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Kinetic Studies of Lipase from *Candida rugosa*

A Comparative Study Between Free and Immobilized Enzyme onto Porous Chitosan Beads

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Abstract

The search for an inexpensive support has motivated our group to undertake this work dealing with the use of chitosan as matrix for immobilizing lipase. In addition to its low cost, chitosan has several advantages for use as a support, including its lack of toxicity and chemical reactivity, allowing easy fixation of enzymes. In this article, we describe the immobilization of *Candida rugosa* lipase onto porous chitosan beads for the enzymatic hydrolysis of olive oil. The binding of the lipase onto the support was performed by physical adsorption using hexane as the dispersion medium. A comparative study between free and immobilized lipase was conducted in terms of pH, temperature, and thermal stability. A slightly lower value for optimum pH (6.0) was found for the immobilized form in comparison with that attained for the soluble lipase (7.0). The optimum reaction temperature shifted from 37°C for the free lipase to 50°C for the chitosan lipase. The patterns of heat stability indicated that the immobilization process tends to stabilize the enzyme. The half-life of the soluble free lipase at 55°C was equal to 0.71 h (K_d = 0.98 h^{-1}), whereas for the immobilized lipase it was 1.10 h (K_d = 0.63 h^{-1}). Kinetics was tested at 37°C following the hydrolysis of olive oil and obeys the Michaelis-Menten type of rate equation. The K_m was 0.15 m*M* and the V_{max} was 51 µmol/(min·mg), which were lower than for free lipase, suggesting that the apparent affinity toward the substrate changes and that the activity of the immobilized lipase decreases during the course of immobilization.

Index Entries: Lipase; immobilization; chitosan; physical adsorption; characterization; hydrolysis.

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Introduction

One of the main limitations to obtaining precursors and products of commercial interest can be associated with the use of chemical catalysts, which are not very versatile and require high temperatures to attain satisfactory reaction rates. In addition to possessing low specificity, generally these catalysts provide mixed chemical compounds or byproducts that require further purification steps *(1,2)*. The cost of energy is increasing, and this will increase the cost of existing energy chemical processes, making the enzyme-catalyzed processes an alternative route to compete with the current practice of chemical synthesis *(3)*. Enzymes are being examined intensively for the preparation of new classes of reagents, especially sugars, chiral synthons, metabolites, and food components *(1,3)*. Emerging technology for the production of such compounds employs lipases that are abundant, stereospecific, stable, and versatile enzymes. In addition to the lipolytic reactions, lipases catalyze a variety of synthetic transformations such as esterifications and interesterifications *(4–7)*.

The industrial use of lipases as catalysts depends on their efficient immobilization and the employment of appropriate supports in such a way that the initial investment in raw material (enzyme and support) is compensated by the high activity and stability of the derivative *(7)*. The high cost of popular supports (silica-based carriers and synthetic polymers) leads many researchers to search for cheaper substitutes such as $CaCO_3$ *(8)*, rice husk *(9)*, chitin, and chitosan *(10,11)*. Of these alternatives, the derivative of chitin, chitosan, appears to be more attractive since chitin is the second most abundant biopolymer in nature after cellulose *(10)*. In addition, this support presents several advantages as enzyme immobilization carrier. Among the most prevalent are versatility in the physical forms that are available (flakes, porous beads, gel, fiber, and membrane); scarce biodegradability, low cost; ease of handling; high affinity toward the proteins, and above all nontoxicity *(11)*. Moreover, good results were obtained in a number of previous studies in which chitosan was used to immobilize lipase *(12,13)* and other hydrolases such as amyloglucosidase, papain, β-glucosidase, and α-L arabinofuranosidase *(10,11)*.

In pursuing our interest in the immobilization and subsequent use of lipases *(14–16)*, we have investigated the feasibility of using chitosan as the matrix for immobilizing microbial lipases. The immobilization criteria were based on the use of a low-cost method of loading enzyme into the support. The enzyme used was nonspecific lipase from *Candida rugosa*. The chosen method of immobilization was simple adsorption, whereby the enzyme adheres to the surface of the support particles by van der Waals forces of attraction.

