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Lipase Immobilized on Hydrophobic Microporous Polypropylene for the Hydrolysis of Palm Kernel Olein

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ABSTRACT

Lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) from *Rhizopus arrhizus* was immobilized in this work by adsorption on microporous polypropylene and employed for the lipolysis of palm kernel olein. The optimum operating temperature for the lipolysis reaction was determined. The reaction follows Michaelis-Menten kinetics with product competitive inhibition for substrate concentrations in the range of 0.175–0.877*M*. The apparent K_M and V_{max} were 0.42*M* and 691 U/mg protein, respectively. A dissociation constant of the enzyme-product complex, K_I = 29.73 m*M*, for the product inhibition was also determined. Additionally, the time-courses of the reaction for various substrate concentrations were obtained and correlated sufficiently with those predicted from the theoretical rate equation for a period of up to 2 h. Experimental results indicated that discrepancies between the observed results and the predicted ones increase with reaction time.

Index Entries: Lipolysis; palm kernel olein; product inhibition; immobilized lipase; *Rhizopus arrhizus.*

Nomenclature: ES*, complex of enzyme and substrate molecules; E_t, total mass of enzyme in the reactor (mg); k_{cat}, rate constant in the Michaelis-Menten equation (mmol/min mg protein); K_I, dissociation constant for the complex EP* (*M*); K_M, apparent Michaelis constant (*M*); P, oleic acid concentration (*M*); P_0, initial concentration of oleic acid (m*M*); S, substrate concentration based on ester bond (*M*); S_0, initial value of S (*M*); t, reaction time (min); V_I, initial velocity of reaction (μmol/min); V_{max}, apparent maximal velocity (μmol/min); *X*, conversion of substrate.

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INTRODUCTION

Enzymatic hydrolysis of fats or oils for fatty acid production has long been performed in aqueous system (*1*). Enzymatic hydrolysis of fat in organic two-phase systems has received extensive attention, since fats or oils are not soluble in water, and lipase is known to be active only at the oil–water interface. Brink et al. (*2*) and Dordick (*3*) gave various reasons to justify the use of organic media instead of aqueous solutions, i.e., increased solubility of nonpolar substrates, enhanced thermostability of enzyme, elimination of microbial contamination, and shift of reaction equilibria. Immobilized enzyme has certain proven benefits, e.g., facilitated product/enzyme separation, increase in conformational stability, and compatibility with continuous process and ease of recovery. Bar indicated that the immobilization could provide additional stability to the enzyme by protecting it against denaturating effects of the organic at phase level (*4*).

A solid phase is present for immobilized biocatalyst in the organic-water biphasic system. In case the organic phase is continuous, the aqueous phase is nondiscrete and the biocatalyst is insoluble; the interfacial area available for mass transfer is determined by the specific surface area of the biocatalyst. Consequently, a liquid dispersed in another liquid is deemed unnecessary (*5*). Synthetic reaction, e.g., esterification or transesterification, if favored since water activity of the system is considerably lower than unity. Muderhwa et al. (*see* ref. *6*) indicated that hydrolytic reaction of triacylglycerol increases rapidly with the increase in water activity of media, and hydrolysis is preferred as water activity approaches to approx 0.9. *n*-Hexane is typically employed as a solvent because of its low denaturing effect on lipase (*7*). Furthermore, Valivety et al. (*8*) discovered that the activity of the solubilized water in *n*-hexane approaches 0.9 when its concentration in *n*-hexane solution is roughly 0.14*M*. Cassells and Halling (*9*) pointed out additionally that a shift of equilibrium from hydrolysis to synthesis reaction will not occur if the activity of water on enzyme molecules is close to unity.

Lipase immobilized on hydrophobic microporous supports yields a higher lipolytic activity than on hydrophilic supports (*10–12*). Supports with a lower aquaphilicity (Aq) value are generally recommended (*13*). Commonly used hydrophobic microporous supports include polypropylene (PP), polyethylene (PE), polystyrene divinylbenzene (PSDVB), etc. Lipase is generally immobilized on hydrophobic microporous supports by physical adsorption; in addition, leakage of lipase may be made negligible (*14*).

The purpose of this work is to investigate the hydrolysis reaction of palm kernel olein by lipase immobilized by physical adsorption on hydrophobic microporous polypropylene in the presence of *n*-hexane. The Michaelis constant K_M, maximum velocity V_{max}, and dissociation constant K_I of the enzyme–product complex are examined. In addition, the time-course analysis of hydrolytic reaction is described. The discrepancy between

the experimental results and theoretical rate equation is observed, and possible reasons for the deviation are also outlined.

