0273-2289/88/1902—0163$02.60

Immobilization/Stabilization of Lipase from *Candida rugosa*

CHRISTINA OTERO, ANTONIO BALLESTEROS, AND JOSÉ M. GUISÁN*

Instituto de Catálisis y Petroleoquimica, CSIC, 28006 Madrid, Spain

Received July 1, 1988; Accepted July 20, 1988

ABSTRACT

With the aim of fixing the enzyme to the maxtrix by multiple covalent linkages, lipase from *Candida rugosa* (formerly *cylindracea*) has been insolubilized through its amino groups on Sepharose 6B previously activated with 2,3-epoxy-1-propanol. Two main variables that are known to control the number of bonds formed have been tested: the contact time between enzyme and activated support, and the temperature at which the immobilization reaction is carried out. Studies on activity and stability of the different derivatives prepared showed that higher temperatures and longer contact times lead to insolubilized enzymes that are more resistant to inactivation by temperature and the presence of organic solvents. At 50°C and pH 7.2, the insoluble lipase was found to be 140 times more stable than its soluble counterpart.

Index Entries: Glycidol-activated agarose; sepharose(aldehyde); lipase from *Candida cylindracea;* multipoint attachment, enzyme insolubilization by covalent; lipase insolubilization; activity-stability; organic solvents, effect of.

INTRODUCTION

Lipases (EC 3.1.1.3) comprise a group of enzymes whose biological function is to catalyze the hydrolysis and synthesis of triacylglycerols. They are of low cost and do not require water-soluble substrates (*1*). At present, there is an increasing interest in the development of applications

*Author to whom all correspondence and reprint requests should be addressed.

for these enzymes, particularly in the detergents, oils and fats, and dairy industries (*2*). Since the demonstration that enzymes can catalyze in organic solvents (*3*), lipases have received a growing attention in research, especially for synthetic purposes, taking advantage of their regio- and stereoselectivity of action. Their low specificity for substrates has even been exploited by Klibanov for synthesizing peptides (*4*).

Pancreatic lipase has been thoroughly studied by Desnuelle and colleagues (*5*), but microbial lipases are now becoming very popular because of their availability. The lipase from *Candida rugosa* (formerly *cylindracea*) is an interesting enzyme because of its high activity in hydrolysis as well as in synthesis; although it is positionally unspecific for triglycerides, however, it discriminates among different fatty acids (*6*). Therefore, stabilization of this enzyme against the harmful effects of temperature and organic solvents should greatly improve its possibilities as an industrial catalyst.

It is commonly accepted that enzyme-support multipoint attachment may exert an important stabilizing effect on the insolubilized enzyme derivatives (*7*). However, strategies for the preparation of enzymes linked by multicovalent bonds to supports have not been very successful. In a previous paper, we have proposed an adequate "immobilization/stabilization" system: the attachment of enzymes, through their amino groups, to monolayers of aldehyde groups built on the hydroxyl groups of agarose gels (*8*). This approach, which presents important advantages in order to get very intense, but not distortive, enzyme-support multipoint attachments, has been successfully applied to the stabilization—more than 1000 times—of tripsin (*9*) and penicillin G acylases (*10*); these studies have led us to establish that, within certain limits, the number of bonds formed increases with the surface density of active groups in the matrix, the contact time between enzyme and support, the pH, and the temperature.

In this paper, this strategy is applied to the stabilization of *C. rugosa* lipase. Because of our previous experience (*11*), this study has been carried out using a highly activated agarose gel and a pH of 10 (bicarbonate buffer) and investigating the influence of contact time between activated support and enzyme, and the temperature in the immobilization mixture. We have tested the activity and the temperature stability of the various insoluble derivatives prepared by selecting different combinations of these variables. The stability of the derivatives in the presence of organic solvents, and the influence of insolubilization on esterasic and lipasic activities, has also been addressed.

EXPERIMENTAL

Material

Lipase from *Candida rugosa* (formerly *cylindracea*) (Type VII, containing 700 U/mg solid, using olive oil as substrate), was purchased from Sigma. Sepharose CL-6B was from Pharmacia, *p*-nitrophenyl butyrate

and tributyrin were also from Sigma, and glycidol (2,3-epoxy-1-propanol) was from Merck.