Two physical forms of chitosan (flakes and porous beads) were tested as supports following previously described methodology *(14)*, and the immobilization efficiency was assessed with respect to the recovery of both protein and hydrolytic activity. The best chitosan form with the highest

immobilization efficiency was selected for further studies, including full characterization of the immobilized derivative under aqueous medium (hydrolysis of olive oil as a model). Comparative studies of free and immobilized enzyme were conducted in terms of pH, temperature, and thermal stability. The enzymatic hydrolysis with the immobilized enzyme in the framework of the Michaelis-Menten mechanism was also analyzed.

Materials and Methods

Materials

Commercial *C. rugosa* lipase (type VII) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). The lipase was a crude preparation with a nominal activity of 1440 U/mg and 16.2 mg of protein/g of powder based on the Bradford *(17)* protein assay method. Chitosan in two different forms was tested: flakes (analytical grade chitosan C-3646 obtained starting from crab shell containing 85% deacetylation; Sigma) and porous beads (pharmaceutical grade supplied by SP Chemical Farma, SP, Brazil) having a purity of 93%, moisture of 6%, and 40-mesh granulometry according to the manufacturer's information. Olive oil (low acidity) was purchased at a local market. Solvents were standard laboratory grade and other reagents were purchased from either Aldrich (Milwaukee, WI) or Sigma.

Immobilization of Lipase onto Chitosan

Lipase was immobilized by physical adsorption on chitosan following previous methodology *(14)* with slight modifications. Chitosan (3 g) was initially soaked in hexane, under agitation (100 rpm) for 1 h. Then, the excess of hexane was drained and 0.5 g of lipase previously dissolved in 10 mL of distilled water was added. The fixation of lipase onto support proceeded under agitation for 3 h at room temperature followed by 18 h at 4°C without agitation. The derivative was filtered (Whatman filter paper 41) and thoroughly rinsed with hexane. The enzyme activity before and after immobilization was determined by measuring the hydrolytic activities of the supernatant liquid solutions, which allowed calculation of the activity yield of the immobilized enzyme preparation.

The effect of enzyme loading on immobilized enzyme activity was studied by varying the amount of lipase offered (0.1–1.0 g of lipase) to a fixed amount of support (3.0 g of chitosan).

Hydrolysis Assay

Hydrolytic activities of free and immobilized lipase were assayed by the olive oil emulsion method according to the modification proposed by Soares et al. *(16)*. The substrate was prepared by mixing 50 mL of olive oil with 50 mL of gum arabic solution (7% [w/v]). The reaction mixture containing 5 mL of the emulsion, 2 mL of 100 m*M* sodium phosphate

buffer (pH 7.0), and either free (1 mL, 5 mg/mL) or immobilized (250 mg) lipase was incubated for 5 min at 37°C. The reaction was stopped by adding 10 mL of acetone-ethanol solution (1:1). The liberated fatty acids were titrated with 25 m*M* potassium hydroxide solution in the presence of phenolphthalein as an indicator. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of free fatty acid/min under the assay conditions.

Catalytic Properties of Lipase Preparations

The estimation of free and immobilized hydrolytic activities at different pH values were carried out with reaction mixtures containing 100 m*M* of the sodium phosphate buffer at a pH range from 3.0 to 9.0 at 37°C. The effect of temperature in both lipase activities was determined from 30 to 60°C under the assay conditions. For the determination of thermal stability, either free or chitosan lipase preparations were incubated in 2 mL of sodium phosphate buffer (pH 7.0) at different temperatures (40–60°C) for 1 h. Samples were withdrawn and assayed for residual activity as previously described, taking an unheated control to be 100% active.

Protein Assay

Protein was determined according to Bradford's *(17)* method using BSA as the standard. The amount of bound protein was determined indirectly from the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein in the filtrate and in the washing solutions.

Results and Discussion

Selection of Chitosan Form and Determination of Lipase Loading on Support

Two chitosan forms (flakes and porous beads) were tested as supports for immobilization of *C. rugosa* lipase by physical adsorption. The structure of chitosan form significantly interfered with both the protein fixation and activity yield, as shown in Table 1. The highest percentage of protein recovery (78.4%) and catalytic activity (14.7%) was obtained by using porous chitosan beads (PCB). It appears that the structure in the form of small granules, characteristic of the pharmaceutical chitosan grade, provided a better distribution of the lipase on the support surface, improving the contact between the interface water/oil, which is necessary for the expression of the activity of immobilized lipases.