MATERIALS AND METHODS

Materials

R. arrhizus lipase (EC 3.1.1.3; triacylglycerol ester hydrolase) was obtained from Sigma (St. Louis, MO) and used without purification in the hydrolysis of palm kernel olein. Palm kernel olein with a saponification value and an acid value of 246 and 0.25, respectively, was donated by Namchow Chemical Inc. (Taiwan). Microporous PP (Accurel EP100, particle size: 200–1000 μm) was a kind gift of AKZO (Obernburg, Germany) and used as a carrier for enzyme immobilization. Particle size in the range of 300–500 μm was selected by using a Tyler sieve. Hexane purchased from J. T. Baker (Phillipsburg, NJ) was employed as a solvent because of its low denaturing effect on lipase. The calibration curve for fatty acid was constructed by using analytical-grade oleic acid (Merck, Germany). Oleic acid was also used in the study of product inhibition. Olive oil emulsion purchased from Sigma was utilized as a substrate for lipase assay.

Methods

Lipase Immobilization

Lipase was immobilized on PP by adsorption. An appropriate amount of concentrated lipase solution (6.5 U) was injected to 0.1 mL of 0.1*M* phosphate buffer (pH = 7.0) on a hydrophobic plastic sheet. Twenty milligrams of microporous polypropylene prewetted with 0.05 mL of ethanol were added to the mixture and flattened by using a small wood bar. This was then immediately placed into a vacuum oven. Water and ethanol in the mixture were evaporated in a vacuum oven at 30 mmHg and 25°C.

Hydrolysis Reaction

The hydrolysis of palm kernel olein by immobilized lipase was carried out in a screw-cap test tube (22 mm diameter) with shaking (150 rpm) at 37°C. Five milliliters of *n*-hexane solution containing palm kernel olein with a concentration of 0.04–0.2 g/mL (0.175–0.877 *M*, based on the ester bond concentration defined in Eq. [1]) were placed into a test tube, and 2 mL of phosphate buffer were added. This mixture was incubated in water bath for 20 min at 37°C, and immobilized supports were added into the test tube. The reaction mixture was shaken for 1 min by vortex (600 rpm), then returned to the water bath, and shaken at 150 rpm as the reaction proceeded. The initial concentration of substrate, S_0, based on the ester bond in palm kernel olein, was defined as follows:

$$S_0\,(M) = [246\,S_0\,(g/mL)] / 56.1 \quad (1)$$

Moreover, the degrees of conversion can be calculated from:

$$X = \mu mol\ of\ fatty\ acids\ liberated\ in\ test\ tube / [5000\,S_0\,(M)] \quad (2)$$

Lipase Assay

The lipolytic activity of the immobilized lipase was assayed using olive oil emulsion. Ten milliliters of 10% v/v olive oil emulsion were incubated at 37°C for 20 min. Immobilized lipase was then added, and the reaction proceeded with for 20 min. The reaction was terminated by adding 10 mL of acetone–ethanol (50% v/v). The liberated free fatty acid was titrated with 0.1*N* NaOH using a METROHM 686 Titroprocessor. A control test was performed by the same procedure as described above, except that no immobilized lipase was added to the emulsion. One unit of activity is equivalent to 1 μmol of free fatty acid liberated/min at 37°C.

Analytical Methods

The initial rate of lipase-catalyzed lipolysis reaction was determined by measuring the liberated free fatty acid content. In the measurement of initial rate, the degrees of conversion for the substrate were maintained below 4%. 0.2 mL of solution in the supernatant of the reaction mixture was removed and added to a test tube containing a mixture of 4.8 mL benzene and 1 mL cupric acetate–pyridene. Next, the reaction was stopped immediately by vigorous shaking of the mixture. Following centrifugation for 5 min the upper layer was recorded at 715 nm (*15*).

RESULTS AND DISCUSSION

Effects of Temperature on Initial Rate and Stability of the Immobilized Lipase

The effect of temperature on lipase activity was studied and those results are shown in Fig. 1. The immobilized lipase shows its maximal activity at 45°C, and denaturing occurred at 55°C. In the stability study, the immobilized lipase (6.5 U) was incubated in 0.1*M* phosphate buffer (pH = 7.0) for 1 h at desired temperatures. The residual activity of lipase drastically declined at 45°C (Fig. 2). An optimum operating temperature of 37°C was chosen by a careful examination of Figs. 1 and 2.

