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Hydrolysis of Palm Kernel Olein in AOT-Isooctane-Water Reversed Micelles

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ABSTRACT

Rhizopus arrhizus lipase (EC 3.1.1.3; triacylglycerol hydrolase) was used in this study to investigate the hydrolysis of palm kernel olein in AOT-isooctane-water reversed micelle system at W_o = 13, pH = 7, and T = 30°C. The hydrolytic reaction obeys Michaelis-Menten kinetics for substrate concentrations in the range (0.175 and 0.877*M*). The apparent K_m and V_{max} for the substrate were 0.397*M* (equivalent to 9.06% w/v) and 5523 U/mg protein, respectively. Product inhibition with a dissociation constant of the enzyme-product complex, K_I = 9.74 m*M*, was confirmed. Experimental results from the change of product concentrations with respect to time correlated sufficiently with those predicted theoretically from the rate equation for a reaction time up to 100 min. However, the discrepancy between the observed results and the predicted ones would increase with reaction time. Possible reasons for this deviation were discussed.

Index Entries: Reversed micelles; lipase; *Rhizopus arrhizus*; palm kernel olein; product inhibition; hydrolysis.

Nomenclature: E_t, total mass of enzyme in the reactor (mg); k_{cat}, rate constant in the Michaelis-Menten equation (mmol/min mg enzyme); K_I, dissociation constant for the complex EP*; K_m, Michaelis constant (M); P; oleic acid concentration (M); P_o, initial value of P; V_I, initial rate (μmol/min); V_m, maximum rate (μmol/min); S, substrate concentration based on ester bond (M); S_o, initial value of S (M); t, reaction time (min); W_o, molar ratio of water to AOT.

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INTRODUCTION

Fatty acids and glycerol, which are essential chemicals in the oleochemical industry, are products of fat hydrolysis. Fat and steam are fed continuously into the reactor, and the hydrolytic reaction occurs at 250°C and 50 atm for 2 h in the available process conventionally used for the production of these chemicals. Yields of this reaction for fatty acid and glycerol are 97 and 12%, respectively. Several drawbacks arise from this (high-temperature and pressure) process. For example, capital investment required for setting up the process is high, downstream purification is expensive, and degradation of unsaturated fatty results in higher environmental pollution. Enzymatic hydrolysis of fat is apparently an attractive alternative, since the reaction is carried out at ambient temperature and pressure. Industrial applications of this enzymatic process are still at their initial stages owing to a lack of clarity regarding the fundamental as well as the engineering aspects associated with the process.

Many investigators have actively participated in the research of enzymatic hydrolysis of fat after Lieberman and Ollis carried out triolein hydrolysis in a fixed-bed reactor with *Candida rugosa* lipase (*1*). Both fat and water are reactants in the hydrolytic reaction. Since fat is not soluble in water, organic solvent is normally required for dissolving the fat. Most early studies involving the enzymatic hydrolysis of fat empolyed emulsion systems (*2*), since lipase is known to be active only when it is present at the oil–water interface (*3,4*). The emulsion system has not found extensive application in the study of fat hydrolysis, since a vigorous agitaiton effect, which causes shear deactiviation of the enzyme, is typically required in maintaining a stable emulsion. Product purificaiton poses yet another challenge in applying emulsion systems to fat hydrolysis. The reversed micellar system has been actively studied as an alternative for carrying out fat hydrolysis, since the nanometer size of the reversed micelles provides a large interfacial area required for the reaction. Lipase is also found to posses superactivity in reversed micelle (*5*). Most of the recent studies involving fat hydrolysis in a reversed micellar system have focused on the effect of parameters on the maximum reaction rate. For example, Han and Rhee (*6*) found optimal reaction conditions for the hydrolysis of olive oil by *Candida rugosa* lipase in AOT-isooctane reversed micelles. Tsai and Chiang (*7*) further investigated the reaction mechanism, the effects of product inhibition, and the time-course of the reaction for the same system.

Palm kernel olein is an important source for many industiral raw materials. The hydrolysis of palm kernel olein was recently studied by Kim and Chung (*8*) in AOT-isooctane reversed micelles. In that study, optimum operating conditions of the reaction and effects of additives on the initial rate were determined. In this study, the hydrolysis of palm kernel olein in AOT-isooctane reversed micelles under optimum water content, pH, and temperature as suggested by Kim and Chung is investigated.

Details regarding the reaction kinetics, product inhibition, and time history of the reaction are presented.