The PCB were used to obtain PCB-lipase preparations of different lipase loadings by changing the concentration of lipase in aqueous solution in which chitosan was immersed. The influence of lipase loading in the range of 0.1–1.0 g of lipase/g of chitosan was studied; Figure 1 presents the results.

Table 1
Immobilization of *C. rugosa* Lipase onto Different Physical Forms of Chitosan[a]

Chitosan form	Bound protein (%)	Activity yield (%)	Hydrolytic activity (μmol/[mg·min])
Flakes	73.10	7.10	22.88
Porous beads	78.40	14.70	42.67

[a]Total amount of protein offered to the immobilization: 16 mg; lipase loading: 360 U/g of support.

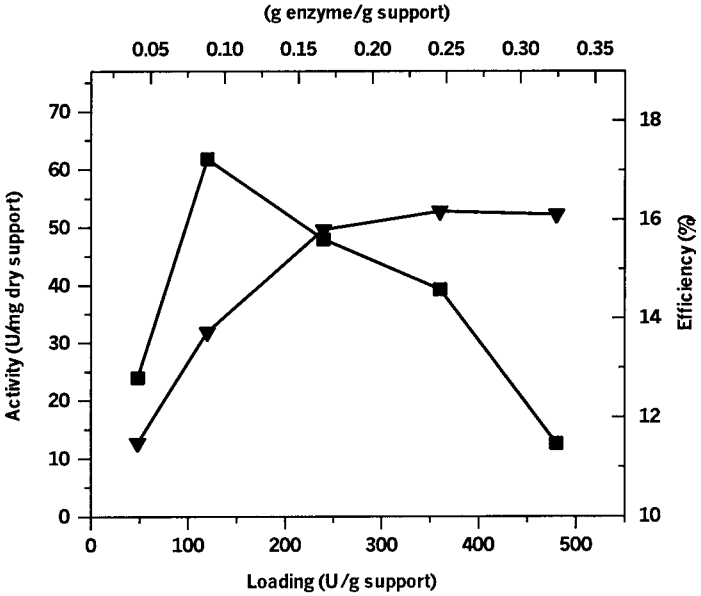

Fig. 1. Effect of lipase loading on hydrolytic activity (■) and activity yield (▼) for *C. rugosa* immobilized onto PCB. Efficiency was calculated by dividing the catalytic activity by lipase loading (see Materials and Methods).

The hydrolytic activity of the PCB-lipase increased from 12.7 to 52.2 U/mg of dry support as more lipase was loaded onto the support (48–480 U/g of support). However, when converted to an efficiency plot (activity/loading), higher efficiencies were obtained at lower lipase loading (120 U/g of support). This may suggest that at loadings over 240 U/g of support, instead of obtaining high lipase fixation on the support surface, multilayer adsorption might occur that could block or inhibit the substrate access (emulsion of olive oil) to the lipase-active sites at the lower layers. It is likely that only the enzyme molecules fixed onto the external layers of chitosan beads are responsible for the detected activity. Therefore, most of the other experiments were carried out using immobilized preparation at the lower lipase loadings of 120 U/g of dry chitosan.

These results are favorable compared with those reported in the literature *(6)* in terms of activity yield. Such yields are probably owing to the

methodology used; i.e., the use of an organic medium (hexane) on the coupling step of the enzyme on the support. Similar results have been described by several researchers, indicating a new trend in the use of organic nonpolar solvents as dispersion media for lipase immobilization on different supports *(13,17,18)*. The mechanism responsible for such an improvement has been not fully understood. According to Oliveira et al. *(15)*, two hypotheses can be put forward. First, an expansion of the supports may occur in nonpolar solvents that can promote better distribution of the enzyme on the support surface. Second, the low polarity of solvents such as hexane helps maintain the enzyme's protective layer of water.

Physicochemical Properties and Kinetics for Free and Immobilized Lipase

Methods for immobilizing enzymes should preserve, as much as possible, their original activities and specificities. However, immobilization seems to either inhibit or enhance the action of lipase. To verify changes occurring in the original kinetics and physicochemical properties of the free lipase on immobilization on chitosan, a comparative study between free and immobilized lipase was carried out in terms of pH, temperature, and thermal stability.