Effect of Lipase Loading

The effect of lipase loading on the relative initial rate of hydrolysis is shown in Fig. 3. The initial rate of enzyme with 6.5 U loading is chosen as unity. The initial rates of various enzymes loading are roughly the same,

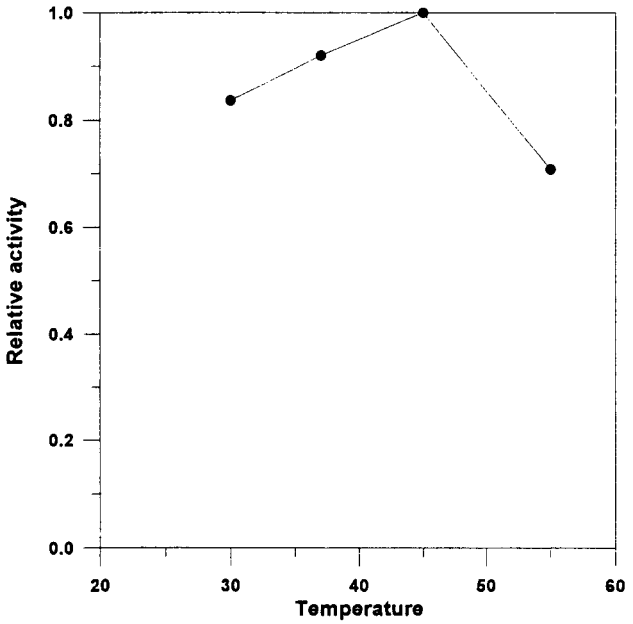

Fig. 1. Effect of temperature on the activity of the immobilized lipase. Reaction conditions: enzyme content 6.5 U, shaking speed 150 rpm, substrate concentration 20% (w/v).

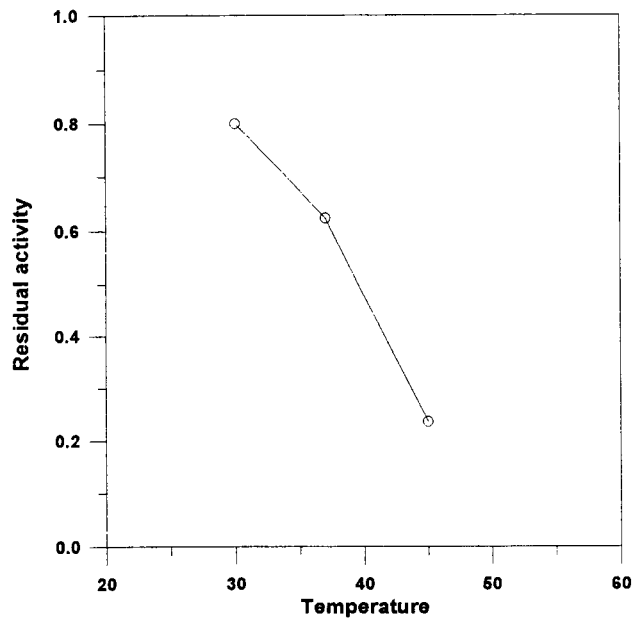

Fig. 2. Effect of temperature on the stability of the immobilized lipase. The immobilized lipase was incubated in 0.1*M* phosphate buffer for 1 h at the indicated temperature. Reaction conditions are the same as those in Fig. 1.

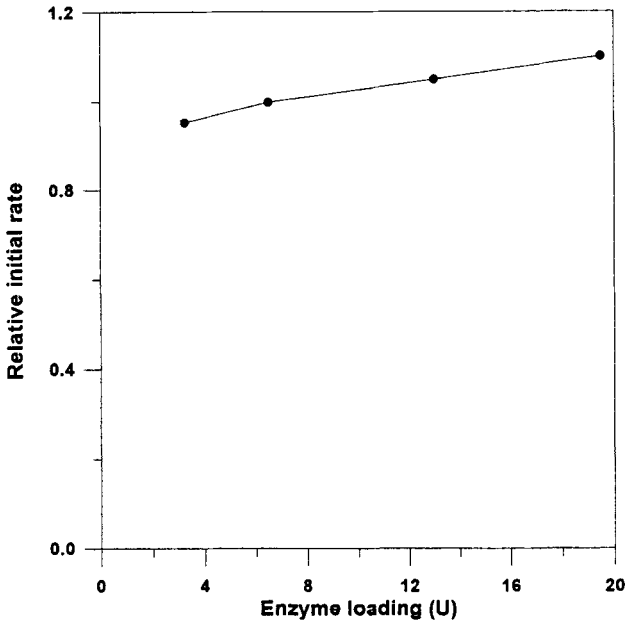

Fig. 3. Effect of lipase loading on the relative initial rate of hydrolysis. Reaction conditions are the same as those in Fig. 1, except for a temperature of 37°C.

indicating that lipase remains close to the surface of microporous PP particle after being immobilized at 30 mmHg vacuum for 30 min. An enzyme loading of 6.5 U is employed in the following studies, unless otherwise specified.

