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Kinetic model for the enzymatic hydrolysis of tributyrin in O/W emulsions

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Abstract

The enzymatic hydrolysis of triglycerides in emulsion form, which involves a heterogeneous system and furthermore unstable over time, is a difficult task. This work proposes a kinetic model for the enzymatic hydrolysis of tributyrin, using the enzyme Lipolase® 100 of Novozymes. This model considers the hydrolysis reaction to take place between the enzyme and the surface ester-bonds of tributyrin. For this, the enzyme penetrates the droplet surface, bonds to a surface-ester group, and performs the hydrolytic step, this latter stage of the process being irreversible. The concentration of the hydrolysable surface bonds in the emulsion has been calculated for the application of the model. The model proved valid for low enzyme concentrations, at which the interface was not found to be saturated, and it has been applied at different temperatures. In all cases, the model succeeded in predicting the initial reaction rates with a relative deviation of less than 5%. The model also reproduces the results found for enzymatic hydrolysis as a function of reaction time up to conversions of 0.25.

Keywords: Enzyme; Kinetics; Lipases; Tributyrin; Emulsion; Biochemical engineering

1. Introduction

Lipases (triacylglycerol acylhydrolases E.C. 3.1.1.3) catalyse the hydrolysis of triglycerides to yield diglycerides, monoglycerides, and free fatty acids. As a result of remarkable progress in biochemical industries, lipases have been developed and detergents containing lipases have been marketed in many countries (Varanasi et al., 1997). Alkaline lipases can be effectively used to enhance the removal of oily soils from fabrics and hard surfaces as well as to increase the detergency of commercial products, especially at low temperatures (Xia et al., 1996).

Furthermore, enzymatic processes using lipases are useful in the production of nutritionally valuable fats and oils because of their specificity and high activity at low temperatures. Unlike the traditional process of chemical hydrolysis (Colgate–Emery process) the enzymatic hydrolysis of triglycerides takes place at ambient temperature and air pressure as well as at near neutral pH, with the consequent improvement in security and energy savings. Also, it becomes possible to produce thermolabile fatty acids that would otherwise decompose under the extreme conditions of the chemical process (Gandhi, 1997). Functional fats and oils, medium-chain triglycerides, polyunsaturated fatty acids, and low-calorie triglycerides are receiving attention for their nutritional benefits. These medium-chain triglycerides are more easily hydrolysed by pancreatic lipase and are commonly used for medical applications (Merolli et al., 1997), such as a caloric supply for people with problems of lipid absorption.

A main feature of lipases is that in water they require only substrates existing as interfacial structures such as oil-in-water emulsions, liposomes, micelles, or monomolecular films. Common to all of these structures is the orientation of lipid molecules at an interface.

The kinetics of enzymatic hydrolysis using lipases has been studied primarily in monolayers (Ivanova et al., 2002; Verger et al., 1973), micellar systems (Berg et al., 1991) and in biphasic systems with or without organic solvents (Kawano et al., 1994; Tsai et al., 1991). Ample research has also been devoted to the field of reverse micellar systems for enzymatic triglyceride hydrolysis (Kim and Chung, 1989; Talukder et al., 2004). However, due to the difficulty of adequately characterizing the interface, few kinetic models have been developed and applied

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to the hydrolysis of triglycerides in O/W emulsion, despite the practical importance of this system (Martinez et al., 1992; Tsai et al., 1991).

In the present work, for the enzymatic hydrolysis of tributyrin in emulsion form, we propose a kinetic model which takes into account the concentration of hydrolysable bonds on the surface of droplets of the tributyrin emulsion, as a way of quantifying the concentration of substrate that is accessible to the enzyme. This model, applied to the initial reaction rates of the enzymatic process, can also be used to explain the kinetic behaviour of the action of lipases with other triglycerides. We have also investigated the effect of temperature on the kinetic constants obtained as well as the progress of the hydrolysis reaction over time.

2. Materials and methods

2.1. Products and reagents

The enzyme used was the commercial lipase Lipolase® 100, from *Thermomyces lanuginosus* (supplied by Novozymes A/S, Denmark) with a molecular weight of 31 700 Da. The protein content in the commercial enzymatic preparation was determined gravimetrically, precipitating the protein with acetone, separating it by centrifugation (8000 rpm, 30 min), and drying the resulting precipitate (60 °C, 24 h). The value was 3.01% by weight.

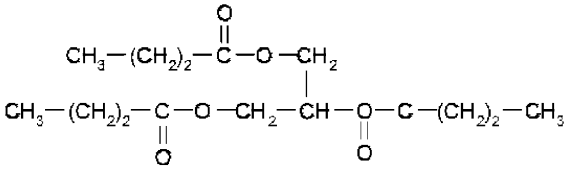
The tributyrin (99% richness) was supplied by Sigma (USA) and used without purification. Its structural formula and physico-chemical properties are listed in Table 1.

To prepare the aqueous emulsion of tributyrin, we used an emulsification reagent, the active agent of which was gum arabic (supplied by Merck, Germany). The quantity of gum arabic incorporated in the final emulsion was 0.1 g/mL of tributyrin. The rest of the components of the emulsification reagent were: sodium chloride (NaCl) 17.90 g/L; potassium dihydrogen phosphate (KH_2PO_4) 0.410 g/L; glycerine ($C_3H_8O_3$) 540 mL/L, and deionised water (Milli-Q® quality). All the reagents (supplied by Panreac, Spain) were of 99% purity or higher.

A phosphate buffer solution (0.5 mM) was used to prepare the initial solution of enzyme from the commercial product, as well as the subsequent dilutions used in the hydrolysis experiments.

Table 1
Structural formula and physico-chemical properties of tributyrin

Structural formula:



Molecular weight:	302.37
Melting point:	−75 °C (1 atm)
Boiling point:	307.5 °C (1 atm)
Density:	1.0350 Kg/L (20 °C)
Water Solubility[a]:	0.412 mM

[a]Ferrato et al. (1997).