Figure 2 shows the variation in relative activity as a function of the pH for free lipase and PCB-lipase. On immobilization, the optimum pH (7.0) for free lipase was shifted for more acidic values (pH 6.0), indicative of the matrix behaving as a polycation *(11)*. It may be assumed that hydrogen and hydroxyl ions are distributed differently between the area close to the surface and the remainder of the solution, with negative charges clustering close to the immobilized enzyme. A microenvironment is thus formed close to the immobilized enzyme with a higher pH than that of the external solution so that the optimal pH becomes lower than that of the free enzyme. It can also be seen that the immobilization procedure conferred higher stability to pH for the immobilized enzyme, since at pH 3.0 the immobilized enzyme still presented 21% of its activity. This fact corroborates with that observed in the literature, as in many cases the immobilization procedure increases pH stability *(20)*.

Figure 3 illustrates the dependence of temperature on both free and immobilized lipase. Maximum activity of the free lipase occurred at 37°C (3400 U/mg of solid), and PCB-lipase showed a maximum activity at 45°C (70.7 U/mg of dry support). It was observed that the immobilization procedure increased the optimum temperature of the enzyme, and this is highly desirable, because higher operational temperatures would lead to lower risks of microbial contamination.

The temperature data were replotted in the form of Arrhenius plots (Figs. 4A,B), from which the energy of activation was calculated by Eq. 1, according to the fitted Eqs. 2 and 3 giving 9.90 and 7.26 kcal/mol for the free and immobilized enzyme, respectively. Therefore, in the immobilized

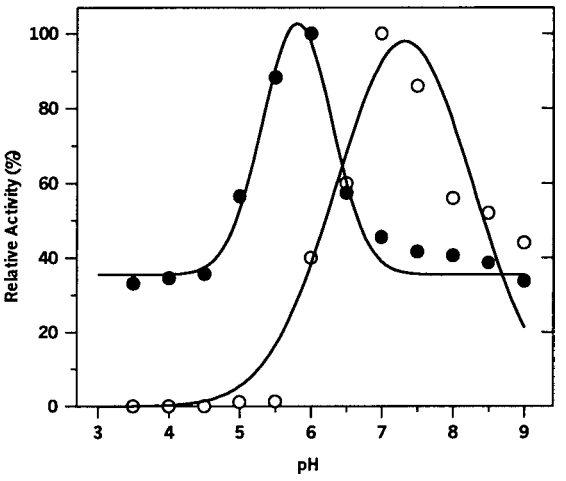

Fig. 2. Effect of reaction pH on hydrolytic activities of lipase preparations. Enzymes were assayed with olive oil emulsion as substrate at 37°C: (○) free lipase; (●) PCB-lipase. Starting activities (free lipase: 3400 U/mg; PCB-lipase: 51.4 U/mg) were taken as 100%.

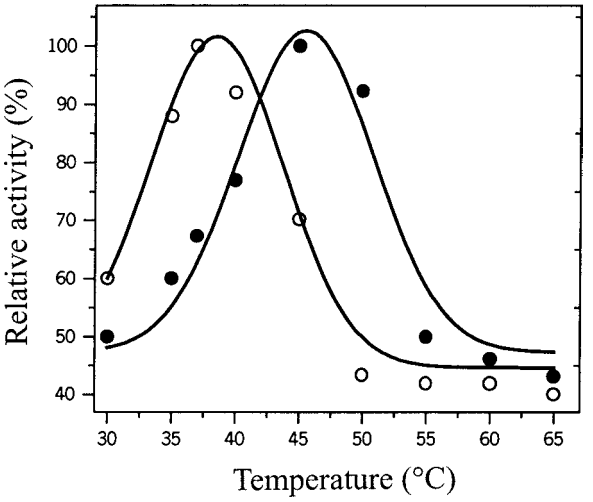

Fig. 3. Effect of reaction temperature on hydrolytic activities of lipase preparations. Enzymes were assayed with olive oil emulsion as substrate at pH 7.0: (○) free lipase; (●) PCB-lipase. Starting activities (free lipase: 3400 U/mg; PCB-lipase: 51.4 U/mg) were taken as 100%.

enzyme form the energy of activation of lipase is 27% lower. Lower activation energy in relation to the free enzyme can be considered an indicative of diffusion resistance of product and substrate in the case of the immobilized enzyme *(21)*. The values of activation energy found for free and immobilized lipases are in the range found for most of the enzymes.