Kinetic Studies

The reaction mechanism of lipolysis is complicated because of the presence of various forms of the substrate, e.g., triglyceride and diglyceride. Michaelis-Menten kinetics is usually employed to describe the hydrolysis reaction by assuming that the reaction involves only a single substrate and a single product (*16*). Results obtained from the initial rates are shown in Fig. 4 as a function of substrate concentrations for the lipolysis of palm kernel olein with immobilized lipase. The reaction follows Michaelis-Menten kinetics for substrate concentrations up to 20% (w/v). This result is noteworthy since the batch hydrolysis of triglycerides is subject to substrate inhibition for substrate concentrations as low as 3–5% (w/v) in an emulsion system (*17,18*). The apparent K_M and V_{max} values are determined according to the Lineweaver-Burk plot with P_0 = 0 m*M* (Fig. 5). K_M and V_{max} values for the substrate were 0.42*M* and 691 U/mg protein, respectively.

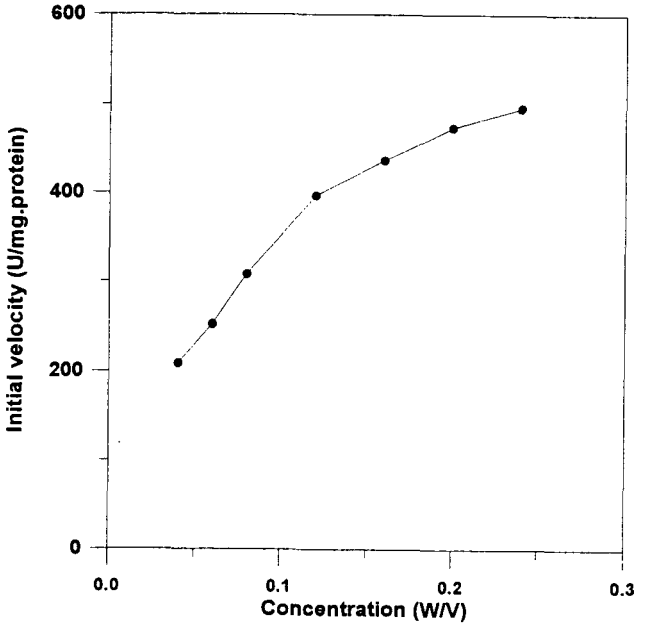

Fig. 4. Relationship between the initial rate and substrate concentration. Reaction conditions: enzyme content 6.5 U, shaking speed 150 rpm, temperature of 37°C.

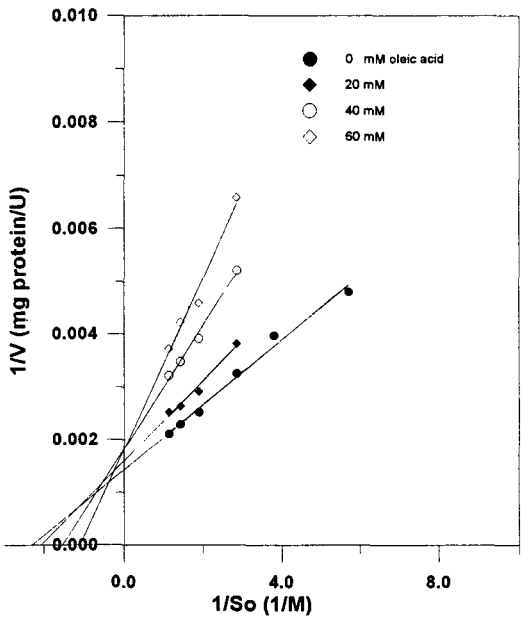

Fig. 5. Lineweaver-Burk plot for palm kernel olein hydrolysis with various oleic acid concentration. Reaction conditions are the same as those in Fig. 4.