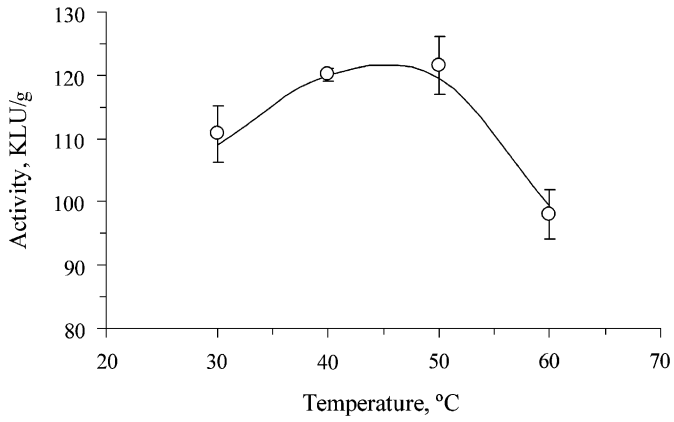


Fig. 1. Influence of temperature in the activity of the enzyme Lipolase 100 (the error bars represent ± the standard deviation of three replicates).

2.2. Preparation of the substrate emulsion

The tributyrin emulsions were prepared at a fixed volume fraction (0.05) diluting them afterwards with a phosphate buffer solution to the final desired concentration. The emulsification was carried out by mechanical dispersion using an UltraTurrax® stirrer (Ika-Werke, Germany) at 13 000 rpm for 1 min in glass beakers.

2.3. Enzymatic activity

The enzymatic activity was evaluated by the pHstat method (Brockman, 1981; Hoppe and Theimer, 1996) at 30 °C and pH 7.0 on a tributyrin emulsion, using an automatic titrator 718 Stat Titrino (Metrohm, Switzerland). This method involves the continuous titration of the fatty acids released during the enzymatic hydrolysis of the triglycerides. The enzymatic activity was calculated from the slope of the titration curve in the interval 3–8 min, of a total of 10 during which the reaction was followed, using

$$A = \frac{v \cdot N_{\mathrm{NaOH}} \times 10^6}{e_{\mathrm{exp}}}, \tag{1}$$

where A is the enzymatic activity expressed in lipase units per gram of the commercial preparation (LU/g); v is the mean rate of addition of NaOH in the interval 3–8 min, in mL/min; N_{NaOH} is the normality of the titrating agent and e_{exp} is the concentration of the enzyme solution which is added (1 mL) to the substrate emulsion (15 mL) to begin the hydrolysis reaction, in g/L.

The influence of temperature on the activity of Lipolase 100 was investigated between 30 and 60 °C. The enzymatic activity (see Fig. 1) reached a maximum between 40 and 50 °C but declined at higher temperatures, possibly due to partial denaturation of the enzyme.

2.4. Droplet-size distribution of the tributyrin emulsions

The droplet-size distribution of the tributyrin emulsions was determined by laser diffraction with a Coulter LS230 instrument

Table 2
Initial hydrolysis rates according to the different criteria used

e_0 (μmol/L)	Criterion 1		Criterion 2		Criterion 3	
	v_{ini} (μmol/ min)	C.V. (%)	v_{ini} (μmol/ min)	C.V. (%)	v_{ini} (μmol/ min)	C.V. (%)
0.0029	4.99	3.73	5.54	4.05	5.62	4.48
0.0060	9.71	1.49	12.12	5.07	12.34	8.61
0.0593	29.29	14.56	101.94	2.02	112.86	4.62
0.5934	12.78	1.57	419.67	4.48	443.16	11.72

e_0 is the concentration of enzyme in the reaction medium, v_{ini} the initial reaction rate (expressed as an extensive variable), C.V. the coefficient of variation, calculated as (standard deviation/mean) ∗100 of three replicates.

(Beckman–Coulter). So that the emulsion preparation would be as repeatable as possible, all were prepared under the same conditions and at a fixed tributyrin volume fraction (0.05), diluting them afterwards to the test concentration. The distribution of droplet sizes was not affected by the dilution.

The Sauter diameter (average volume/surface area) was used as the most representative mean diameter for the kinetic study of the enzymatic hydrolysis of the tributyrin.

2.5. Enzymatic hydrolysis of tributyrin

The enzymatic hydrolysis of tributyrin, at pH 7.0, was monitored according to the pH-stat method.

Solutions of NaOH at concentrations of 0.05–1.0 N, depending on the experiment performed, were used as the titrating agent. For a continual proper dosage of the titrating agent, the automatic titration system was adjusted in each experiment by fixing the maximum and minimum addition rate and the dynamics parameter that permits the selection of the pH range outside of which the dosage is continual. The intervals of these parameters used in the experiments were: dynamics parameter of 0.05–0.2; maximum addition rate of 0.01–30 mL/min; and minimum rate of 0.01–300 μL/ min.

For the hydrolysis reaction, 15 mL of prepared emulsion were placed in the reactor and, when the test temperature was reached, the reaction was initiated by adding 1 mL of the enzyme solution while keeping the reaction system continually stirred and thermostatically controlled. The volume of the titrating agent added to the reaction vessel was recorded for 20 min.

The volume fraction of tributyrin in the aqueous emulsion was changed between 0.0023 and 0.0469 and the enzyme concentration was varied between 0.0006 and 1.484 μmol/L.

2.6. Estimation of the initial reaction rates

For the calculation of the initial reaction rates of the enzymatic hydrolysis of tributyrin, three different criteria were tested and afterwards the most suitable one was chosen.

Under criterion 1, the initial rate was calculated from the slope of the titration curve between 3 and 8 min of reaction. This criterion is used by Novozymes A/S to calculate enzymatic activity (Novo-Nordisk, 1991).

Criterion 2 estimates the slope of the hydrolysis curve at the beginning of the reaction. For this, the count begins at time 0 and a growing number of experimental points are incorporated, making straight-line adjustments by the least-squares method until the value of the slope of these straight lines begins to decrease (Hass et al., 1995).

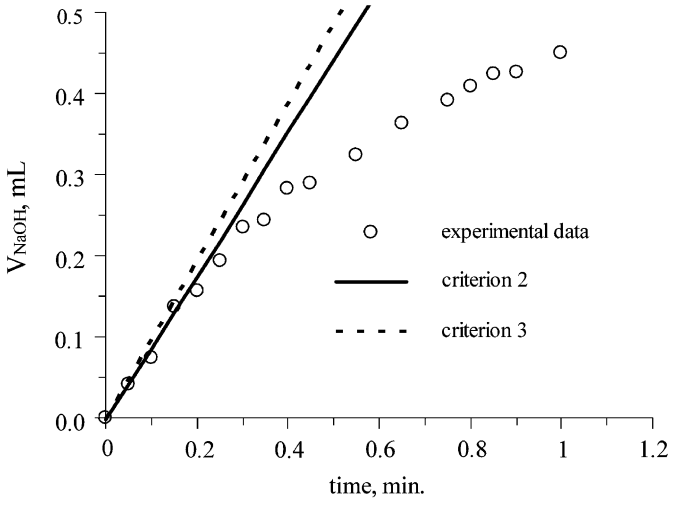


Fig. 2. Comparison of the initial rates calculated according to criteria 2 and 3 for an enzyme concentration of 0.593 μmol/L.