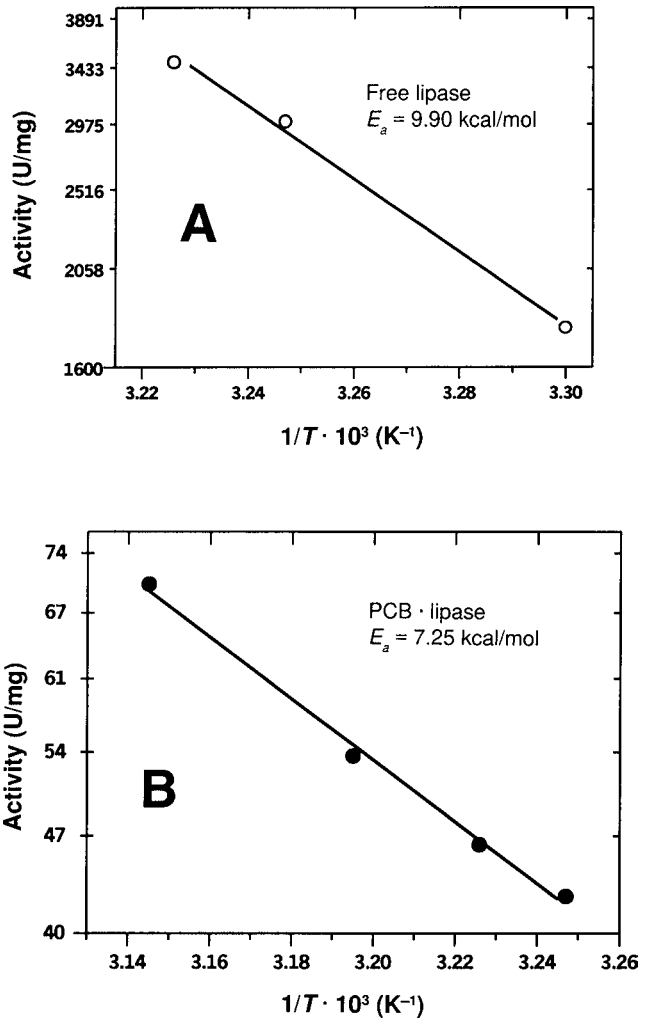

Fig. 4. Arrhenius plots for **(A)** free lipase and **(B)** PCB-lipase using olive oil emulsion 50% (v/v), pH 7.0.

$$A = A_0 \exp(-E_a/RT) \quad (1)$$

$$\text{Free lipase: } A = 1.33 \times 10^6 \exp(-9900/RT) \quad r^2 = 0.999 \quad (2)$$

$$\text{PCB-lipase: } A_i = 6.47 \times 10^6 \exp(-7260/RT) \quad r^2 = 0.954 \quad (3)$$

in which A_0 is the constant of Arrhenius, A is the activity of the enzyme, E_a is the energy of activation of the hydrolysis reaction (cal/g-mol), R is the universal gas constant (1.987 cal/g-mol K), and T is the absolute temperature (K).

Figure 5 presents a comparison of thermal stability for both lipase forms. The immobilized lipase in PCB is more stable than the free one at temperatures ≥50°C. For temperatures ≤45°C, both preparations exhibited

