Olive Oil Hydrolysis by Celite-Immobilized *Candida rugosa* Lipase

Sibel Fadıloğlu* and Zerrin Söylemez

Department of Food Engineering, Faculty of Engineering, Gaziantep University, 27310 Gaziantep, Turkey

Candida rugosa lipase was immobilized on Celite by acetone precipitation and adsorption. The immobilized enzyme was used for catalyzing olive oil hydrolysis in an aqueous medium without any added emulsifiers. Adsorption on Celite gave better results in terms of effectiveness factor, reuse, and stability compared to acetone precipitation. Adsorbed lipase exhibited good hydrolytic activity but was never more efficient than soluble enzyme. The effectiveness factor of lipase adsorbed on Celite was found to be 0.90. The fatty acid formation was linearly increased ($P < 0.05$) up to an enzyme concentration of 0.5 mg. The progress curve of the olive oil hydrolysis gave a typical hyperbolic form. After immobilization, the optimal reaction pH was shifted from 7 to 6.5 and the optimal reaction temperature from 40 to 45 °C. Although the lipase immobilized on Celite exhibited higher stability, the rapid loss of enzyme activity appeared to be the main problem during repetitive use. The parameters, specific production rate, and maximum product value were estimated for each experimental data set by applying the Logistic-2 model.

Keywords: *Candida rugosa lipase; adsorption; immobilization; olive oil; hydrolysis*

INTRODUCTION

Recently, microbial lipases have gained considerable interest in industrial applications, particularly in fat and dairy industries (Chen and Yang, 1992; Cho and Rhee, 1993). Other areas of interest include lipase-catalyzed triglyceride hydrolysis (Han and Rhee, 1986; Tsai et al., 1991; Tsai and Chiang, 1991) and esterification (Jaeger et al., 1994; Osada et al., 1990; Berger and Schneider, 1992) as energy saving processes. As the practical applications increased, the requirement for bulk amounts of enzyme became a limiting factor. Therefore, immobilization of the lipase is considered to be one possible way to allow reuse of the enzyme and a possibility for continuous operations.

Many immobilization techniques of lipases have been employed recently, each involving a different degree of complexity and efficiency (Ruckenstein and Wang, 1993; Shaw et al., 1990; Malcata et al., 1990). The physical immobilization of lipase on solid support can offer several advantages over chemical methods and gel-entrapment. In contrast to the rather difficult chemistry of chemical methods (Stark and Holmberg, 1989; Otero et al., 1988) and diffusional limitations on entrapment (Chen and Yang, 1992), adsorption is the most simple and least expensive method and is also known to retain high catalytic activity (Shaw et al., 1990; Basri et al., 1994)

In the present study the hydrolytic activity of immobilized lipase has been studied in an organic solvent free system that is rarely used in the oils and fat industry, especially for systems employing immobilized enzymes. Two methods, adsorption by acetone precipitation and by vacuum-drying, have been applied to obtain the most suitable procedure for immobilization of lipase for olive oil hydrolysis. Diatomaceous earth (Celite, Hyflo supercel) was used as a support material because of its high adsorption capacity due to the presence of large amount of micropore space (Wisdom et al., 1984). Nonspecific *Candida rugosa* (*C. rugosa*) lipase was selected because of its high activity in hydrolytic reactions (Ruckenstein and Wang, 1993).

Nonlinear equations were used to fit the data, and the best model was selected to describe the progress curve of olive oil hydrolysis and the exact influence of adsorbed enzyme concentration on product formation rate (Zwietering et al., 1990).

MATERIALS AND METHODS

Lipase (triacylglycerol acyl-hydrolase (EC 3.1.1.3) from *Candida rugosa*, type VII, specific activity, 4865 units/ mg protein) was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The protein content of the commercial preparation was nearly 10% by mass, and the fraction of pure lipase in the mixture was 1.1% by mass. Celite used for immobilization, was also purchased from Sigma. Commercial olive oil was supplied from a local market and purified with charcoal as described by Benzonana et al. (1971). All other reagents and chemicals used were of analytical grade (Merck or Riedel de Haen).