With criterion 3, the rate is calculated using the initial points of the hydrolysis curve, but adjusting them to fourth-order polynomials and calculating the derivative at time 0. The number of points considered in the fit was increased until the derivative at time 0 for two consecutive polynomials differed by less than 0.5%.

The corresponding calculations necessary to apply criteria 2 and 3 were made by programs carried out in MatLab®.

Table 2 indicates the initial reaction rates calculated for different enzyme concentrations and a volume fraction of tributyrin of 0.0094 at 30 °C. Each value is the mean of three replicates and their coefficient of variation is also indicated.

Criterion 1, the simplest one, caused the least dispersion in the results at low enzyme concentrations and thus is appropriate for evaluating the enzymatic activity. However, at high concentrations, it does not represent the initial reaction rate because the reaction rate decreases rapidly at first (Fig. 2). This problem disappeared with the application of criteria 2 and 3, which do represent the initial reaction rate, this increasing with the enzyme concentration until reaching a maximum value, and in no case decreasing.

However, given that the dispersion of the rates calculated by criteria 2 on the same set of replicates was less, and that at high concentrations the initial rate calculated according to criterion 3 represents the slope of the straight line that joins only the first two or three experimental points (see Fig. 2), criterion 2 was therefore chosen for determining the initial reaction rates.

3. Results and discussion

3.1. Evolution of the droplet-size distribution during enzymatic hydrolysis

Fig. 3A shows the droplet-size distribution, before and after the reaction, for an emulsion with a volume fraction of tributyrin of 0.0094 and an enzyme concentration of 0.593 μmol/L. Also, the results of a control assay without enzyme are presented (Fig. 3B). The results rule out that the emulsion was appreciably destabilized in the reactor in the absence of enzyme.

The addition of enzyme did affect the droplet-size distribution which moved towards smaller diameters. This variation in the size distribution during hydrolysis was not reflected in the mean Sauter diameter calculated, which remained practically constant or increased slightly after the reaction. This slight increase can be attributed to the higher relative importance of the droplets of greater size after the hydrolysis (see Fig. 3A). Therefore the mean Sauter diameter will be considered to have remained constant, taking the first value measured in the emulsion for subsequent calculations.

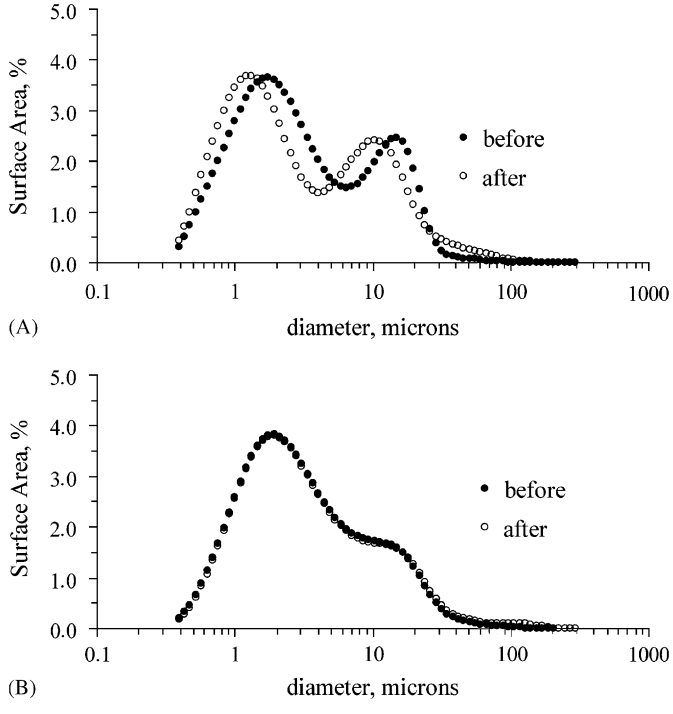


Fig. 3. Droplet-size distributions of tributyrin emulsions (volume fraction of 0.0094) before (0 min) and after 20 min of enzymatic hydrolysis. (A) $e_0 = 0.593$ μmol/L; and (B) control experiment without enzyme.

3.2. Concentration of surface active centres

Lipases catalyse the hydrolysis of triglycerides according to the process:

$$\text{TG} \rightarrow \text{DG} + \text{FFA} \rightarrow \text{MG} + \text{FFA} \rightarrow \text{Glycerol} + \text{FFA},$$

where TG, stands for the triglyceride, DG for the diglyceride, MG for the monoglyceride, and FFA for the free fatty acids released during the process.

As almost all triglycerides are practically insoluble in water and the lipases are mostly soluble, the above reactions occur in a heterogeneous system and therefore require the prior adsorption of the enzyme at the lipid–water interface. Benzonana and Desnuelle (1965) proposed, for the hydrolysis of long-chain triglycerides in emulsion, a two-stage mechanism consisting of the adsorption of the enzyme and the subsequent reaction catalysed by the enzyme, assuming a kinetic of the Michaelis–Menten type. The role represented by the substrate concentration in the homogeneous enzyme kinetic is played in this case by the specific interfacial area of the emulsion. Verger et al. (1973) reached a similar equation to that of Michaelis–Menten for the hydrolysis of phospholipid monolayers but in which the kinetic constants are a function of the interface properties. Afterwards, several authors applied this model to determine the apparent parameters K_M and $v_{\max}$ (Martinez et al., 1992; Jones and Weatherley, 2003). Nevertheless, the kinetic analysis of soluble enzymes acting at interfaces cannot be treated by the classical M–M theory because of the two-dimensional state of the substrate involved (Mogi et al., 1999).

To be consistent with the fact that the enzyme reaction occurs at the interface, we expressed the substrate concentration in a two-dimensional way, using the term "concentration of surface active centres" for the concentration of substrate at the interface, and thus accessible to the enzyme. The hydrolysis of tributyrin in emulsion form implies the direct contact between the enzyme and the ester bonds in the tributyrin molecules. For the hydrolysis reaction to occur, the ester bond must be situated on the surface of the droplet in contact with the aqueous phase containing the lipase. The tetrahedral arrangement of the central carbon of the glycerol in the tributyrin molecule and the three *n*-butyl hydrophobic groups bonded to three of these positions suggest that the tributyrin molecule will appear more frequently with the edge formed by the hydrogen and an ester group in contact with the aqueous phase (Fig. 4A). That is, only one of the ester groups is accessible from the aqueous phase. Also, the same reasoning indicates that when hydrolysis occurs the diglyceride formed may be retained in the organic phase, and if it is found on the surface it will be preferentially arranged with the hydrogen and the free alcohol group towards the aqueous phase, orientating its remaining ester groups towards the core of the organic phase (Fig. 4B).