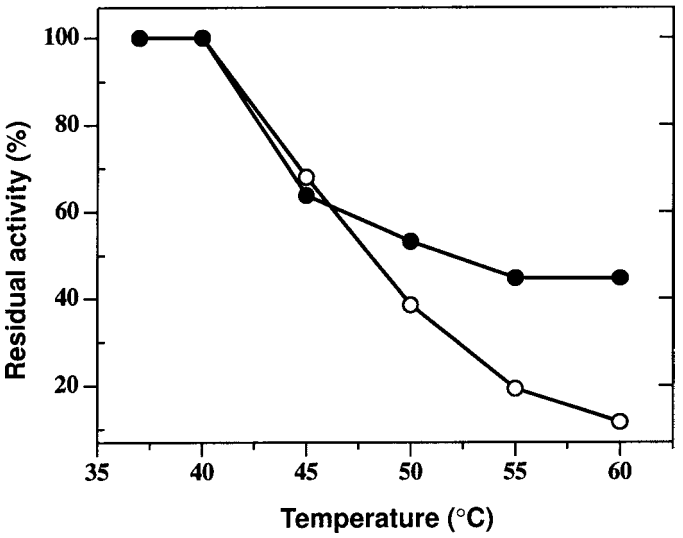

Fig. 5. Temperature deactivation for (○) free lipase and (●) PCB-lipase at different temperatures. Experiments were carried out in 100 m*M* phosphate buffer (pH 7.0). Starting activities (free lipase: 3400 U/mg; PCB immobilized lipase: 51.4 U/mg) were taken as 100%.

Table 2
Thermal Deactivation of *C. rugosa* Lipase Thermally Denatures in Olive Oil Emulsion (50% [v/v]), pH 7.0, at Different Temperatures for 1 h

Temperature (°C)	Free lipase		PCB-lipase	
	K_d (h^{-1})	$t_{1/2}$ (h)	K_d (h^{-1})	$t_{1/2}$ (h)
45	0.52	1.33	0.45	1.54
50	0.98	0.70	0.63	1.10
55	1.69	0.41	0.81	0.85
60	2.45	0.28	0.83	0.83
65	2.50	0.28	1.00	0.69

a similar trend. The PCB-lipase treated at 60°C for 1 h still held significant activity (on the order of 36% in relation to its activity at 37°C), whereas the free lipase lost 94% of its original activity.

Based on these results, thermal inactivation constants were calculated (K_d) for free and immobilized lipase by using Eq. 4, and the results are given in Table 2:

$$A_{in} = A_{in_0} \exp(-K_d \cdot t) \quad (4)$$

As can be seen, the loss of the catalytic activity for the free enzyme is higher than for the immobilized enzyme. This fact demonstrates that the immobilization procedure on chitosan gave to the lipase higher thermal stability (i.e., lower values of $-K_d$). Note, however, that the higher thermal

stability of the immobilized lipase could be a consequence of mass transfer resistance. Since the activity yield was 14.7% for lipase immobilized onto chitosan beads, it is likely that mass transfer resistances are contributing to lowering the activity yield and increasing the apparent thermal stability of the immobilized enzyme.

Data of thermal inactivation constants (K_d) as a function of the temperature allow the calculation of the energy of deactivation by applying a similar approach to the one used for the energy of activation. In most of the available literature *(21)*, it is normally assumed that the enzyme thermal denaturation is a reaction with the rate of enzyme deactivation (r_d) being first order in relation to the concentration of the active enzyme (E):

$$r_d = -K_d E \tag{5}$$

and the deactivation constant (K_d) being a function of temperature as given by the Arrhenius equation:

$$K_d = K_d^0 \exp(-E_d/RT) \tag{6}$$

in which E_d is the energy of deactivation, R is the universal gas constant (1.987 cal/mol K), and T is the absolute reaction temperature.

Values obtained for K_d for various test temperatures are plotted in the form of an Arrhenius plot, i.e., log of K_d against the inverse of absolute temperature, yielding the energy of deactivation (E_d), as the angular coefficient of the adjusted straight lines, multiplied by R, the universal constant. Curves were plotted for the free and immobilized lipase (Figs. 6A,B). Analysis of Fig. 6A shows that lipase thermal deactivation as either free or immobilized enzyme is more accentuated at higher temperatures and a single straight line cannot be fitted to the data, showing that the simple assumptions leading to Eqs. 4–6 do not hold in these enzyme preparations. The Arrhenius type of equation fitted to the experimental data shown in Fig. 6A for the temperature range from 40 to 50°C is as follows:

$$K_d = 9.96 \times 10^{18} \exp(-29{,}221/RT) \quad r^2 = 0.9999 \tag{7}$$

It was observed that the PCB-lipase shows thermal inactivation at temperatures higher than 55°C, and for this case the Arrhenius type of equation adjusted to the results shown in Fig. 6B for the temperature range from 50 to 60°C is as follows:

$$K_d = 5.15 \times 10^{18} \exp(-29{,}615/RT) \quad r^2 = 0.9032 \tag{8}$$

The value of the energy of deactivation for free lipase (E_d = 29.22 kcal/mol) was practically equal to the PCB-lipase (E_d = 29.62 kcal/mol), indicating that the lipase is quite sensitive to denaturation temperatures higher than 50°C.