Lipase Immobilization. *Adsorption by Acetone Precipitation.* The method involves precipitation of the enzyme from solution onto the solid support. The preparate was obtained by first dissolving enzyme in 0.1 M KP buffer, pH 7.0, and then mixing throughly with support material (200 μg of solid/ mg of Celite). The enzyme was precipitated by the slow addition of chilled acetone (20 mL) at −20 °C. The immobilizate was left at −20 °C for 15 min, and then acetone was removed by decantation.

Adsorption by Vacuum-Drying. Candida rugosa lipase was dissolved in doubly distilled water. The enzyme solution was added to support material (200 μg of solid/mg of Celite), and the mixture was shaken slowly. Water was removed by evaporation under vacuum at room temperature (20−25 °C) for 7 h. The immobilized enzyme was then desiccated and stored in a refrigerator. The moisture content of the preparate was determined as 0.88% using an oven drying at 105 °C.

Lipase Activity. The activities of both free and immobilized lipase were determined using olive oil as the

* To whom correspondence should be addressed (fax 0342 3601100; e-mail fadılogluet.alpha.bim.edu.tr).

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Table 1. Models Used for Fitting Experimental Data

model	eq	model	eq
Gompertz	$y = a \exp[-\exp(b - cx)]$	Logistic-2	$y = [(a - d)/(1 + (x/c)^b)] + d$
Logistic-1	$y = a/[1 + \exp(b - cx)]$	quadratic	$y = a + bx + cx^2$

substrate. The hydrolysis was carried out in 100 mL conical plastic-stoppered flasks at 37 °C with shaking at 120 strokes/min. The reaction was initiated by adding various amounts of free or immobilized lipase into the reaction mixture (4 mL) containing a known amount of olive oil in 25 mM KP, pH 7.0, unless otherwise stated. Before incubation at 37 °C, 1 min of vortex mixing was applied (Wang et al., 1988). The reaction was terminated by the addition of 5 mL of ethanol. When immobilized lipase was used, the enzyme was separated by centrifugation. Then the amount of free fatty acid released in the reaction mixture was estimated by titration with 0.5 N KOH using phenolphthalein as an indicator. The reaction mixture operated without the enzyme was titrated in the same way and used as a blank. Lipase adsorbed Celite after centrifugation was immediately introduced in a new reaction mixture for repetitive assays.

One unit of lipase activity was defined as the amount which liberated 1 μmol of fatty acid/min at 37 °C. The percentage hydrolysis was calculated from the acid value of the oil in the mixture after complete hydrolysis on the basis of the saponification value of the oil used. The effectiveness factor was estimated as the ratio of the rate of reaction for an immobilized enzyme to the rate of reaction for the same amount of enzyme in solution. Initial reaction rates were considered in order to make a reliable comparison.

Mathematical Modeling. The nonlinear equations (Table 1) were fitted to experimental data that reflect the time and enzyme concentration dependency of olive oil hydrolysis by nonlinear regression analysis. The fitting procedure was done by using a computer package program, Sigma Plot (Jandel Scientific, San Francisco, CA). The nonlinear regression analysis were carried on the Marquardt−Levenberg algorithm. The algorithm calculates the set of parameters, predicted and residual data at 95% confidence intervals.

Predicted data obtained by using various models were compared with the experimental data statistically by the linear regression coefficient (R^2). The best fitting is observed when R^2 values reach 1.

Statistical Analysis. Statistical analysis (ANOVA) was carried out by using a computer program, Statgraphics (Stsc, Rockville, MD). Anova test was performed for all experimental runs to determine significancy at the 95% confidence interval.

RESULTS AND DISCUSSION

The hydrolytic activity and stability of *Candida rugosa* lipase immobilized with two different technique were compared. Acetone precipitated lipase exhibited lower hydrolytic activity and stability than simply adsorbed lipase. Fatty acid (FA) release was 198 μmol and 18 μmol of FA for two subsequent batches within 60 min reaction time under the assay conditions of 12.5% (v/v) olive oil and 2 mg of adsorbed enzyme preparation in 25 mM potassium phosphate buffer, pH 7.0, at 37 °C.

Although noncovalent immobilization of several enzymes was achieved by acetone precipitation, such enzyme preparation usually showed good stability when operated in organic solvents (Wisdom et al., 1984; Wehtje et al., 1993).

