first-order deactivation mechanism for the enzyme, seems not adequate because it does not fit the data, and authors need to add an influence of product on lipase stability to fit suitably the data. This excessively complex model could probably be simplified by using a second-order deactivation for the enzyme.

4. Conclusions

A kinetic model has been proposed based on the mechanism of the reaction of the lipase-catalyzed hydrolysis of the olive oil without any added emulsifier. Results show that the rate-controlling step in the hydrolysis of olive oil in a well-stirred reactor is not the penetration of the enzyme at the interface or the formation of the enzyme-substrate complex but the breakdown of the enzyme-substrate complex to the product. The product inhibition observed in the enzyme kinetic analysis is not rate-limiting either. Hydrolysis modeled as Michaelis-Menten kinetics with a simultaneous second-order enzyme deactivation reports a good agreement between the model prediction and the experimental data. The rate constants in the mathematical model were determined numerically from the experimental results. This study shows that simple models can be used to accurately predict the rate of lipase hydrolysis of oils in a batch reactor. Although complex

models are often better than simplistic ones because they include more relevant details, from the results presented here, it has been proved that a simple Elovich kinetic model equation better describes the enzymatic hydrolysis of oil. This is an exciting conclusion because a basic rule in modeling should not be forgotten, that a model should be as simple as possible. The proposed kinetic model may find promising applications in assessing, optimizing, and designing the enzymatic hydrolysis of oils.

Author Contributions

The author did all the research work of this study.

Competing Interests

The author has declared that no competing interests exist.

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