All enzymatic reactions yield data that can be analyzed in the framework of the Michaelis-Menten mechanism. To examine whether or not the rate of hydrolysis obeys this type of kinetics, a study was carried out by varying the proportion of olive oil in the substrate emulsion from 10 to

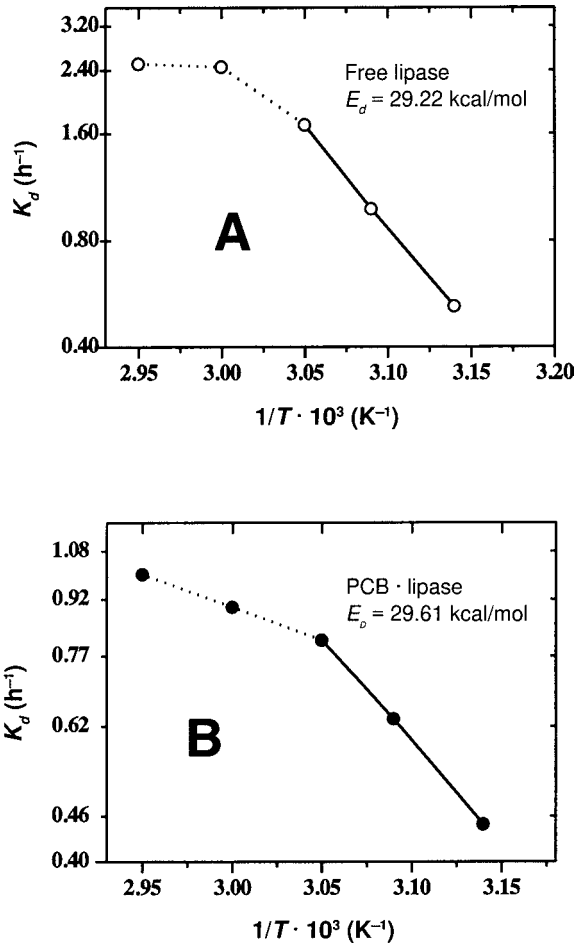

Fig. 6. Energy of deactivation for **(A)** free lipase and **(B)** PCB-lipase.

70% (v/v). This gave a concentration in fatty acids from 280 to 2000 m*M*. K_m and V_{max} values were determined from the Lineweaver-Burke plots derived from Fig. 7A and Fig. 7B, in which hydrolytic activities for free and immobilized lipase, respectively, are presented as a function of the substrate concentration. Values of V_{max} were 38.46 and 51 μmol/(mg·min) for free and immobilized lipase, respectively, while the K_m value for free enzyme was 0.42 m*M* and for the immobilized lipase was 0.15 m*M*. The results suggest that the activity of the free lipase as a function of the concentration of fatty acid follows kinetics of the Michaelis-Menten type, indicating that in the range studied, a possible inhibition by reaction products was not detected. For the case of the PCB-lipase, different behavior was observed and a slight reduction in the activity was detected for substrate concentration >50% (v/v). This can be indicative of substrate inhibition or diffusional resistance. We chose to study the immobilized lipase with 120 U/g of support since it showed the highest catalytic efficiency (Fig. 1);