Immobilization of lipase by simple adsorption using vacuum evaporation seemed to be more favorable to the retention of the enzyme activity. Probably the enzyme got forced onto the support physically and dried onto it. Fatty acid produced with 2 mg of adsorbed enzyme preparation and 12.5% (v/v) olive oil was 340 μmol and 154 μmol FA for the first and second use within a 60 min reaction time, respectively. At 25% (v/v) substrate concentration, the effectiveness factor was found to be 0.90 for the adsorbed enzyme. This is in agreement with the findings of Lie and Molin (1991) but in contrast to those of Cho and Rhee (1993) who found that immobilization on a hydrophobic carrier enhances the lipase activity. The adsorbed lipase was also quite stable and retained about 56% of the initial activity during the course of 6 months storage in the refrigerator.

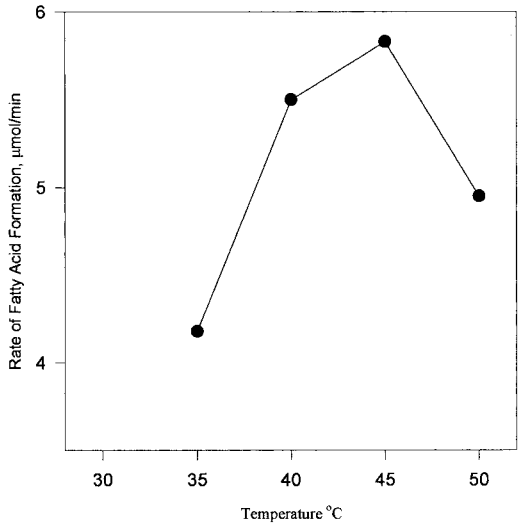

Figure 1. Effect of temperature on activity of immobilized lipase on Celite. Reaction conditions: olive oil, 12.5% (v/v); enzyme, 12.5 μg of protein/mL; 25 mM potassium phosphate buffer, pH 7.0; reaction time, 20 min.

Owing to better hydrolytic activity and stability, lipase adsorbed on Celite by vacuum evaporation was selected for further experiments.

Optimum Temperature for the Hydrolysis of Olive Oil. The optimum temperature for free lipase was determined as 40 °C (data not shown). The temperature dependency for the immobilized enzyme is shown in Figure 1. The optimum temperature shifted to 45 °C due to immobilization. A significant ($P < 0.05$) increase in the product formation rate was observed at this temperature. A similar change in the optimum temperature has been reported for *Candida* lipase coupled to agarose and chitin. It has been suggested that lipase immobilized in the matrix with multiple-point attachment or with higher hydrophobicity exhibits greater thermal stability (Shaw et al., 1990).

Optimum pH for the Hydrolysis of Olive Oil. As shown in Figure 2, a significant ($P < 0.05$) increase in the product formation rate was observed at pH 7.0 for the free enzyme and pH 6.5 for the immobilized enzyme. Yang and Chen (1994) reported no change in the pH optima of *C. rugosa* lipase entrapped in ENTP-4000 prepolymer, while a shift in pH optima of lipase immobilized on PVC, Sepharose, chitin, and agarose has been reported (Shaw et al., 1990).

Effect of Adsorbed Enzyme Concentration on Olive Oil Hydrolysis. The rate of olive oil hydrolysis was significantly ($P < 0.05$) affected by the amount of enzyme adsorbed on Celite (Figure 3). At a fixed substrate concentration (12.5% (v/v)), the amount of fatty acid produced was linearly proportional to the enzyme concentration up to 0.5 mg of enzyme (50 μg of protein) within 20 min reaction time showing that the

