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KINETICS OF HYDROLYSIS OF TRIBUTYRIN BY LIPASE

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Abstract

Kinetics of the enzymatic hydrolysis of tributyrin using lipase has been investigated. The initial rate of reaction was determined experimentally at different substrate concentration by measuring the rate of butyric acid produced. Michaels-Menten kinetic model has been proposed to predict the initial rate of hydrolysis of tributyrin in micro-emulsion system. The kinetic parameters were estimated by fitting the data to the model using three methods, namely, the Lineweaver-Burk, Edie-Hofstee and Hanes methods. The Michaels-Menten model with the constant predicted by Edie-Hofstee and Hanes methods predicted the initial rate of reaction at various substrate concentrations better than the model with the constant predicted Lineweaver-Burk method, especially at high substrate concentrations.

Keywords: Lipase hydrolysis, Tributyrin, Michaelis-Menten kinetics.

1. Introduction

The applications, importance and significance of lipase in oleochemical industry have been thoroughly demonstrated in literature [1-4]. The most important among these applications is the use of lipase for the production of fatty acids from oils. It is recently attempted as an energy-saving method, especially for producing high value-added products or heat sensitive fatty acids [1-4].

Nomenclatures

a_t	Total specific interfacial area [m^{-1}]
$[E]$	Free enzyme concentration [kLU m^{-3}]
$[E]_t$	Total active enzyme concentration [kLU m^{-3}]
k^*_{cat}	Catalytic rate constant [min^{-1}]
k_d	Desorption rate constant [min^{-1}]
k_p	Adsorption rate constant [$m^2.min^{-1}$]
K_e	Equilibrium constant of ES [mol m^{-3}]
K_i	Product inhibition constant [mol m^{-3}]
LU	Lipase Unit
$[P]$	Product concentration [mol m^{-3}]
$[S]$	Substrate concentration [mol m^{-3}]
Greek Symbols	
υ	Reaction rate [mol m^{-3} min^{-1}]

Lipase catalysed reactions take place at the interface between the aqueous phase containing the enzyme and the oil phase, where the enzyme has to penetrate the interface as a first step in the reaction [1,2]. Al-Zuhair *et al*. [1] derived a mathematical model to predict the hydrolysis rate of oils by lipase, from a proposed kinetic mechanism of the reaction, assuming low enzyme concentration (Eq.1).

$$\upsilon = \frac{k^*_{cat}\,[E]_t \cdot [S]}{K_e\left[\frac{k_d}{k_p\,a_t^2} + 1\right] + [S]} \tag{1}$$

Where the substrate [S], represents the concentration of ester bonds in the oil. Comparison of Eq(1) and the Michaelis-Menten kinetic equation (Eq.2) shows that the K_m in Eq(1) is not a constant and is a function of the total specific interfacial area, a_t.

$$\upsilon = \frac{k^*_{cat}\,[E]_t \cdot [S]}{K_m + [S]} \tag{2}$$

In a mechanically stirred bi-phasic systems, the total free interfacial area changes with changes in operating conditions, such as substrate concentration, agitation speed and temperature [1]. That is the reason behind the discrepancy found in previous models that assumed Michaelis-Menten kinetics in the prediction of the hydrolysis rate of oils by lipase in mechanically agitated systems [3]. However, oil-water micro-emulsions, aided by emulsification reagent, are stable systems with much larger interfacial area. This would result in a constant total free interfacial area, independent on operating conditions. Therefore, based in Eq (1), the Michaelis-Menten model could successfully be adopted to such systems as found in the work of Knezevic et al [4] on the hydrolysis of palm oil in lecithin/isooctane reversed micelles.

On the other hand, Malcata et al [5,6] proposed to describe the rate expression of the hydrolysis of oils by lipase by Ping-Pong-Bi-Bi mechanism. This leads to a rate expression associated with a Michaelis-Menten mechanism in the presence of product inhibition, (Eq. 3):

$$\upsilon = \frac{k^*_{cat}[E]_t \cdot [S]}{K_m\left(1 + \frac{[P]}{K_i}\right) + [S]} \tag{3}$$

However, considering the initial rate of reaction, the product concentration [P] is assumed to be negligible with comparison to the initial substrate concentration [S] and the K_m value, hence, Eq (3) is simplified again to Eq (2).

It is therefore clear that the Michaels-Menten kinetic model (Eq. 2) is appropriate to predict the initial rate of hydrolysis of tributyrin in micro-emulsion system. In this paper, experimental data are fitted to the Michaelis-Menten model using three methods, namely, the Lineweaver-Burk, Edie-Hofstee and Hanes methods. The model with the constant predicted are validated against the experimental results.