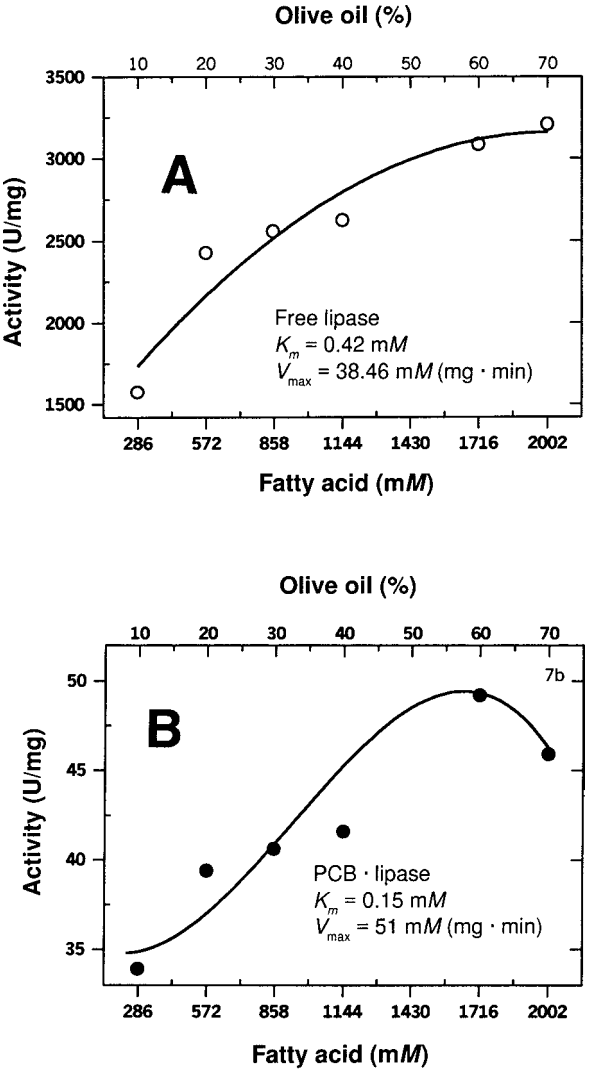

Fig. 7. Hydrolytic activities for **(A)** free lipase and **(B)** PCB-lipase as a function of substrate concentration at pH 7.0 and 37°C.

however, this preparation also has demonstrated the possible presence of mass transfer resistance, and, therefore, it should be emphasized that the kinetic parameters obtained with this preparation can be influenced by enzyme load, particle diameter, porous diameter, and so on. If we were interested in obtaining true intrinsic kinetic parameters of the immobilized lipase and not the ones at the highest catalytic efficiency, a lower enzyme load would have been used. Data of this nature in the literature are lacking; however, such data would help researchers access the relative importance of the phenomena that lower activity yield, such as external and internal mass transfer limitations, sterical hindrances, and conformational changes.

Table 3
Comparison of Physicochemical Properties
and Kinetics Parameters for Free and Immobilized Lipase

Parameter	Free lipase	PCB-lipase
Activity (U/mg)	3700	51.4
Water content (%)	5	20
Optimum pH	7.0	6.0
Optimum temperature (°C)	37	45
Energy of activation (kcal/mol)	9.90	7.26
Thermal inactivation constant (K_d, h^{-1}) at 50°C	0.98	0.63
Half-life (h) at 50°C	0.63	1.1
Energy of deactivation (kcal/mol)	29.22	29.62
K_m (m*M*)	0.42	0.15
V_{max} (μ*M*/[mg·min])	38.46	51

Table 3 summarizes the general characteristics of the free lipase and PCB-lipase.

Conclusion

Enzyme immobilization by physical adsorption traditionally refers to binding of the enzymes via weak attractive forces to an inert carrier that has not been chemically derivatized. Because the carrier is directly involved in binding to the enzyme, both morphologic and chemical characteristics play important roles. Of the two chitosan forms (flakes and porous beads), PCB showed the most favorable morphologic properties for the immobilization of *C. rugosa* lipase. It is likely that internal mass transfer occurred with both chitosan forms, but the flakes displayed the lowest catalytic activity yield. The methodology for immobilizing lipase on PCB by adsorption using hexane as the dispersion medium gave high protein retention (80%), and compared with other lipase immobilization methods, relatively high activity yield of 17% at lipase loading of 120 U/g of dry support. This is still low, however, in comparison with other enzymes and may reflect the presence of mass transfer limitations owing to multilayer enzyme load. The thermal stability of the immobilized lipase was higher than that for the free enzyme, although this effect may be a consequence of multilayer enzyme immobilization and mass transfer resistance. Kinetic parameters obtained based on the curve of the activity as a function of the substrate concentration (fatty acid) indicated that the free enzyme presents a kinetic of the Michaelis-Menten type, while the apparent kinetic behavior of PCB-lipase resulted in enzyme rates at higher substrate concentration, which may be a consequence of diffusional resistance limitations or the presence of inhibition by substrate. These facts need further study, particularly the relative contribution of different phenomena that reduce the yield of immobilized lipase activity.

Acknowledgments

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