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Kinetic study on the hydrolysis of palm olein using immobilized lipase

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Abstract

The application of immobilized lipase (EC 3.1.1.3) is gaining interest in the oleochemical industry as it offers advantages over conventional chemical reactions. In the present study, a commercial immobilized lipase, Lipozyme TL IM, was used to catalyze the hydrolysis of palm olein in an aqueous-organic phase. A ping-pong bi-bi model with substrate inhibition by water was used to describe the hydrolysis reaction. The reaction rate constants of the proposed mechanism were determined by fitting the model into experimental data using a nonlinear curve fitting software. Based on the results of this study, the model proposed was able to fit the data with a correlation coefficient of 0.9586. The rate of formation of fatty acids is limited by the formation of glycerol. The critical water content before inhibition occurs was found to be 3.6% (v/v). No inhibition by palm olein was observed up to a concentration of 874.76 g/l.

Keywords: Aqueous two phase; Immobilized enzymes; Lipase; Heterogeneous reactions; Modeling; Hydrolysis

1. Introduction

The rapid development of enzyme technology has brought considerable attention to the application of lipase (EC 3.1.1.3) in the fat and oil industry [1,2]. Enzymatic reaction using lipase offers a lot of advantages over conventional chemical reaction. Lipases can be used effectively and economically under mild conditions [3]. This is an important characteristic because extreme conditions could cause polymerisation of fat and form by-product [4]. Hence, the use of lipase would reduce the need to remove the colour and the by-products through further separation method such as the distillation process, which is an energy intensive process. However, the application of lipase is still in its infancy due to the high cost of the enzyme [5,6]. This problem can be overcome by employing lipase in immobilized form, where the enzyme can be reutilized easily. Besides that, immobilization of enzyme enables the processes to be operated continuously.

Even though several reports have been published on the use of lipase for the hydrolysis of fats and oils, a reliable kinetic model to predict the hydrolysis rate is still lacking [4]. Besides that, very few data are available on the hydrolysis of palm olein [7]. However, many studies [8–10] reported that the ping-pong bi-bi model could be used to describe the catalytic action of lipases irrespective of the type of biotransformation.

In this study, a ping-pong bi-bi model with substrate inhibition by water was used to describe the reaction mechanism of lipase-catalyzed hydrolysis of palm olein. The reaction was carried out in a biphasic system with *n*-hexane as the organic solvent. *n*-Hexane was used because it poses less inhibition effect on the enzyme [11]. No surfactant or emulsifier was added to the reaction medium to avoid additional separation processes, which are not favourable in the industry.

2. Materials and methods

2.1. Materials

The commercial immobilized lipase, Lipozyme TL IM (bead size 0.3–1.0 mm, wet bulk density 415 kg/m^3), was obtained from Novozyme. Local cooking oil with the brand name Seri Murni (44.3 wt.% saturated fats, 12.1% poly-unsaturated fats and 43.6% mono-unsaturated fats), which is distributed by FFM Marketing Sdn Bhd, Malaysia, was used as the source of palm olein. Palmitic acid, oleic acid and linoleic acid, which were

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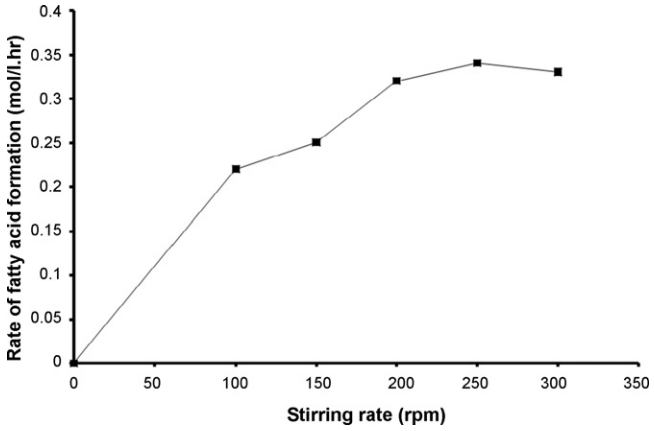

Fig. 1. The effect of stirring rate on the rate of batch reaction.

all of GC standard, were purchased from Sigma–Aldrich. Triolein and *n*-hexane were products of Sigma–Aldrich and Fluka Chemie AG, Switzerland respectively.