2. Chemicals

Liquid lipase (EC 3.1.1.3) from *Mucor miehei* (claimed activity 100 kLU ml^{-1}) was obtained from Novo Nordisk, Denmark. Analytical grade Tributyrin (98%) was obtained from Acros Organics, USA. NaOH was obtained from Mallinckrodt, Sweden. Gum Arabic, NaCl, KH_2PO_4, and glycerol were obtained from Fisher Scientific, UK.

3. Experimental Procedure

The method is based on the hydrolysis of tributyrin by lipase, and titrating the butyric acids produced with 0.05 NaOH in distilled water [7]. The alkali consumption was registered as a function of time at pH 7.0 using an auto titrator (Metrohm 794 Basic Titrino, Swaziland).

3.1 Preparation of emulsifying reagent

8.95 g NaCl and 0.2 g KH_2PO_4 were dissolved in 200 ml demineralised water with 270 ml glycerol. Under vigorous stirring using homogeniser (X120, CAT Ingenieuburo, Germany) 3.0 g gum Arabic was added to the mixture. The homogeniser was capable of stirring up to 30,000 rpm. The mixture was then transferred to a 500 ml measuring flask and demineralised water was added to make up the volume to 500 ml.

3.2. Preparation of substrate emulsion

Under homogeniser stirring of up to 30,000 rpm, 50 ml of emulsifying reagent was mixed with 250 ml of tributyrin-water mixture having different concentrations of tributyrin. The concentrations used were prepared to give final ester bond concentrations, [S], of the following values: 6.8, 13.7, 20.5, 9.11, 27.3, 68.4, 102.6, 136.8 and 171.0 mol m^{-3}.

3.3. Determination of the reaction rate

Small samples of the substrate emulsion having a volume of 20 ml were withdrawn into a small beaker, which was placed in the titration set-up. The pH of the solution was then adjusted to 7.0 ± 0.1 with 0.05 N NaOH. 2 ml of enzyme solution, having a concentration of 1.0 LU ml^{-1} of enzyme, was added to the substrate mixture to initiate the reaction and the pH–stat titration was started. The alkali addition was carried out for 5 minutes. This reaction was slow enough to assume that within the first five minutes the changes in the initial concentration of substrate was negligible (i.e, small amounts of substrate have been reacted). The rate of the alkali addition was used to determine the initial reaction rate, using Eq (4).

$$Rate\ of\ reaction, \upsilon \left(\frac{mol}{m^3 min}\right) = \frac{Slope\left(\frac{ml}{min}\right) \times Normality_{NaOH}\left(\frac{mol}{L}\right) \times 10^{-3}\left(\frac{L}{ml}\right)}{Volume\ of\ the\ sample\left(m^3\right)} \quad (4)$$

The abovementioned procedure was repeated ten times for each prescribed substrate concentration. The results are presented in Fig. 1, where the error bars give an indication of the accuracy and reproducibility of the results.

4. Validations of Kinetic Model Equations

The Michaelis-Menten in its original form (Eq.2), is not well suited to estimate the kinetic parameters k^*_{cat} and K_m. By rearranging Eq (2) the following options for data plotting and graphical parameter evaluation can be derived:

$$\frac{1}{\upsilon} = \frac{1}{k^*_{cat}\left[E\right]_t} + \frac{K_m}{k^*_{cat}\left[E\right]_t}\frac{1}{\left[S\right]} \quad (5)$$

$$\upsilon = k^*_{cat}\left[E\right]_t - K_m\frac{\upsilon}{\left[S\right]} \quad (6)$$

$$\frac{\left[S\right]}{\upsilon} = \frac{K_m}{k^*_{cat}\left[E\right]_t} + \frac{1}{k^*_{cat}\left[E\right]_t}\left[S\right] \quad (7)$$

