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Kinetics of the enzymatic hydrolysis of triglycerides in o/w emulsions Study of the initial rates and the reaction time course

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ABSTRACT

The hydrolysis of emulsified tributyrin by *Thermomyces lanuginosus* lipase (TLL) has been studied by titrimetric and colorimetric measurements as well as by thin-layer chromatography (TLC). A kinetic model have been developed and applied to the initial reaction rates as well as to simulate the time course of the hydrolysis reaction at several temperatures, lipase concentrations and volume fractions of tributyrin. The model successfully predicted the initial reaction rates, even at saturating enzyme concentrations, with a relative deviation of less than 5%. The reaction-progress curves predicted by the model also agree with the experimental results significantly. The combined analysis both of the experimental results and of the simulations suggests that the hydrolysis of tributyrin by TLL proceeds through two separate stages which partially overlap. In the first one, the main reaction is the hydrolysis of one of the outer ester groups (sn-1 or 3) of the tributyrin molecule. The second stage starts when the interface becomes saturated of diglyceride, and involves mainly the hydrolysis of the remaining outer ester bond. The results found offer valuable information on the time course of the enzymatic hydrolysis of emulsified triglycerides, which is useful for some of the applications of these enzymes, such as free fatty acid production, wastewater treatment or washing processes.

1. Introduction

Hydrolysis of emulsified triglycerides by lipases (E.C. 3.1.1.3) has great practical importance, as it represents a more attractive alternative for fatty acid production than the traditional chemical process [1]. Additionally, triglyceride emulsions (O/W) also constitute the environment in which lipases exert their catalytic action in nature as well as in other important and promising applications of these enzymes, such as the treatment of wastewater with a high fat content [2] or in washing processes [3–5], where lipases enable a more efficient removal of oily stains, especially at low temperatures [3,5].

Despite these facts, the most comprehensive studies on lipase kinetics have been conducted with monolayers [6], micelles [7,8] and phospholipid vesicles [9]. These systems, though thermodynamically stable and easier to characterize than emulsions, only partially reproduce the performance conditions of lipases in the aforementioned applications. Thus, in order to optimize lipase performance in hydrolytic and cleaning applications, the kinetics of lipase action in O/W emulsions must be studied in detail.

It is also remarkable that virtually all the kinetic studies on the hydrolysis of emulsified triglycerides by lipases published so far [10–14] consider only the initial reaction rates—that is, the kinetic models developed in these studies are never applied to predict the reaction time course, which is of key importance in simulating the operation of enzymatic bioreactors as well as in gaining a more complete insight into the mechanism of lipase action in washing and wastewater treatment processes. Very recently, Hermansyah et al. [15] have proposed a kinetic model for the lipase-catalyzed hydrolysis of triglycerides which assumes a Ping Pong Bi mechanism with competitive inhibition by the fatty acid at the three stages of the reaction. The model satisfactorily predicts the progress of the reaction over time, but results in a large set of kinetic and adsorption constants which, in addition, are difficult to validate by comparing them to previously publish results. Moreover, and despite the greater complexity of this model, it only allows for a slight improvement of the fittings when compared to those obtained with a Michaelis–Menten-type model. Nevertheless, a better comprehension of the changes taking place in the reaction medium during hydrolysis would allow optimization of working conditions for lipases in each application.

However, analysis of the progress of lipolysis over time is a difficult task, as reaction products, once released, can be distributed between the aqueous or the organic phase, depending on their pre-