2.2. Enzyme assay

The activity of lipase was assayed by using triolein as the substrate. A 50-ml jacketed vessel was first filled with 10-ml triolein and 1-ml water. *n*-Hexane was added to make up the total reaction mixture to 40 ml. A time-zero sample was then taken. The reaction was initiated by adding 1-g immobilized enzyme. The reaction was conducted at 55 °C (which is the optimum temperature for the activity of Lipozyme TL IM) with a stirring rate of 200 rpm (which was chosen based on the experimental results as shown in Fig. 1 to prevent mechanical breakage of Lipozyme and avoid external mass transfer limitations). One-microliter sample was withdrawn every 30 min until the reaction reached equilibrium. Lipase activity is defined here as the initial hydrolysis rate of triolein at the above mentioned conditions. One unit of enzyme activity corresponds to a formation of one micromole of oleic acid/l/min.

2.3. Batch reaction

Batch reaction was carried out using the same method for enzyme assay, with palm olein as the source of triglyceride. In order to study the effect of water and palm olein concentrations on the activity of immobilized lipase, reaction was conducted by varying the concentration of one substrate while maintaining the concentration of the other substrate. First, hydrolysis reaction was repeated using a constant amount of water (2 ml) while the amount of palm olein used was varied at 10, 20, 30 and 38 ml. After that, reactions were carried out using different amount of water (1, 2, 3 and 4 ml) while maintaining the amount of palm olein at 30 ml. For each reaction, appropriate amount of *n*-hexane was added to bring the total reaction volume to 40 ml.

2.4. Sample analysis

All the samples were analyzed using gas chromatography. The Shimadzu GC-17A Version 3 (Kyoto, Japan) used was equipped with a flame-ionization detector (FID). A Nukol column (15 m length × 0.53 mm i.d. × 0.50 μm film thickness, Supelco, USA) was used. The carrier gas was nitrogen at 600 kPa. The injector and detector were set at 220 °C. The column temperature was programmed to rise from 110 to 220 °C at 8 °C/min. The gas chromatography column was linked to the Shimadzu CLASS-VP Chromatography Data System software (Columbia, USA). Calibration curves for palmitic acid, oleic acid and linoleic acid were first developed using external GC standards. All samples were diluted with *n*-hexane at a dilution factor of 100 before 1 μl of the diluted samples were injected into the chromatography column.

3. Results and discussions

3.1. Enzyme assay

In this study, duplicate was made for each experiment to ensure the reproducibility and accuracy of the data. Results reported were the average values of the data. The activity of Lipozyme TL IM was found to be 1.5 kU/g. It was determined by calculating the initial slope of the hydrolysis profile of triolein (plot not shown).

3.2. The kinetic model

The mechanistic model proposed in this study is the same as the ping-pong bi-bi model described by Cleland [12] and Segel [13]. Even though the hydrolysis of triglycerides is a series of directed step-wise reactions with the formation of diglycerides and monoglycerides as intermediates [14], an overall reaction was considered during the development of the proposed model.

Referring to Fig. 2, the first step in this mechanism is the binding of triglyceride (A) to the enzyme (E) forming a lipase–triglyceride complex (AE). This complex then isomerizes into an intermediate complex (F) with the concomitant release of glycerol (P). After that, three molecules of water (B) form another binary complex (FB_3) with the intermediate complex, which isomerizes unimolecularly to a lipase–product complex. Finally the fatty acids (Q) are released and the enzyme is regenerated. Water causes substrate inhibition by competing with triglyceride to form a water–lipase complex.