Product Inhibition by Oleic Acid

Oleic acid was utilized as an inhibitor for investigating product inhibition, since it is one of the principal fatty acids in the hydrolysis of palm kernel olein. The initial rates were calculated from experiments with oleic acid in the range of 20–60 m*M*, and those results are presented in Fig. 5. This figure indicates that the hydrolytic reaction of palm kernel olein catalyzed by the immobilized lipase is competitively inhibited by oleic acid. Consequently, the mechanism of hydrolysis reaction can be expressed as:

$$\begin{array}{ccccc} & & k_1 \quad k_{cat} & & \\ E + S & \rightleftharpoons & ES^* & \rightarrow & E + P \\ + & & k_2 & & \\ P & & & & \\ \downarrow\uparrow K_I & & & & \\ EP^* & & & & \end{array} \qquad (3)$$

where K_I is the dissociation constant of the enzyme-product complex EP*. The initial rate V_I can be described as:

$$V_I = k_{cat}SE_t / \{K_M [1 + (P / K_I)] + S\} \qquad (4)$$

where E_t is the total amount of enzyme, and S and P are concentrations of substrate and product, respectively. A K_I of 29.73 m*M* was determined from Fig. 5. The smaller value of K_I revealed that product inhibition plays a significant role in the hydrolysis of palm kernel olein by immobilized *R. arrhizus* lipase.

Time-Course Analysis

To investigate the time-course of the hydrolysis reaction of immobilized lipase, Eq. (4) is rewritten as:

$$V_I = (dP / dt) = k_{cat}SE_t / \{K_M [1 + (P / K_I)] + S\} \qquad (5)$$

Product concentration as a function of time can be obtained by integrating Eq. (5) with initial condition $P_0 = 0$, thereby yielding:

$$V_{max}t = k_{cat}E_t t = P + K_M \{\ln [S_0 / (S_0 - P)] + (1 / K_I) / [S_0 \ln [S_0 / (S_0 - P)] - P]\} \qquad (6)$$

The time-course of product concentration predicted by Eq. (6) correlates sufficiently with the experimental results for a reaction time up to 2 h as shown in Fig. 6. Deviation between the predicted and experimental results increases with time. This discrepancy may be the result of the following phenomena: (1) The inactivation of immobilized lipase at the oil–water interface: a reversible enzyme adsorption to the oil–water interface (*19*) has been proposed for the lypolysis reaction; additionally, progressive inactivation of lipolytic enzyme at the interface has been pointed out to occur at the triglyceride–water interface (*20*); and (2) solvent effect, which may include: (a) noncompetitive inhibition of solvent (*21*) and (b) inactivation of enzyme at solvent–water interface (*22*).

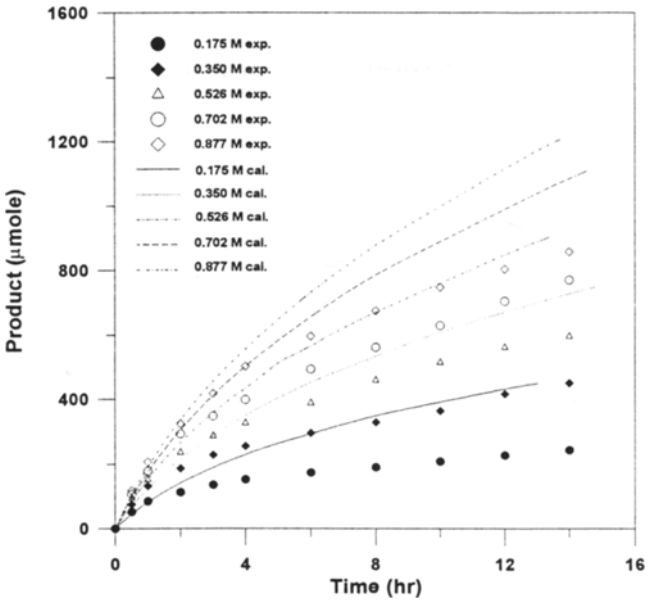

Fig. 6. Product concentrations as a function of time for palm kernel olein hydrolysis. Reaction conditions are the same as those in Fig. 4.

CONCLUSION

Immobilized *R. arrhizus* lipase-catalyzed hydrolysis of palm kernel olein was carried out in organic solvent with suspended immobilized lipase particles at an optimum temperature of 37°C. Shifting of reaction equilibrium from hydrolysis to synthesis was not observed. A small K_I value of 29.73 m*M* indicates significant product inhibition. A Michaelis-Menten mechanism with competitive inhibition was proposed to describe the kinetics of the hydrolytic reaction. Large discrepancies between the predicted and the experimental obtained product concentrations were observed for reaction times longer than 2 h. This discrepancy is possibly the result of the inactivation of lipase at the oil–water interface, noncompetitive inhibition of solvent, and the inactivation of enzyme at solvent–water interface.

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