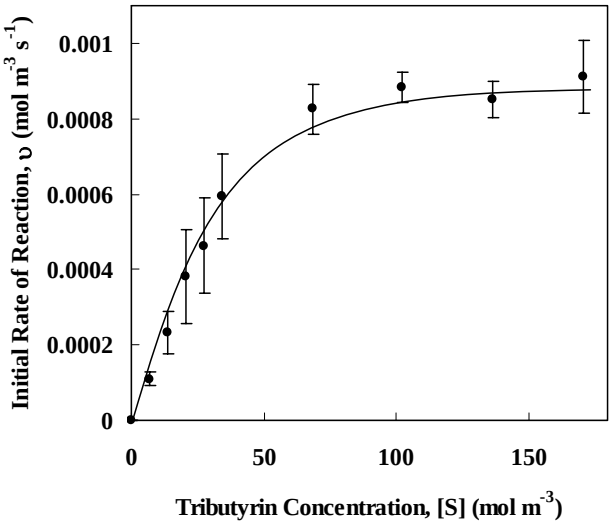

Fig.1. The change of initial rate of reaction at different initial substrate concentration, [S]

Each equation suggests an appropriate linear plot. In evaluation of the kinetic parameters of the model using such plots, however, several points should be noted. Figure 5 shows the plot of Eq (5), as $1/\upsilon$ vs $1/[S]$ (known as a Lineweaver-Burk plot). Note that, the most accurate rate values are those at high substrate concentration. This is because at high substrate concentrations, it is more accurate to assume the change in substrate concentration to be negligible during the course of the experiment. Fig. 2, however, shows the most accurate rate values clustered near the origin, while those less accurate rate values are far from the origin and dominantly determine the slope $K_m/ k^*_{cat}[E]_t$. Therefore, the value of K_m determined by this method is subject to large errors. Substituting the value of enzyme concentration used of 90.0 kLU m^{-3}, the Michaelis-Menten kinetic model equation with the estimated constants from Lineweaver-Burk plot is given by Eq (8):

$$\upsilon = \frac{3.1\times 10^{-5}[E]_t[S]}{160.4+S} \quad \pm 1.4\times 10^{-4} \tag{8}$$

Figure 3 and 4 shows the plots of Eqs (6) and (7) (known as Edie-Hofstee and Hanes plots, respectively). The plots tend to spread out the data points for higher values of [S], so that the slope, and hence the value of K_m can be determined accurately. The Michaelis-Menten kinetic model equations with the estimated constants from Edie-Hofstee and Hanes plots are given by Eqs (9) and (10), respectively,

$$\upsilon = \frac{1.3\times 10^{-5}[E]_t[S]}{47.9+S} \quad \pm 4.6\times 10^{-5} \tag{9}$$

$$\upsilon = \frac{1.3\times 10^{-5}[E]_t[S]}{47.6+S} \quad \pm 4.6\times 10^{-5} \tag{10}$$

It is clearly seen that the constants predicted by the Edie-Hofstee and Hanes methods are very close, however, those found by the Lineweaver-Burk method differs considerably. Figure 5 show the comparison between the experimental results and the Michaelis-Menten kinetic equation with the estimated constants Eqs (8-10), to measure how closely the experimental results are presented. The figure shows that the Michaels-Menten model curves, with the constants determined by the three methods, predicted fairly well the initial rate of reaction at low substrate concentrations. However, at high substrate concentration, the Lineweaver-Burk model curve deviates from the experimental data and did not predict the substrate saturation.

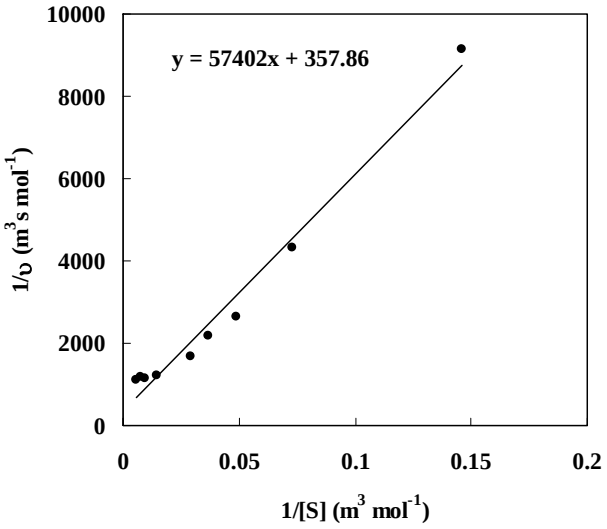

Fig.2. The Lineweaver and Burk method, 1/υ (s m^3 mol^{-1}) versus 1/[S] (m^3 mol^{-1})

On the other hand, the Edie-Hofstee and Hanes model curves followed better the trend of the experimental data at high substrate concentrations. The deviation between the experimental data and the model equations can also be noticed from the absolute error shown in Eqs (8-10). The absolute error of the Lineweaver-Burk equation is $1.4\text{x}10^{-4}$ mol m^{-3} s^{-1}, three times higher than the absolute error of the Edie-Hofstee and Hanes equations, $4.6\text{x}10^{-5}$ mol m^{-3} s^{-1}. It can be concluded therefore, that the Lineweaver-

Burk method is limited to low substrate concentrations, in the regions prior to the substrate saturation.