A material balance was performed for each component involved in the mechanistic model. Two assumptions were made while performing the material balance: (a) mass transfer limitation in the reaction system was negligible; and (b) all the reactions are elementary. Based on the above assumptions, the differential equations for the components are as follows:

$$\frac{d[A]}{dt} = k_2[AE] - k_1[A][E] \quad (1)$$

$$\frac{d[AE]}{dt} = k_1[A][E] - k_2[AE] + k_4[F][P] - k_3[AE] \quad (2)$$

$$\frac{d[F]}{dt} = k_3[AE] - k_4[F][P] - k_5[F][B]^3 + k_6[FB_3] \quad (3)$$

$$\frac{d[B]}{dt} = k_6[FB_3] - k_5[B]^3[F] + k_{10}[EB] - k_9[E][B] \quad (4)$$

$$\frac{d[FB_3]}{dt} = k_5[F][B]^3 - k_6[FB_3] - k_7[FB_3] + k_8[E][Q]^3 \quad (5)$$

$$\frac{d[Q]}{dt} = k_7[FB_3] - k_8[E][Q]^3 \quad (6)$$

$$\frac{d[P]}{dt} = k_3[AE] - k_4[F][P] \quad (7)$$

$$\frac{d[E]}{dt} = k_2[AE] - k_1[A][E] - k_9[E][B] + k_{10}[EB] + k_7[FB_3] - k_8[E][Q]^3 \quad (8)$$

$$\frac{d[EB]}{dt} = k_9[E][B] - k_{10}[EB] \quad (9)$$

The sum of all the intermediate enzyme complexes is equal to the enzyme loading as shown in the following equation:

$$[E_0] = [E] + [AE] + [F] + [FB_3] + [EB] \quad (10)$$

In most enzyme kinetic studies, reaction rate constants are integrated to form simple reaction rate parameters. These parameters can be obtained by fitting experimental data into integrated rate equations using nonlinear regression. However, the solution of rate equations for complicated pathways is tedious and often requires the use of simplifying assumptions [15]. Besides, different results can be obtained depending on the relative rates of each reaction. Therefore, in the present study, a time-course analysis was conducted to determine the rate constant of each reaction or pathway.

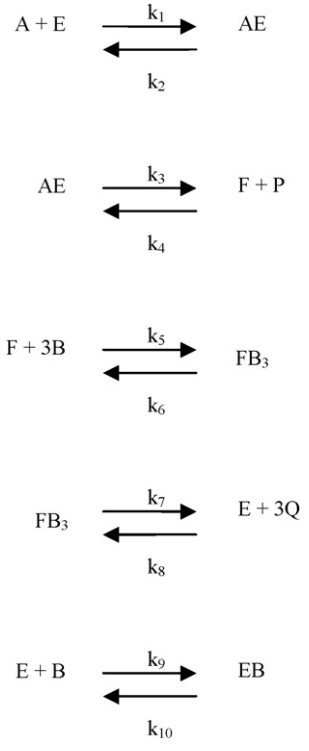

Fig. 2. The proposed ping-pong bi-bi mechanism with inhibition by water during the hydrolysis of palm olein by immobilized lipase.

Table 1
Reaction rate constants for the hydrolysis of palm olein

Rate constants	Reaction step	Unit	Value
k_1	A + E → AE	$l\,h^{-1}\,g^{-1}$ enzyme	0.2
k_2	AE → A + E	$mol\,h^{-1}\,g^{-1}$ enzyme	85.9
k_3	AE → F + P	$mol\,h^{-1}\,g^{-1}$ enzyme	9.8
k_4	F + P → AE	$l\,h^{-1}\,g^{-1}$ enzyme	0.5
k_5	F + 3B → FB_3	$l^3\,mol^{-2}\,h^{-1}\,g^{-1}$ enzyme	3499
k_6	FB_3 → F + 3B	$mol\,h^{-1}\,g^{-1}$ enzyme	24.7
k_7	FB_3 → E + 3Q	$mol\,h^{-1}\,g^{-1}$ enzyme	106.4
k_8	E + 3Q → FB_3	$l^3\,mol^{-2}\,h^{-1}\,g^{-1}$ enzyme	1.7
k_9	E + B → EB	$l\,h^{-1}\,g^{-1}$ enzyme	6.2
k_{10}	EB → E + B	$mol\,h^{-1}\,g^{-1}$ enzyme	38.1

Reaction conditions: 1 g enzyme (Lipozyme TL IM); temperature = 55 °C; stirring rate = 200 rpm.