Therefore, to develop a suitable model that explains the enzymatic hydrolysis of tributyrin, we must evaluate the concentration of the surface ester bonds (or surface active centres) which must be the same as that of surface molecules of tributyrin.

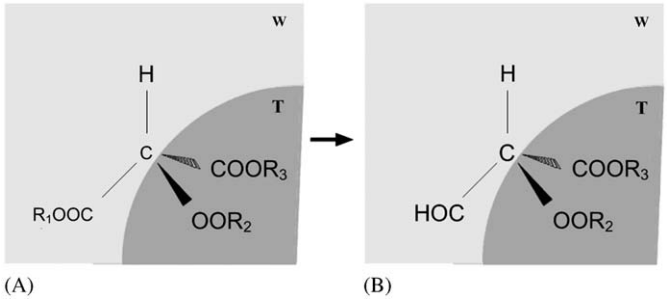


Fig. 4. Arrangement of tributyrin (A) and dibutyrin (B) molecules at the interface. *W* stands for aqueous phase and *T* for tributyrin (oil phase).

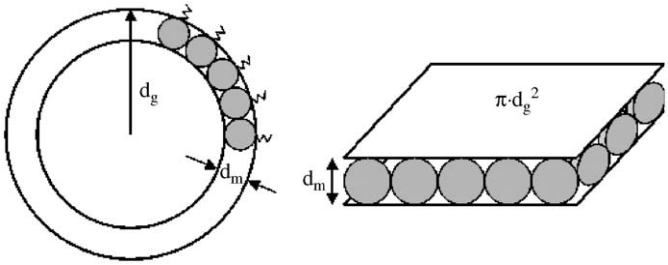


Fig. 5. Evaluation of the concentration of surface active centres.

The concentration of surface molecules on the tributyrin droplets of d_g diameter was calculated by the procedure described below and schematized in Fig. 5.

The diameter of the tributyrin molecule (d_m) in liquid phase was evaluated from

$$d_m = \sqrt[3]{\frac{6V_m}{\pi}} = 9.75 \times 10^{-8}\,\text{cm} \quad \therefore 9.75 \times 10^{-4}\,\mu\text{m}, \tag{2}$$

where V_m is the volume of the tributyrin molecule in liquid phase ($4.85 \times 10^{-10}\,\mu\text{m}^3$) that can be calculated on knowing its molecular weight and its density.

For a spherical droplet of diameter d_g, the surface molecules are those found on the outer spherical crown of thickness d_m, and, given that $d_m \ll d_g$, the number of surface molecules of tributyrin would be

$$N_{ms} = \frac{\text{Volume of the outer spherical crown}}{V_m} = \frac{\pi d_g^2 d_m}{V_m} = 6.32 \times 10^6 d_g^2, \tag{3}$$

where d_g should be expressed in microns. The total number of molecules in the droplet would be

$$N_m = \frac{\pi d_g^3}{6V_m} = \frac{\pi}{6(4.85 \times 10^{-10})} d_g^3 = 1.08 \times 10^9 d_g^3 \tag{4}$$

and the fraction of surface molecules can be determined by:

$$Y_S = \frac{N_{ms}}{N_m} = \frac{5.85 \times 10^{-3}}{d_g}. \tag{5}$$

If the distribution of the droplet sizes in the emulsion is known (Fig. 3) then it is possible to calculate the Sauter diameter, averaging the volume/surface area relationship according to:

$$d_{gS} = \frac{\int_0^\infty f d_g^3 d d g}{\int_0^\infty f d_g^2 d d g}. \tag{6}$$

If the emulsion contains a volume fraction of the dispersed phase equal to α, the concentration of tributyrin per unit of volume of the emulsion (c_0) is given by:

$$c_0 = \frac{\alpha \cdot \rho_T}{M_T} = \frac{1035\alpha}{302.37} = 3.42\alpha\,\frac{\text{mol}}{\text{L}}, \tag{7}$$

where ρ_T is the density of the tributyrin and M_T its molecular weight.

The concentration of molecules at the interface between the organic and aqueous phases (c_{S0}) would be:

$$c_{S0} = Y_S c_0 = 0.0200\frac{\alpha}{d_{gS}}\,\frac{\text{mol}}{\text{L}}. \tag{8}$$

Using this equation is possible to estimate c_{S0} from the volume fraction of the dispersed phase in the emulsion and the mean Sauter diameter.

The calculation of the molar enzyme concentration requires knowing the quantity of active protein present in the commercial enzyme preparation and its molecular weight. On the basis of these two data, it is possible to estimate the molar concentration of the enzyme in the emulsion.

3.3. Mechanism of the process

In any case, for the enzymatic hydrolysis to take place, it is necessary for the enzyme to make contact with a surface ester bond. The hydrolysis of dissolved tributyrin has been assumed to be negligible because the enzyme we are using is known to exhibit a pronounced interfacial activation (Martinelle et al., 1995). Therefore, the kinetic mechanism occurs in three stages:

(1) Adsorption or penetration of the enzyme in the aqueous-organic interface, involving the formation of the activated enzyme

$$E + I \underset{k_d}{\overset{k_a}{\rightleftarrows}} E^*. \tag{9}$$

(2) Joining of the activated enzyme with a hydrolysable surface ester bond and formation of the enzyme–substrate complex

$$E^* + C_s \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E^* C_s. \tag{10}$$

(3) Separation of the intermediate formed in the products and release of the activated enzyme. This step was considered to be irreversible

$$E^* C_s + \text{H}_2\text{O} \overset{k_2}{\rightarrow} E^* + D + A, \tag{11}$$

where E represents the free enzyme, I the free interfacial area, E^* the activated enzyme (adsorbed), C_s a free hydrolysable surface ester bond, E^*C_s the enzyme–substrate

complex, D the diglyceride, and A the fatty acid released in the hydrolysis. The corresponding elemental kinetic constants are k_a, k_d, k_1, k_{-1} and k_2.