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Nomenclature

a	free specific interfacial area of the emulsion (cm^2/L)
A_m	enzyme coverage (cm^2/mol)
a_T	total specific interfacial area of the emulsion (cm^2/L)
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-hydrolyzed ester bonds in the emulsion (mol/L)
c_{MB}	monobutyrin concentration per unit of volume of the emulsion (mol/L)
c^*_{s0}	initial interfacial concentration of surface ester bonds (mol/cm^2)
c_{TB}	tributyrin concentration per unit of volume of the emulsion (mol/L)
d_{gS}	mean Sauter diameter of the emulsion (μm)
ΔE	apparent activation energy (kJ/mol)
[E]	free enzyme concentration (mol/L)
[E*]	interfacial concentration of adsorbed (activated) enzyme (mol/cm^2)
e_o	initial concentration of the enzyme in the emulsion (mol/L)
[E*S]	interfacial concentration of enzyme–substrate complex (mol/cm^2)
G	dimensionless parameter defined in Eq. (35)
ΔH_a	enthalpy change for enzyme-adsorption (kJ/mol)
k_2	rate constant (includes water concentration) (s^{-1})
k_{ap}	apparent rate constant defined in Eq. (12) (s^{-1})
K_{ap}	apparent equilibrium constant defined in Eq. (13) (L^2/cm^4)
K_e	equilibrium constant of enzyme-adsorption (L^2/cm^4)
K^*_M	Michaelis–Menten constant for the interfacial enzyme–substrate complex, defined in Eq. (9) (mol/cm^2)
K_r	surface/bulk partition coefficient for dibutyrin
M_{tb}	molecular weight of tributyrin (302.37 g/mol)
n_{but}	amount of butyric acid released (mol)
r	reaction rate (mol/(L s))
r_0	initial reaction rate (mol/(L s))
[S*]	interfacial concentration of free hydrolysable surface ester bonds (mol/cm^2)
[S*OH]	Interfacial concentration of hydrolyzed surface ester bonds (diglyceride) (mol/cm^2)
y_a	fraction of free interfacial area, defined in Eq. (14)

Greek symbols

α	volume fraction of tributyrin in the emulsion
β	dimensionless parameter defined in Eq. (33)
ρ_{tb}	tributyrin density (1.035 kg/L)

dominantly hydrophilic or lipophilic character. In addition, some of the hydrolysis products, such as free fatty acids, mono- and diglycerides, are surface-active compounds and, consequently, tend to accumulate at the interface, thereby decreasing the interfacial tension and interfering with the action of lipases. This results in a rapid reduction of the reaction rate, found by several authors [5,13–16] on performing the hydrolysis of vegetable oils in O/W emulsions.

Another difficulty frequently found in most of the already published studies on enzymatic hydrolysis of emulsified triglycerides [10–14] is about the determination of the specific interfacial area of the substrate emulsions, which is a key parameter for the kinetic modelling. Estimation is normally accomplished by the use of empiric equations, such as the Calderbank's [17], which implies a loss of accuracy compared to its direct measurement.

In a previous study [18], we also developed a simplified kinetic model for tributyrin hydrolysis, based on the calculation of the concentration of surface ester bonds. This model proved valid for low and saturating enzyme concentrations, although it was necessary to use a different equation for each one of these limiting situations.

In the present work, a kinetic model for the enzymatic hydrolysis of triglycerides in O/W emulsions has been developed, using the same assumptions as in Tsai and Chang's study [12]. This model has been used to perform a systematic kinetic study of the enzymatic hydrolysis of triglycerides in O/W emulsions, varying enzyme concentration, oil phase volume fraction and temperature. Tributyrin was chosen as a model substrate, because butyric acid mainly distributes in the aqueous phase, thus making it easier to describe the surface behaviour of the system as the reaction progresses. To ensure a more accurate determination of the model parameters, a greater number of hydrolysis experiments, as compared to previously published studies, have been carried out. The model, applied to the initial reaction rates, proves valid for low and high (saturating) enzyme concentrations, the relative deviation between the experimental and predicted values being less than 5%.

The kinetic model has also been applied to predict the time course of the hydrolysis of emulsified tributyrin, with useful conclusions being drawn about the changes in the droplet composition during the reaction. Several assumptions, made on the spatial arrangement of tri- and diglyceride molecules at the surface of the organic-phase droplets, were finally confirmed by the analysis of the experimental results.

2. Materials and methods

2.1. Products and reagents

The enzyme used was the commercial lipase Lipolase® 100, from *Thermomyces lanuginosus* (TL), supplied by Novozymes A/S, Bagsvaerd, Denmark, with a reported 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 specific activity of the enzyme is 100,000 LU/g of the commercial product (one LU represents the amount of enzyme that releases 1 μmol/min of butyric acid at 30 °C and pH 7.0). TLL possesses sn-1,3 specificity, i.e., it preferentially cleaves the ester bonds on the positions sn-1 and sn-3 of the triglyceride molecule.

Tributyrin (99% richness) was supplied by Merck (Darmstadt, Germany) and used without further purification. To stabilize the tributyrin emulsions an emulsification reagent, for which the active ingredient was gum arabic (from Merck) was used. The detailed composition of the emulsification reagent has been described elsewhere [18]. The final emulsion consistently contained 0.1 g of gum arabic/mL of tributyrin.

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

The rest of the reagents used in the experiments (supplied by Panreac, Barcelona, Spain) were analytical grade or better.