MATERIALS AND METHODS

Materials

R. arrhizus lipase (EC 3.1.1.3; triacylglycerol 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 a gift from Namchow Chemical Inc. (Taiwan). AOT (bis[2-ethylhexyl] sodium sulfosuccinate) obtained from Sigma was used as surfactant without further purification. Isooctane (HPLC-grade) purchased from J. T. Baker (Phillipsburg, NJ) was selected as a solvent, since it more closely resembles the molecular structure of AOT than other solvents and, thereby, can more easily form stable reversed micelles. The calibration curve for fatty acid was constructed by using analytical-grade oleic acid (Merck, Germany). Oleic acid was also employed in the study of product inhibition.

Methods

Preparation of Reversed Micelles

Appropriate amounts of concentrated lipase solution (depending on the total enzyme activity required) and Tris-maleate buffer (depending on W_o required) were added into a screw-cap culture tube containing a solution of 50 m*M* AOT in isooctane. Stirring was applied until the solution became clear (ca. 30 s).

Lipase Assay

A screw-cap culture tube containing 4.99-mL mixture of palm kernel olein (4–20% w/v) and AOT (50 m*M*) was maintained at a constant temperature of 30°C for 10 min to which 1 μL (13 U) lipase was added. Vortex mixing was applied until the mixture in the tube became clear (ca. 30 s). The tube was then sustained at 30°C, and the reaction was allowed to proceed with for 10 min; 0.2 mL of solution in the reaction mixture was removed and added to a test tube containing a mixture of 4.8 mL benzene and 1 mL cupric acetate-pyridine. The reaction was stopped immediately by vigorous shaking of the mixture. The supernant was removed after the mixture was centrifuged for 5 min and absorbance at 715 nm was measured. The amount of free fatty acids was obtained from the calibration curve (9).

The activity of lipase required for the production of 1 μmol of free fatty acid/min with palm kernel olein as substrate was defined as 1 U. The degree of conversion was defined as follows.

$$\text{Degree of conversion (\%)} = [\mu\text{mol of free fatty acid in reaction mixture} / 5000\, S_o\,(M)] \quad (1)$$

where S_o (M) is the initial substrate concentration in M (mol/L).

$$S_o\,(M) = [246 S_o\,(\text{g/mL}) / 56.1] \quad (2)$$

RESULTS AND DISCUSSION

Kinetic Studies

Lipase-catalyzed hydrolysis of fat is a reversible, multistep reaction. The reaction mechanism is complicated owing to the presence of various forms of the substrate, e.g., triglyceride and diglyceride. Michaelis-Menten kinetics can be employed to describe the reaction if the hydrolysis reaction is simplified as a reaction involving only a single substrate and a single product. The activity of lipase depends on W_o (molar ratio of water to AOT), pH, and temperature of the reversed micelle system. W_o = 13, pH = 7.0, and T = 30°C were selected as optimum operating conditions in this work according to Kim and Chung (*8*). Oleic acid was employed as an inhibitor for investigating product inhibition, since it is one of the principal fatty acids produced in the hydrolysis of palm kernel olein. The Lineweaver-Burk plots for substrate concentrations of 0.175–0.877M (4–20% w/v) and oleic acid concentrations of 0–80 mM are presented in Fig. 1. The degrees of conversion for the substrate were maintained below 4% in preparing the initial rate presented in Fig. 1. This figure revealed that the initial rate of reaction increases with initial substrate concentration (S_o) in the range of S_o studied. Substrate concentrations higher than 0.88M were avoided to ensure that the reaction follows Michaelis-Menten kinetics (*8*).

Figure 1 further indicated the presence of competitive inhibition by oleic acid in the lipase-catalyzed hydrolysis of palm kernel olein. The reaction mechanism can be described as:

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

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

$$V_I = (k_{cat} S\, E_t) / \{K_m[1 + (P/K_I)] + S\} \quad (4)$$

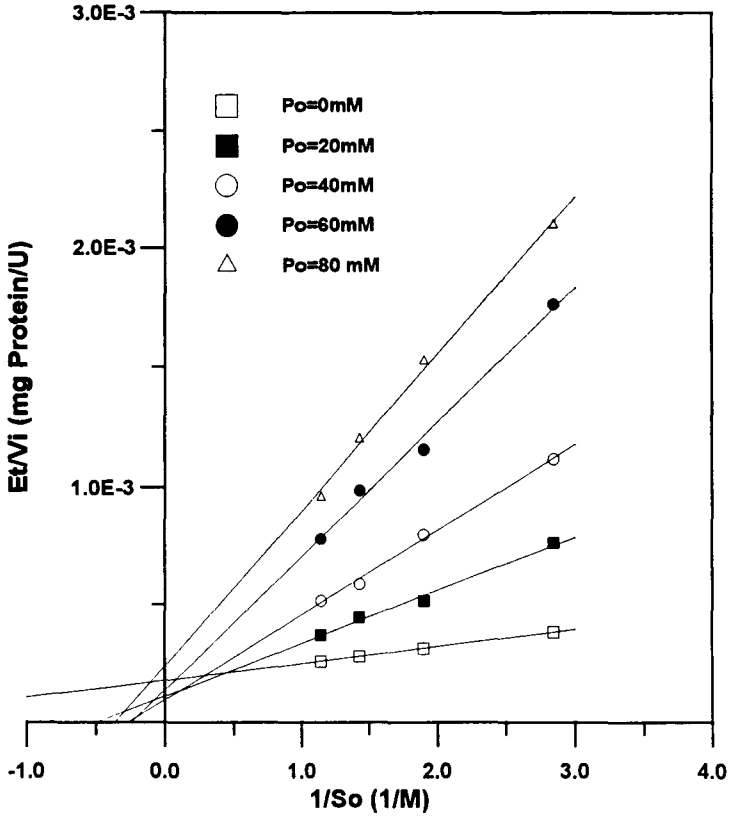

Fig. 1. Lineweaver-Burk plot for palm kernel olein hydrolysis at W_o = 13, pH = 7.0, T = 30°C, 50 m*M* AOT-isooctane soluction and enzyme activity = 13 U.

where E_t is the total amount of enzyme, and S and P are concentrations of substrate and product, respectively. The apparent K_m and V_m were determined from the straight line with P_o = 0 in Fig. 1 as 9.06% and 5523 U/mg protein, respectively, which compare favorably with 11.4% and 5714 U/mg protein reported in previous literature (*8*). K_I can be determined from a plot of the slopes of the straight lines in Fig. 1 as functions of oleic acid concentration. K_I = 9.74 m*M* was obtained, which is only around one-tenth of that reported by Tsai and Chiang (*7*) for the hydrolysis of olive oil. This revealed that product inhibition is more important in the hydrolysis of palm kernel olein than the hydrolysis of olive oil.

Effects of Oleic Acid on the Stability of Lipase

The effects of oleic acid on the stability of lipase are shown in Fig. 2. Lipase activity decays quite rapidly in the absence of oleic acid. As the reaction proceeds, substrate and product vie for the active sites of lipase.

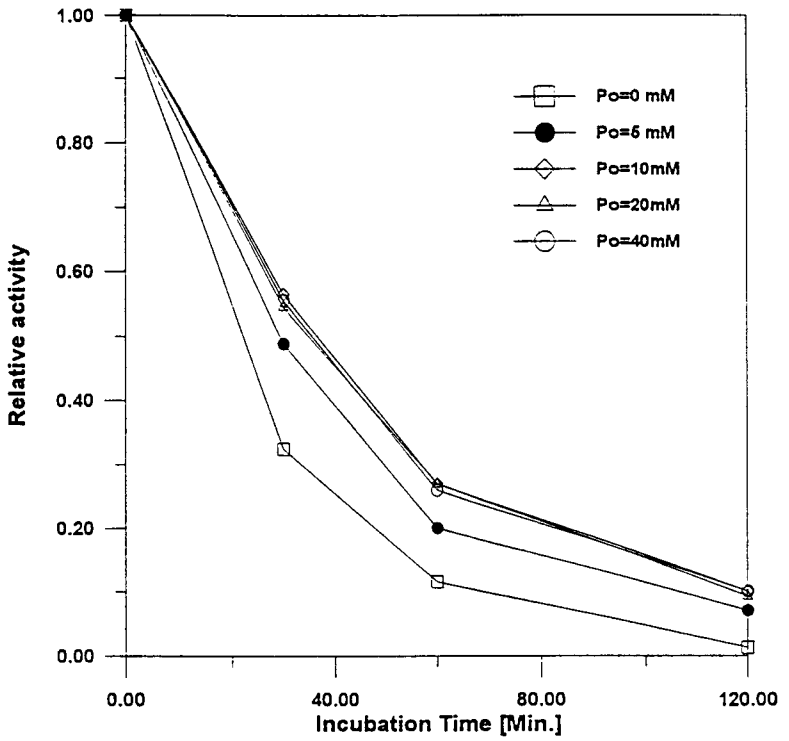

Fig. 2. The effects of oleic acid on the stability of lipase for S_o = 0.877*M*. Conditions as in Fig. 1.