The enzyme adsorption stage is considered to conform to the Langmuir isotherm. Thus, the adsorbed enzyme concentration could be expressed as a function of the free enzyme in the aqueous phase and of the interfacial area of the emulsion:

$$[E^*] = \frac{K_A[E^*]_{\max}[E]}{1 + K_A[E]}, \tag{12}$$

where K_A represents the equilibrium constant associated with the adsorption of the enzyme and $[E^*]_{\max}$ the maximum concentration of absorbed enzyme which in turn depends on the total interfacial area of the emulsion.

However, Eq. (12) can be simplified in two limiting situations:

- For low, and thus non-saturating, enzyme concentrations $K_A[E] \ll 1$, and therefore Eq. (12) becomes:

$$[E^*] = K_A[E^*]_{\max}[E]. \tag{13}$$

- For saturating enzyme concentrations $K_A[E] \gg 1$, and consequently the absorbed enzyme concentration will be constant and equal to its maximum value:

$$[E^*] = [E^*]_{\max}. \tag{14}$$

When the approximation of the stationary state is applied to the intermediate formed, we get:

$$\frac{\mathrm{d}[E^*C_s]}{\mathrm{d}t} = k_1[E^*][C_s] - (k_{-1} + k_2)[E^*C_s] \approx 0, \tag{15}$$

where the brackets represent the molar concentrations of the corresponding species (moles per unit of emulsion volume). Solving the concentration of the enzyme–substrate complex of Eq. (15) gives:

$$[E^*C_s] = \frac{k_1}{(k_{-1} + k_2)}[E^*][C_s]. \tag{16}$$

The reaction rate can be calculated from the second stage:

$$r = k_2[E^*C_s] = \frac{k_2k_1}{(k_{-1} + k_2)}[E^*][C_s]. \tag{17}$$

The balances of enzyme and of the hydrolysable surface ester bonds are given by Eqs. (18) and (19), respectively

$$[E^*C_s] + [E^*] + [E] = e_0, \tag{18}$$

$$[E^*C_s] + [C_s] = c_{S0}. \tag{19}$$

3.4. Initial reaction rates

If we apply Eq. (17) to the initial reaction rates, we get:

$$r_0 = \frac{k_2k_1}{(k_{-1} + k_2)}[E^*]_0[C_s]_0. \tag{20}$$

The model can be solved for the two limiting situations previously described:

Non-saturating enzyme concentrations: The Langmuir isotherm reduces to Eq. (13) and, in addition, Eq. (19) is simplified to:

$$[C_s] \approx c_{S0}. \tag{21}$$

Considering Eqs. (13), (16) and (21) the enzyme balance, Eq. (18), can be expressed as:

$$e_0 = [E^*]\left[\frac{k_1}{k_{-1} + k_2}c_{S0} + \left(1 + \frac{1}{K_A[E^*]_{\max}}\right)\right]. \tag{22}$$

Isolating the absorbed enzyme concentration from Eq. (22) and replacing it in Eq. (20) yields Eq. (23), which is valid for initial reactions rates at low enzyme concentrations

$$r_0 = \frac{k_2e_0c_{S0}}{K_M\left(1 + \dfrac{1}{K_A[E^*]_{\max}}\right) + c_{S0}}. \tag{23}$$

The Michaelis–Menten constant, K_M, has been introduced in Eq. (23). It should be pointed out that the factor multiplying the Michaelis–Menten constant in the above equation is a function of the interfacial area of the emulsion. Thus, we can define a modified Michaelis constant in the following form:

$$K_M^* = K_M\left(1 + \frac{1}{K_A[E^*]_{\max}}\right). \tag{24}$$

Saturating enzyme concentrations: The absorbed enzyme concentration is equal to its maximum value. Therefore, replacing Eq. (14) and (16) in the balance of surface esters bonds (Eq. (19)), we get:

$$[C_S]\left(1 + \frac{k_1}{k_{-1} + k_2}[E^*]_{\max}\right) = c_{S0}. \tag{25}$$

Isolating $[C_s]$ from Eq. (25) and replacing it in Eq. (20) we obtain Eq. (26) which is valid for initial reaction rates and high enzyme concentrations

$$r_0 = \frac{k_2c_{s0}}{\dfrac{K_M}{[E^*]_{\max}} + 1}. \tag{26}$$

Therefore, to apply the model to the experimental data, we should know the range of the enzyme and substrate concentrations with which we are faced in order to decide in which of the two limiting situations we are working.

The smaller volume fraction of tributyrin and the greater enzyme concentration used in the same experiment were 0.0094 and 1.563 g/L, respectively, which is equivalent to a concentration of surface active centres of 3.28×10^{-5} mol/L and a molar concentration of enzyme of 1.484×10^{-6} mol/L, referring to the total emulsion volume. Therefore, the enzyme concentration is less than that of surface ester bonds, at least by one order of magnitude, throughout the experimental domain. However, the analysis of the experimental results obtained at 30 °C, which are plotted in Fig. 6, clearly indicates that the saturation of the

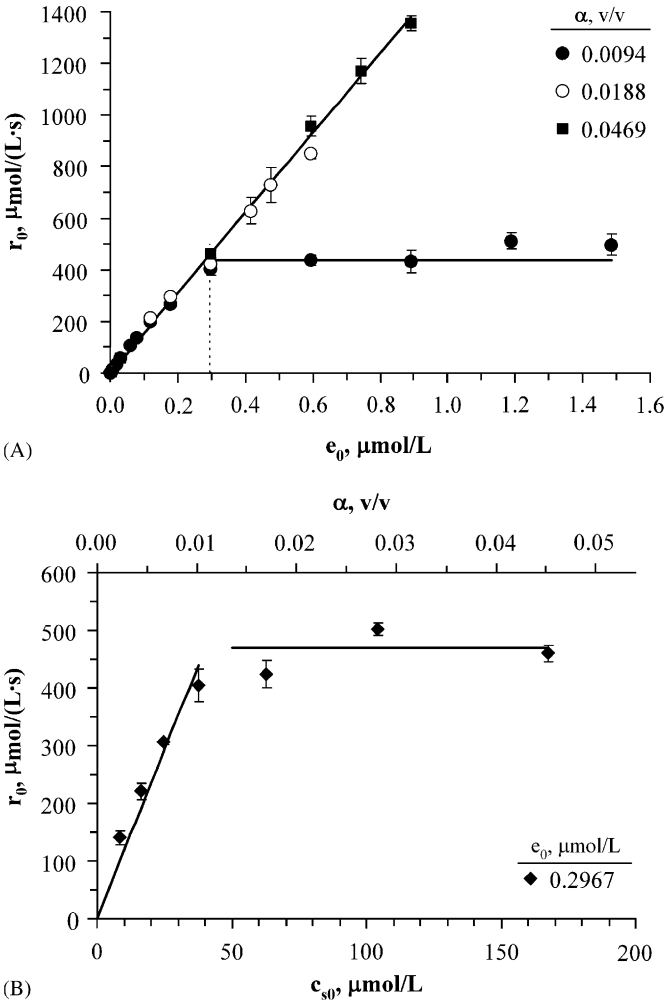


Fig. 6. Experimental reaction rates obtained at 30 °C: (A) experiments performed at a constant volume fraction of tributyrin (0.0094, 0.0188 and 0.0469); and (B) experiments carried out at a constant enzyme concentration (0.2967 μmol/L). The vertical broken line approximately indicates the saturation limit for $\alpha = 0.0094$. Solid lines correspond to the model predictions.