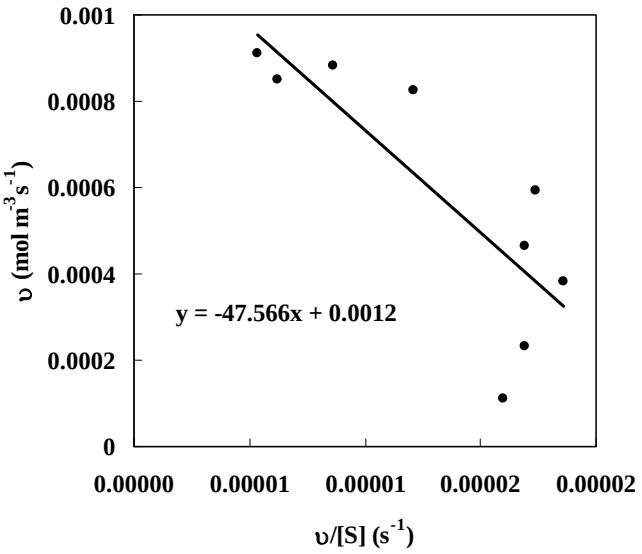

Fig. 3. The Edie-Hofstee method, υ (mol m^{-3} s^{-1}) versus υ/[S] (s^{-1})

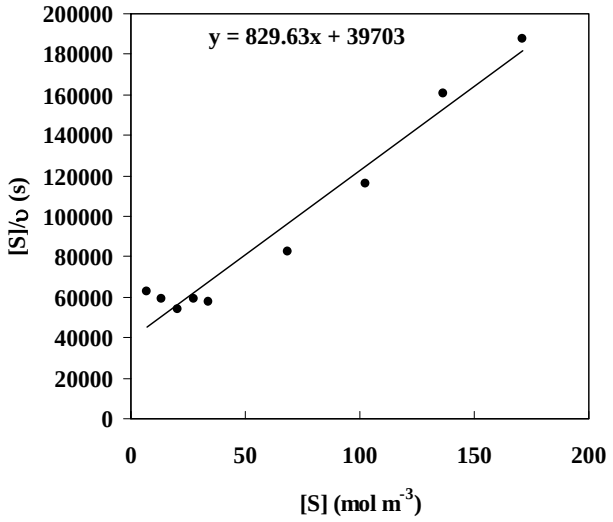

Fig. 4. The Hanes method, [S]/υ (s) versus [S] (mol m^{-3})

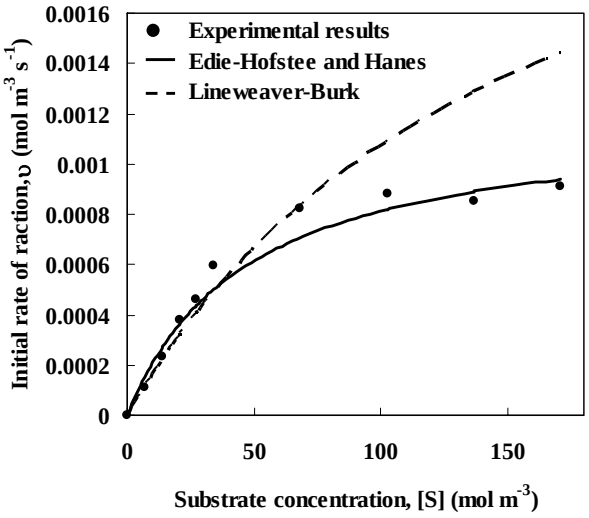

Fig. 5. Comparison between the experimental results and the Michaelis-Menten kinetic equation with the estimated constants Eqs (5-7)

5. Conclusions

An experiment was conducted to determine the initial rate of enzymatic hydrolysis of tributyrin in micro-emulsion system using lipase at different substrate concentration. Michaels-Menten kinetic model has been considered to predict the initial rate of reaction. Lineweaver-Burk, Edie-Hofstee and Hanes methods were used to estimate the kinetic parameters of the Michaels-Menten model by fitting the experimental data. It was shown that Edie-Hofstee and Hanes models predicted the initial rate of reaction at various substrate concentrations better than the Lineweaver-Burk model, especially in the substrate saturation region.

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