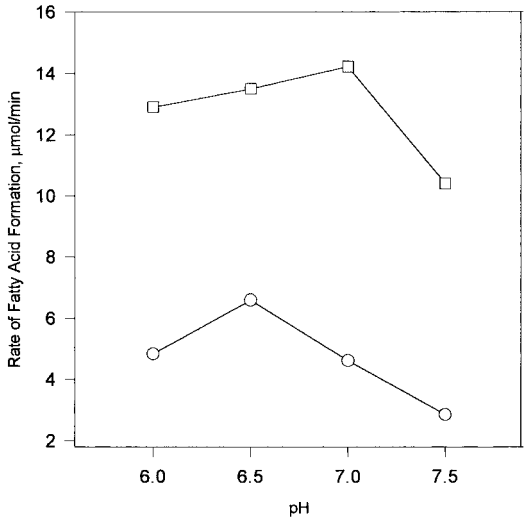

Figure 2. Effect of pH on activity of free and immobilized lipase on Celite. Reaction conditions: 25 mM potassium phosphate buffer, 37 °C; for free enzyme (□−), olive oil, 25% (v/v) and enzyme, 25 μg of protein/mL; reaction time, 30 min; for immobilized enzyme (○−) as in Figure 1.

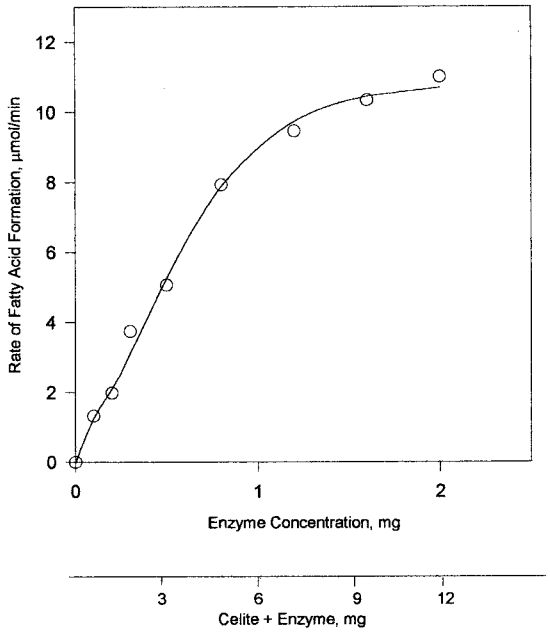

Figure 3. Effect of the amount of adsorbed enzyme on olive oil hydrolysis. Reaction conditions: olive oil, 12.5% (v/v); 25 mM potassium phosphate buffer, pH 7.0; temperature, 37 °C; reaction time, 20 min. Symbol represents the experimental data points; solid line is the fitted curve.

system was in steady state. After this point linearity deviates and the percent hydrolysis exceeds 5% and reaches 16% under the catalytic activity of 2 mg of adsorbed enzyme preparation.

Time Course of Olive Oil Hydrolysis. Figure 4 shows the fatty acid formation as a function of reaction time at fixed substrate (12.5% (v/v)) and enzyme (2 mg of solid/assay or 0.2 mg of protein/assay) concentrations. The progress curve of the olive oil hydrolysis gave a typical hyperbolic form. The fatty acid formation was linearly proportional to the reaction time up to 10 min.

Under optimum conditions (pH = 6.5; T = 45 °C) with 25% (v/v) olive oil and 0.2 mg of protein/assay, the immobilized preparation showed 90% hydrolysis after 12 h reaction time (Figure 5). Deactivation of lipase was found to be biphasic with a low $t_{1/2}$ of 100 min and a high $t_{1/2}$ of 8 h.

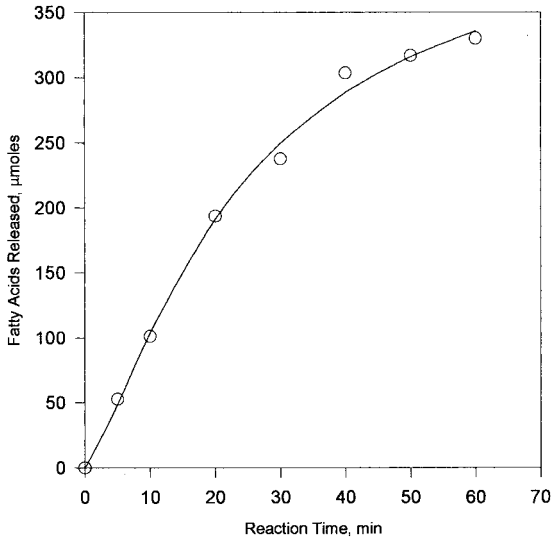

Figure 4. Time course of olive oil hydrolysis by immobilized lipase on Celite. Reaction conditions as in Figure 3. Enzyme, 2 mg of solid/assay (12 mg Celite + enzyme per assay). Symbol represents the experimental data points; solid line is the fitted curve.