The values of the reaction rate constants were determined by fitting the system of Eqs. (1)–(10) into experimental data using the nonlinear curve fitting software Matlab 7.0. The optimization method used was nonlinear least square while Runge–Kutta 4,5 was used as the ODE (ordinary differential equation) solver. The rate constants, as determined using the software, are listed in Table 1.

Some conclusions can be made on the kinetic rate of each step by comparing the values of the rate constants in Table 1. However, comparisons can only be made among constants with the same units. Comparing k_1, k_4 and k_9, the value of k_9 is significantly larger compared to the other two constants. This means that the formation of a water–enzyme complex is faster than the formation of a triglyceride–enzyme complex. Thus, the inhibition rate by water is quite high. The value of k_2 is very much higher than k_3, indicating that the triglyceride–enzyme complex favours the reverse reaction back to enzyme and triglyceride instead of forming the first product. On the other hand, k_7 is larger than k_6, which shows a favourable reaction to form the second product. Since the reaction favours the formation of the second product ($k_7 > k_6$) but not the first product ($k_3 < k_2$), it can be concluded that the formation of the final product (fatty acids) is limited by the formation of the first product (glycerol). In other words, once the glycerol is formed, it is easier for the fatty acids to be produced.

Fig. 3 shows the comparison between modeled and experimental data. The sum of square errors between the modeled data and all the experimental data in Fig. 3 as reported in the Matlab output report is 0.2 while the correlation coefficient is 0.9586. Therefore, the ping-pong bi-bi model with inhibition by water as proposed in this study was adequate to describe the kinetic behavior of palm olein hydrolysis.

The total palm olein conversion obtained in this study was only about 28% for each reaction in 6 h, except for the reaction with an initial water amount of 1 ml, which achieved a slightly lower conversion (21%) because water had become the limiting substrate. Roy et al. [16] also reported the same low conversion using olive oil as the source of triglyceride. However, Knezevic et al. [17] managed to obtain a hydroly-

Table 2
Percentage conversion of different lipase-hydrolyzed systems

Study	Substrate	Types of immobilized lipase	System	Operation time (h)	Percent conversion (%)
Current study	Palm olein	*Thermomyces lanuginosus* lipase on granulated silica carrier (commercial Lipozyme TL IM)	Hexane two-phase system	6	28
[16]	Olive oil	*Corynebacterium* lipase in calcium alginate	Polyvinyl alcohol system	7	29.16
[17]	Palm oil	*Candida rugosa* lipase in calcium alginate	Lecithin/isooctane system	3	74

sis conversion of approximately 74%. A summary is tabulated in Table 2.

Based on Table 2, the conversion achieved could be affected by the types of lipase used. Lipase from *Candida rugosa* seems to give a high conversion. However, a commercial immobilized lipase from Novozyme, Lipozyme TL IM, was used in this study to reflect the industry. Table 2 also shows that a lecithin/isooctane system gives a high conversion in a very short time. However, emulsifier is not favourable in the industry as it requires further separation processes in the downstream, which is the reason this system was not selected for the current study.

3.3. Effect of substrate concentration

An initial rate analysis was performed on the modeled curves in Fig. 3 to study the effect of substrate concentration on the hydrolysis rate. The effect of palm olein is presented in Fig. 4. According to Fig. 4, the rate of reaction increased with the palm olein concentration. No inhibition was observed up to a palm olein concentration of 874.76 g/l. On the other hand, inhibition of lipase activity by water has been observed. Yadav and Devi [18] reported that water inhibits the activity of lipase with a critical water content of 3.6% (v/v). Using the rate constants in Table 1, simulation was carried out for water content of 3.6% (v/v). Fig. 5 shows the effect of water content on the hydrolysis rate (data taken from Fig. 3 and the simulation result at 3.6% (v/v)). The rate of reaction increased when the water content