Occupation of an active site by oleic acid apparently provides protection for lipase from deactivation. The increase of oleic concentration actually slows down the rate of lipase deactivation up to a concentration of approx 10 m*M*, as is clearly indicated in Fig. 2. The association of oleic acid with the active site probably reaches the saturation point at an oleic concentration of 10 m*M* such that little additional protection of lipase from deactivation was observed for an oleic acid concentration higher than 10 m*M*.

Time-Course Analysis

Equation (4) can be rewritten as:

$$V_I = (dP / dt) = \{k_{cat}SE_t / K_m (1 + (P / K_I) + S\} \quad (5)$$

Product concentrations as a function of time can be obtained by integrating Eq. (5), in which the result is:

$$V_m t = k_{cat}E_t t = P + K_m\{\ln(S_o / S_o - P) + (1 / K_I) [S_o \ln (S_o / S_o - P] - P)\} \quad (6)$$

The predicted time-course of product concentration as well as the experimental results are presented in Fig. 3. Experimental results correlated sufficiently with those predicted by Eq. (6) for a reaction time up to 100 min.

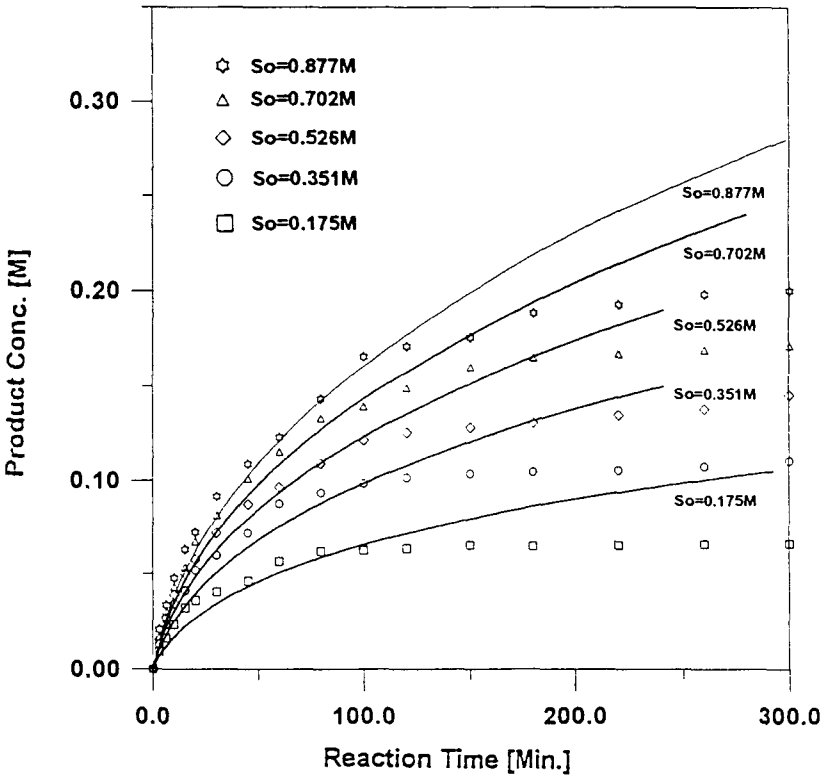

Fig. 3. Product concentrations as function of time for palm kernel olein hydrolysis. Conditions as Fig. 1. Solid lines are calculated results based on Eq. (6).

However, the deviation increases with time. Similar results for the hydrolysis of olive oil in AOT-isooctane-water reversed micelles with *C. rugosa* lipase were reported by Tsai and Chiang (*7*). The decrease in W_o, the change in the microenvironment, and the enzyme deactivation as the reaction proceeds were proposed as possible reasons for the observed discrepancy.