interface is reached within the experimental domain. As can be seen in Fig. 6A, in the experiments performed with a volume fraction of tributyrin of 0.0094, a maximum reaction rate is attained when the enzyme concentration is increased over a critical value (0.3 μmol/L approx). As expected, when the volume fraction of tributyrin is increased the saturation phenomenon does not occur at the same critical concentration of enzyme (see Fig. 6A). Another interesting fact is that the slope of the linear region of the plots in Fig. 6A does not depend on the volume fraction of tributyrin, which, according to Eq. (23), indicates that the concentration of surface ester bonds (c_{s0}) must be higher than the modified Michaelis constant in all the experiments displayed in Fig. 6A.

Consequently, using Eq. (23) to adjust the experimental results obtained at $\alpha > 0.0094$ and at $\alpha = 0.0094$ and $e_0 < 0.3$ μmol/L, we can determine the value of k_2 and an approximate value of K_M^*. To do that a program in MatLab®, designed to minimize the sum of the absolute value of the residuals, was used. The calculated values are listed in Table 3 together with the relative deviation between the experimental results and those predicted by the model, calculated according to Eq. (27)

$$\text{Relative deviation} = \frac{\sum |(r_0)_{\text{exp}} - (r_0)_{\text{cal}}|}{\sum (r_0)_{\text{exp}}}. \tag{27}$$

It is noteworthy that the results of experiments performed with different volume fractions of tributyrin (0.0094, 0.0188, 0.0469) can be satisfactorily predicted using a single value of K_M^*. According to Eq. (23) this can only happen if the value of K_A is very high, which indicates a large affinity of the enzyme for the interface.

On the other hand, experiments carried out in saturating conditions ($\alpha = 0.0094$ and $e_0 > 0.3$ μmol/L) can be fitted to Eq. (26), which predicts a constant value of r_0 in the conditions referred to. Having determined the value of r_0 (4.565×10^{-4} mol/L s according to the experimental results plotted in Fig. 6A) and using the previously calculated value of k_2, it is possible to estimate the value of $K_M/[E^*]_{\max}$ for $\alpha = 0.0094$ (see Table 3).

The initial reaction rates obtained while keeping the enzyme concentration constant ($e_0 = 0.297$ μmol/L) and varying the concentration of surface ester bonds (i.e., the volume fraction of tributyrin) are shown in Fig. 6B. The linear part of the plot, which corresponds to volume fractions of tributyrin lower than 0.0094, can be fitted to Eq. (26), which, taking into account the previously estimated value of $K_M/[E^*]_{\max}$, reduces to Eq. (28)

$$r_0 = \frac{[E^*]_{\max} k_2}{K_M} c_{s0}. \tag{28}$$

From Eq. (28) it is possible to estimate an average value of $K_M/[E^*]_{\max}$ (see Table 3). Obviously, this value is applicable only for volume fractions of tributyrin between 0.0023 and 0.0094.

The results shown in Fig. 6 also confirm that the hydrolysis of dissolved tributyrin is not significant. As can be seen in Fig. 6B, when the concentration of surface ester bonds is lower than 40 μmol/L, and thus lower than the concentration of dissolved tributyrin (which remains constant and equal to 412 μmol/L as long as the oily phase is present), the initial reaction rate varies almost linearly with the concentration of surface ester bonds. This could not happen if the dissolved tributyrin were significantly hydrolysed by the enzyme.

The proposed model therefore explains the enzymatic hydrolysis of tributyrin with the enzyme Lipolase 100 in both limiting situations: saturating and non-saturating enzyme concentrations. The agreement between the experimental and predicted results can also be observed in Fig. 7, where they are plotted against each other.

Given that the molar concentration of the enzyme was less than that of surface-ester bonds throughout the experimental domain, the enzymatic saturation can only be explained by the lack of interface available for the enzyme, as it occupies a far greater space than that of a surface-ester bond. Thus, from a certain enzyme concentration that depends on the interface area

Table 3
Model parameters calculated from the fit of experimental results at 30 °C

Equation	Data fitted	Relative deviation (%)	k_2 (s^{-1})	$\bar{K}^*_M$ (mol/L)	$\frac{K_M}{[E^*]_{max}}$
(23)	$\alpha = 0.0094$ and $e_0 < 0.3$ μmol/L $\alpha > 0.0094$	4.5	1580.2	2.926×10^{-6}	—
(26)	$\alpha = 0.0094$ and $e_0 > 0.3$ μmol/L	7.8	—	—	115
(28)	$\alpha < 0.0094$	11.5	—	—	135

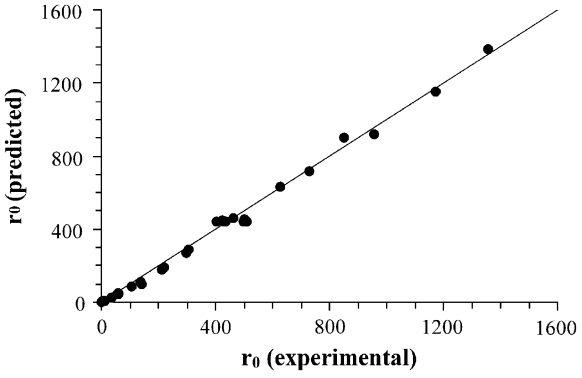


Fig. 7. Comparison of the experimental reaction rates at 30 °C and those predicted by the model (Eqs. (23) and (26)).

available, the addition of new enzyme does not imply an increase of the initial hydrolysis rate.

Therefore, the enzyme concentration beyond which interface saturation occurs is a critical value that should be taken into account in the practical application of enzymatic hydrolysis of triglycerides, given that the use of higher enzyme concentrations than this will not speed up the reaction. This value of course depends on the specific interfacial area of the emulsion.