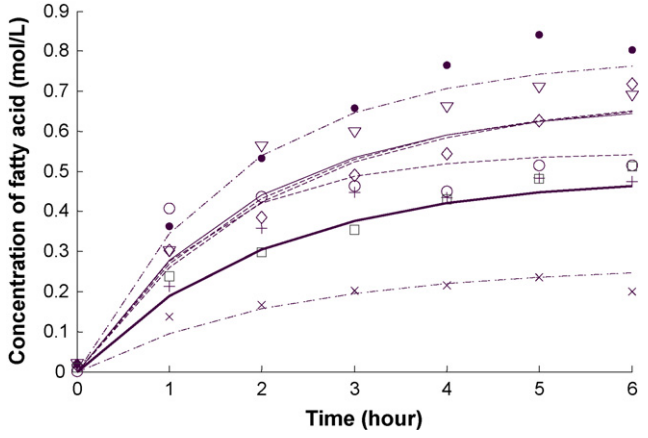

Fig. 3. Comparison between modeling results (lines) and experimental data (symbols). At constant amount of water (2 ml): (a) 10 ml palm olein (×); (b) 20 ml palm olein (+); (c) 30 ml palm olein (▽); (d) 38 ml palm olein (●). At constant amount of palm olein (30 ml): (a) 1 ml water (◯); (b) 3 ml water (◇); (c) 4 ml water (□).

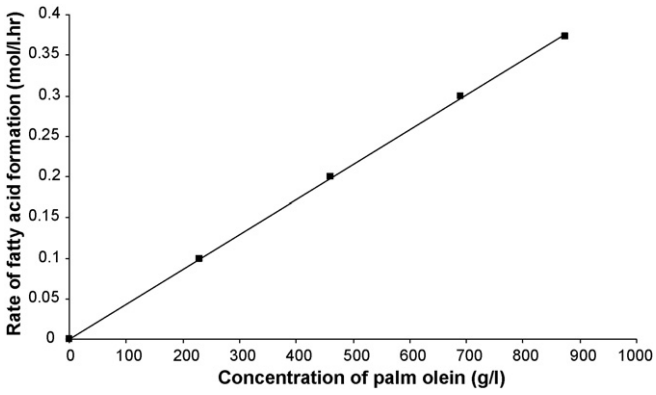

Fig. 4. The effect of palm olein concentration on the rate of reaction.

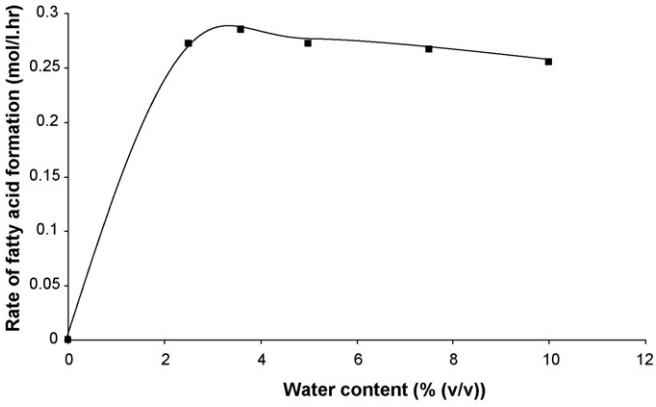

Fig. 5. The effect of water content on the rate of reaction.

increased up to 3.6% (v/v), beyond which any increase in the amount of water caused the rate of reaction to drop. According to Yadav and Devi [18], the addition of water beyond a critical amount could increase the thickness of water layer formed around the enzyme. This causes organic reactants and products with poor solubility in aqueous medium to diffuse with difficulty through the water layer to the active centers of the enzyme.

4. Conclusions

The ping-pong bi-bi model with substrate inhibition by water was proposed to describe the kinetic characteristics for the hydrolysis of palm olein by immobilized lipase. A good agreement was obtained between the proposed model and the experimental data, which means that the model was adequate

to reflect the reaction mechanism. No inhibition by palm olein was observed in the range of concentration used in this study. However, water content above 3.6% (v/v) inhibited the activity of lipase.

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