Yamada et al. (*10*), pointed out that hydrophobic and electrostatic interactions between enzyme and AOT molecules may lead toward lipase deactivation in reversed micelles. From a structural prediction based on far-UV CD spectral data for *R. arrhizus* lipase in AOT-isooctane reversed micelles, Brown et al. (*11*) demonstrated the structural reorganization of *R. arrhizus* lipase on incorporation into reversed micelles. The reorganized structure was characterized by a dramatic increase in β-sheet and overall accountable secondary structure. The effect of glycerol on lipase stability in reversed micelles remains controversial (*8*). In this study, however, glycerol is ruled out as a factor in affecting lipase stability, since it was not present in the *R. arrhizus* lipase-catalyzed hydrolysis of palm kernel olein. The deviation observed in Fig. 3 for a time greater than 100 min is

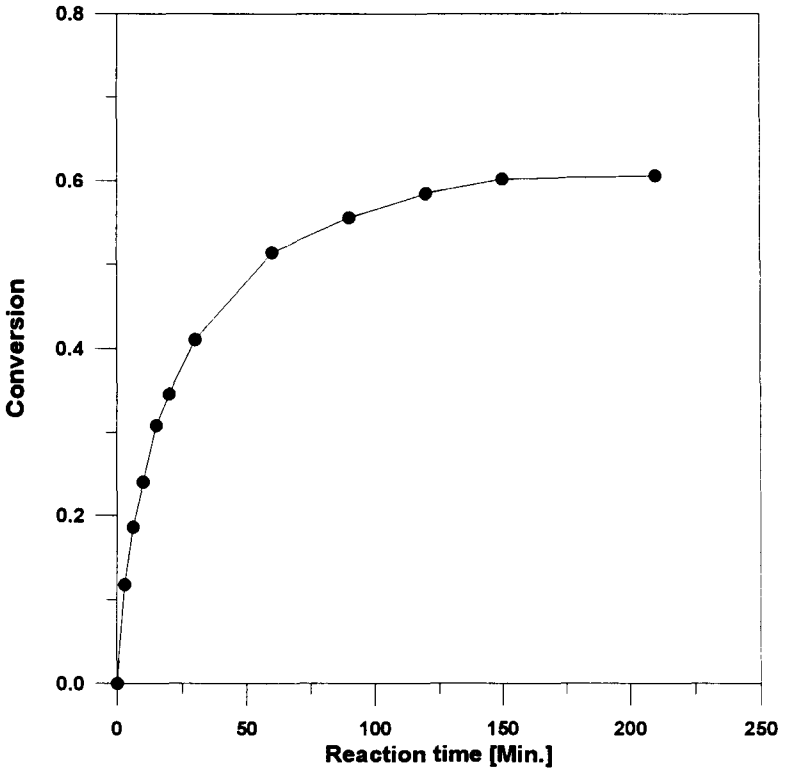

Fig. 4. Relationship between the degree of conversion and reaction time for S_0 = 0.175*M*. Conditions as in Fig. 1, except enzyme activity = 26 U.

possibly the result of several factors. Equilibrium shifts toward the reactant side as water content decreases with time. A decrease in W_0 reduces the enzyme activity. Enzyme activity can also be reduced when conformational change of lipase occurs as 2-monoglyceride and reversed micelles approach each other, which cause a change of hydrogen bonding in the β-sheet structure of lipase.

Prazeres et al. (*12*) contended that three molecules of fatty acid combine with each of the lipase molecules, since each lipase molecule possesses three subcenters. They proposed a third-order nonlinear model with product inhibition to describe fat hydrolysis in reversed micelles and found better agreement between experimental and predicted results even for a time period > 100 min.

The degree of conversion increases as initial substrate concentration decreases. The maximum degree of conversion attainable theoretically is 66.7% for 1,3-specific *R. arrhizus* lipase-catalyzed hydrolysis of palm kernel olein. The degree of conversion as a function of time is given in Fig. 4 for S_0 = 0.175*M* and a lipase activity of 26 U. A maximum degree of conversion of 61%, which is close to the theoretical value of 66.7%, can be attained indicating the presence of 2-monoglyceride (not 1- or 3-monoglyceride) in the end products.

CONCLUSION

R. arrhizus lipase-catalyzed hydrolysis of palm kernel olein was carried out in AOT-isooctane reversed micelles under optimum W_o, pH, and temperature. A small K_I of 9.74 m*M* indicated significant product inhibition. A Michaelis-Menten mechanism 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 100 min. This deviation is the result of shifting, which occurs in the reaction equilibrium and enzyme deactivation. A decrease in W_o in the presence of 2-monoglyceride possibly contributes toward the deactivation of lipase.

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