3.5. Progress of the reaction over time

Given that the model suitably reproduced the results corresponding to the initial reaction rates, we have applied it to the progress of the reaction over time for the experiments made at non-saturating enzyme concentrations. In this case, the balance of the surface centres should include the hydrolysed bonds, and thus would present the following form, where C_{SOH} represents a hydrolysed surface ester bond

$$[E^*C_S] + [C_S] + [C_{SOH}] = c_{S0}. \tag{29}$$

When we consider the balance in the way specified, Eq. (23) becomes:

$$r = \frac{k_2 e_0 (c_{S0} - [C_{SOH}])}{K^*_M + (c_{S0} - [C_{SOH}])}. \tag{30}$$

The relation between c_{s0} and $[C_{SOH}]$ is given by the conversion, which, referred to the initial total concentration of ester bonds in the emulsion, can be calculated as:

$$x = \frac{3c_0 - c_e}{3c_0} \quad c_e = 3c_0(1 - x), \tag{31}$$

where c_0 represents the initial molar concentration of tributyrin, and c_e the total concentration of non-hydrolysed ester bonds in the system, from which the reaction rate can be expressed as:

$$r = -\frac{dc_e}{dt} = 3c_0 \frac{dx}{dt}. \tag{32}$$

According to the previous definition, the full conversion is achieved when all the ester bonds in the system are hydrolysed. As the enzyme is sn-1,3 specific, the reaction cannot progress beyond $\frac{2}{3}$ of the total ester bonds in the system, unless acil-migration phenomenon would take place which is unlikely to occur in the time scale of our experiments.

As previously stated probably not all of the ester groups in the tributyrin molecule are accessible from the aqueous phase. If we take into account the tetrahedral arrangement of the central carbon of the glycerol in the tributyrin molecule (Fig. 4A), only one of the ester groups will be accessible from the aqueous phase. When the hydrolysis occurs, the diglyceride formed is retained fundamentally in the organic phase; if it is on the surface, it will be arranged preferentially with the hydrogen and the alcohol group free towards the aqueous phase, orientating its remaining ester groups towards the core of the organic phase (Fig. 4B). This signifies that under normal conditions the process hydrolyses around $\frac{1}{3}$ of the ester groups present, which agrees with the experimental results obtained (Fig. 8).

Given that the diglyceride and a small fraction of the butyric acid released can remain in the organic phase, an acceptable approach would in principle be for the droplets to remain practically constant during the process in the same way as for the volume fraction of the organic phase and the specific total interface area, as confirmed experimentally (Fig. 3). If we admit that the ester group of position 2 is not hydrolysed, since the enzyme is 1,3-specific, at each time, it holds that:

$$c_0 = c_{TB} + c_{DB} + c_{MB}, \tag{33}$$

$$c_e = 3c_{TB} + 2c_{DB} + c_{MB}, \tag{34}$$

where c_{TB}, c_{DB} and c_{MB} are, respectively, the molar triglyceride, diglyceride, and monoglyceride concentrations in the emulsion.

In the first stage of the hydrolysis, it may be assumed that practically no monoglyceride was formed and the above equations are simplified to:

$$c_0 = c_{TB} + c_{DB}, \tag{35}$$

$$c_e = 3c_{TB} + 2c_{DB}. \tag{36}$$

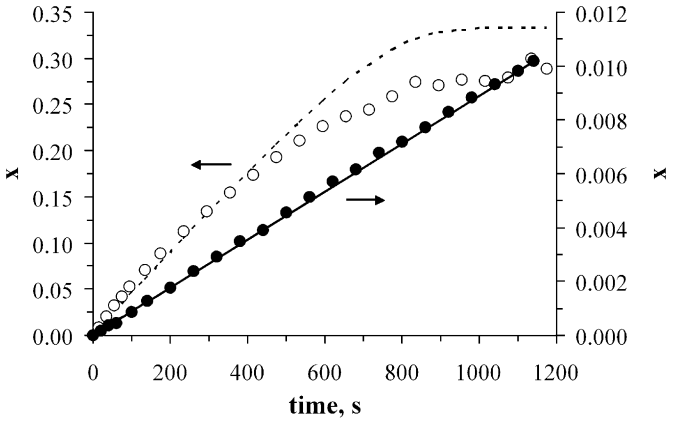


Fig. 8. Comparison between the experimental and calculated conversions. Experiments carried out at a volume fraction of tributyrin of 0.0094 and two different enzyme concentrations: (•) 0.0006 μmol/L; and (○) 0.0297 μmol/L.

Subtracting both equations to eliminate the dibutyrin concentration, we get

$$c_{TB} = c_e - 2c_0 = c_0(1 - 3x) \tag{37}$$

and therefore

$$c_{DB} = 3c_0 x. \tag{38}$$

If we admit that the droplet composition is uniform during the hydrolysis, the molar concentration of the free surface alcohol groups (diglycerides) will be:

$$[C_{SOH}] = 3c_{S0}x. \tag{39}$$

Replacing this latter equation in the expression of the reaction rate, Eq. (30), we get:

$$r = 3c_0\frac{dx}{dt} = \frac{k_2 e_0 c_{S0}(1 - 3x)}{K_M^* + c_{S0}(1 - 3x)}. \tag{40}$$

If we express c_0 as a function of the volume fraction of tributyrin (Eq. (7)), we get the following expression that describes the progress of the reaction over time:

$$\frac{dx}{dt} = \frac{k_2}{10.26\alpha} \cdot \frac{e_0 c_{S0}(1 - 3x)}{K_M^* + c_{S0}(1 - 3x)}. \tag{41}$$

The foregoing equation can be used to simulate the progress of the reaction with time using the values of the kinetic constants calculated from the initial reaction rates. For this, the MatLab® program was used. The simulations made coincided very acceptably with the experimental values for low enzyme concentrations at the different temperatures studied, as observed in Fig. 8 where the simulations are plot together with the experimental values for two different enzyme concentrations at 30 °C.

However, at higher enzyme concentrations, deviations occurred when reaching conversion values higher than 0.25, as reflected in Fig. 8. These deviations can be ascribed to the accumulation of the diglyceride in the interface as the reaction progresses to conversions of nearly one third of the total ester bonds.