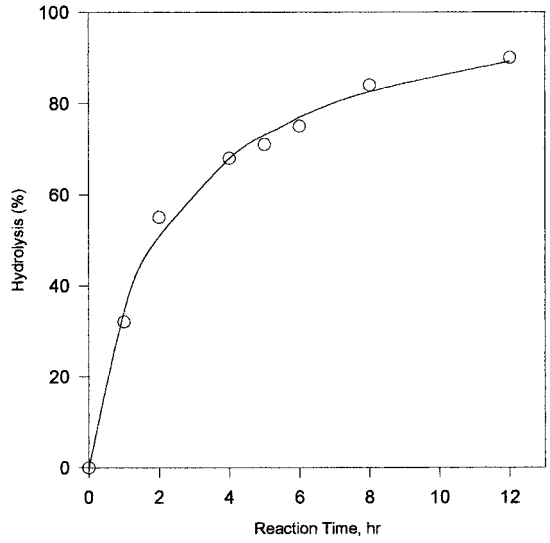

Figure 5. Percentage hydrolysis as a function of time by Celite adsorbed lipase. Reaction conditions: olive oil, 25% (v/v); 25 mM potassium phosphate buffer, pH 6.5; enzyme, 2 mg of solid/assay; temperature, 45 °C. Symbol represents the experimental data points; solid line is the fitted curve.

Table 2. Results of Linear Regression Analysis (R^2 Values) of Models

exptl set[a]	Gompertz	Logistic-1	Logistic-2	quadratic
1	0.993	0.985	0.996	0.995
2	0.992	0.991	0.999	0.997
3	0.957	0.940	0.995	0.932

[a] Experimental set: (1) rate of fatty acid formation vs enzyme concentration; (2) fatty acid released vs reaction time; (3) hydrolysis (%) vs reaction time.

Results of Mathematical Modeling. To check the goodness of fit of each model (Table 1), linear regression analysis was performed between experimental and predicted data to determine R^2 values. R^2 values of each model for experimental runs are given in Table 2.

R^2 values indicate that the Logistic-2 model gave the best fitting for all experimental sets. Experimental data

Table 3. Derived Parameters

exptl set	specific prodn rate	max product value
1	12.19 ± 0.65 μmol/(min mg of solid)	13.38 ± 1.19 μmol/min
2	11.38 ± 0.28 μmol/min	435.20 ± 53.49 μmol
3	47.97 ± 2.21% hyd/h	108.10 ± 13.36% hyd

Table 4. Repetitive Use of Immobilized Lipase[a]

no. of use	μmol of FA	retained activity (%)
1st	295	100
2nd	84	29
3rd	31	11

[a] Reaction conditions: enzyme, 2 mg of solid/assay; olive oil, 25% (v/v); 25 mM potassium phosphate buffer, pH 6.5; temperature, 45 °C; reaction time, 10 min.

and predicted curves were plotted by this model and are shown in Figures 3−5. In this model, d is the asymptotic value in which the product formation rate decreases and the maximum value of product is reached. SPR was derived from this model by calculating the first derivative at the inflection point (Dalgıç, 1998).

The function of SPR is

$$\mathrm{SPR} = [(d - a)/(4bc)](b^2 - 1)[(b + 1)/(b - 1)]^{1/b}$$

The derived parameters are given in Table 3.

Repetitive Use of Immobilized Lipase. The results of repeated use of immobilized lipase preparation in the shaking flask system are shown in Table 4.

The preparation demonstrated a rapid drop in activity. This could probably be due to the discharge of enzyme from the carrier during the course of the reaction. This seemed to be the main problem in these applications, and further investigations are needed to determine the origin of the activity loss.

In conclusion, the hydrolysis of olive oil could be carried out successfully in an aqueous medium by adsorbed lipase on Celite in which nonpurified lipase preparation were used for good hydrolytic activity. The immobilized *Candida rugosa* lipase obtained by vacuum-drying on Celite seems to be feasible for various applications of triglyceride hydrolysis. However, it offers the benefit of applying economically if a much better ability of repetitive use could be achieved.

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