Table 4
Kinetic parameters for the enzymatic hydrolysis of tributyrin with Lipolase 100 at different temperatures

Temperature (°C)	Relative deviation (%)		k_2 (s^{-1})
30	4.6		1580.2
40	3.4		1761.2
50	2.7		1898.5
60	1.3		1960.0
Parameters of Arrhenius equation			
$\Delta E/R$, K	ΔE (kcal/mol)	B (s^{-1})	R^2
899.4	1.79	3.09×10^4	0.993

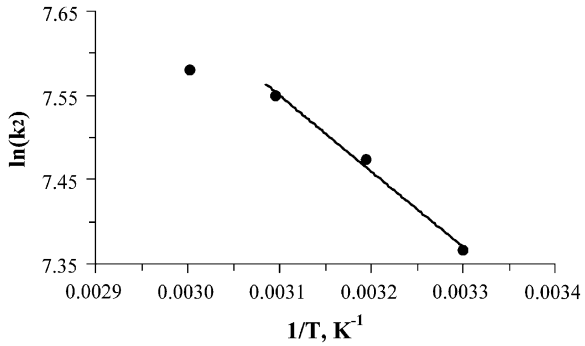


Fig. 9. Variation of the constant k_2 with temperature. Fit of the Arrhenius equation.

The remarkable slowing down of the reaction as it approaches one third of the total ester bonds must be overcome in order to make the enzymatic reaction a viable process. One possible approach to solving this problem is the addition of surfactants to the reaction medium. According to some of our results (data not shown), the anionic surfactant LAS (sodium salt of the dodecyl benzenesulfonic acid) helps to overcome this problem, partially reducing the slowing down of the reaction. Another possible approach would be reducing the water content of the medium in which the enzymatic reaction is carried out by using organic solvents.

3.6. Effect of temperature in the initial rate of hydrolysis

The application of the model for low enzyme concentrations (Eq. (23)) to the rest of the temperatures assayed, enabled us to determine the variation of the kinetic constant k_2 with temperature (see Table 4). To do that, we have used an average value of K_M^* in order to avoid solutions without physical meaning. The fit proved satisfactory with relative deviations consistently lower than 5%.

The fit of the Arrhenius equation of the constant k_2 is shown in Fig. 9, where the k_2 values are aligned except at a temperature of 60 °C. The greater importance of the enzymatic deactivation at 60 °C (Fig. 1) was very probably the cause of this behaviour. The application of the Arrhenius equation up to 50 °C led to the determination of the apparent activation energy of the enzymatic reaction (ΔE) and of the frequency factor (B). These values are also reflected in Table 4.

The value found for the activation energy of the reaction (1.79 kcal/mol) is of the same order of magnitude as that found by Al-Zuhair et al. (2003) for the hydrolysis of palm oil by lipase of 1.2 kcal/mol. In addition, it proves comparable with the values found by other authors for the enzymatic hydrolysis of triglycerides, both in emulsion, 5.3 kcal/mol (Desnuelle, 1961) an in reverse micelles, 7.0 kcal/mol (Kim and Chung, 1989).

4. Conclusions

A kinetic model has been developed for the enzymatic hydrolysis of tributyrin in the form of O/W emulsion. The model takes into account the molar concentration of the surface ester bonds that are accessible to the enzyme.

The model gives a satisfactory fit, with a relative deviation of less than 5%, for the initial reaction rates determined at low (non-saturating) enzyme concentrations and at temperatures of up to 60 °C, enabling the determination of the kinetic constant k_2 and its variation with temperature. The results obtained at saturating enzyme concentrations can also be predicted by the proposed model. In addition, the kinetic model can also be applied to the course of the reaction over time up to conversions of 0.25, i.e., when the interface is presumably saturated with diglyceride.

From a practical standpoint, low enzyme concentrations, for which saturation of the interface does not occur, are the suitable ones for enzymatic hydrolysis of triglycerides in aqueous emulsions and also for the use of the enzyme in detergent formulas, as a higher dosage of the enzyme will not contribute to an increase in detergency. Therefore, the model we have presented in this work represents a rather simplified approach that is useful for the sizing of enzymatic reactors for the hydrolysis of triglycerides in aqueous emulsion, even in the presence of an organic solvent as the diluent. It also provides useful information as regards the addition of lipases to enzymatic detergent formulations. It should be remarked that despite the results presented in this work where obtained with tributyrin, the proposed model can also be applied to the hydrolysis of other triglycerides of greater practical importance.

Notation

A	lipolytic activity, LU/g
B	frequency factor (Arrhenius equation), s^{-1}
c_0	initial concentration of tributyrin per unit of volume of the emulsion, mol/L
c_{DB}	dibutyrin concentration per unit of volume of the emulsion, mol/L
c_e	total concentration of non-hydrolysed ester bonds in the emulsion, mol/L
c_{MB}	monobutyrin concentration per unit of volume of the emulsion, mol/L
c_{s0}	initial concentration of surface ester bonds, mol/L
c_{TB}	tributyrin concentration per unit of volume of the emulsion, mol/L
C_S	free hydrolysable surface ester bond
C_{SOH}	hydrolysed surface ester bond
d_g	diameter of a tributyrin droplet, μm
d_{gS}	mean Sauter diameter of the emulsion, μm
d_m	diameter of a tributyrin molecule, μm
e_{exp}	concentration of the enzyme solution which is added to the substrate emulsion to begin the hydrolysis reaction, g/L
e_0	concentration of the enzyme in the emulsion, mol/L
E	free enzyme
E^*	adsorbed (activated) enzyme
$[E^*]_{max}$	maximum concentration of absorbed enzyme, mol/L
E^*C_S	enzyme–substrate complex
ΔE	apparent activation energy, kcal/mol
I	free interfacial area
k_2	kinetic constant (includes water concentration), s^{-1}
K_A	equilibrium constant associated with the adsorption of the enzyme
K_M^*	modified Michaelis–Menten constant, Eq. (24), mol/L
M_T	molecular weight of tributyrin(302.37 g/mol)
N_m	total number of molecules in the tributyrin droplet
N_{ms}	total number of surface molecules in the tributyrin droplet
N_{NaOH}	sodium hydroxide normality
r	reaction rate, mol/(L s)
r_0	initial reaction rate, mol/(L s)
v	mean rate of addition of NaOH in the interval 3–8 min
v_{ini}	initial reaction rate (expressed as an extensive variable), μmol/ min
V_m	volume of the tributyrin molecule in liquid phase, μm^3
Y_s	fraction of surface molecules of tributyrin in the emulsion

Greek letters

α	volume fraction of tributyrin in the emulsion
ρ_T	tributyrin density, g/mL

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