Methods

Storage of the Native Enzyme

A weighed amount of the commercial preparation is dissolved at room temperature (ca. 20°C) in 0.1*M* phosphate buffer, pH 7.2, 0.1*M* NaCl (standard buffer) and left with gentle stirring. After 2 h, the mixture is centrifuged at 17,000×g during 15 min to discard the insoluble material, if any. This enzyme solution, when not used immediately, was stored at 4°C.

Preparation of Insolubilized Derivatives

ACTIVATION OF SEPHAROSE CL-6B. Glyoxyl–agarose gels, Ag–O–CH_2 CHO, were prepared by etherification with glycidol of agarose gels and further oxidation with periodate of the resulting glyceryl–agarose gels, Aga–O–CH_2–CHOH–CH_2OH, as previously described (*8*). With this methodology, Sepharose–aldehyde gels containing 76.5 μmol aldehyde/mL of gel were prepared. This activation of Sepharose 6B corresponds—taking 80 nm as average pore diameter and 25 m^2/cm^3 as specific surface area (*12*)—to 18 aldehyde residues/10 nm^2 of gel surface.

PREPARATION OF SEPHAROSE(ALDEHYDE)–(AMINE)LIPASE. A solution of lipase in standard buffer is diluted 1:1 with 0.2*M* Na_2CO_3–$NaHCO_3$ buffer, pH 10.0, the pH adjusted to 10.0, and the enzyme activity determined (A_o). To this enzyme solution, an equal volume of activated agarose gel was added. (A typical total volume for an immobilization mixture was 8 mL; and the amount of lipase used was always the same: 1.25 mg/mL of gel, well below the maximal loading of the activated support). The mixture was gently stirred and at different times, aliquots of the whole suspension and of the supernatant were withdrawn to determine the enzyme activity. After the desired contact time between enzyme and support, solid sodium borohydride (to a final concn. of 1 mg/mL of immobilization mixture) was added in order to reduce the Schiff's base formed; after 30 min of gentle stirring at room temperature, the insoluble enzyme derivative was washed with 0.1*M* phosphate buffer, pH 7.2, and with water. (In a thorough study, these conditions were found to be the best ones for the $NaBH_4$ reduction of tripsin(amine)–agarose(aldehyde) derivatives (*13*)).

Enzyme Assays

As standard assay in this work the hydrolysis of *p*-nitrophenyl butyrate (pNPB) has been followed graphically at 400 nm and 30°C in a Varian Cary 210 spectrophotometer provided with magnetic stirring. To increase the solubility of the substrate, a mixture containing, per mililiter, 0.05 μL of pNPB and 30 μL of acetonitrile, was prepared in standard buffer, and submitted to disruption in a Branson sonifier for 10 min. Three mL of this solution were deposited in the cuvet in the spectrophotometer, left 5 min,

and the reaction started with an appropriate amount (25–50 μL) of soluble or immobilized enzyme.

In some cases, the activity was also evaluated following the hydrolysis of tributyrin, in the absence of added emulsifier or acetonitrile, at 30°C and in 1 m*M* phosphate, 0.1*M* NaCl. The acid released was continuously titrated to pH 7.2 with the aid of a pHstat Radiometer model TTT80.

Thermal Stability

Samples of soluble and insolubilized lipase were incubated in various pH and temperature conditions. At different times, aliquots were drawn and the residual activity measured in the standard conditions (pH 7.2, 30°C, with pNPB).

RESULTS

Stability of the Native Enzyme in the Conditions of Insolubilization

Since the reaction between amine and aldehyde groups is reversible at neutral pH (*14*), immobilization on aldehydic supports through the amino groups of proteins must be carried out at alkaline pH. The number of covalent bonds formed between protein(amino) and agarose(aldehyde) increases substantially with pH between 7.5 and 10 (*11*). Furthermore, in preliminary experiments we found that this lipase only immobilizes on agarose–aldehyde gels at pHs higher than 9.5. Therefore, we carried out the immobilization reaction at pH 10. Figure 1 presents the stability of soluble lipase in bicarbonate buffer, pH 10.0, at various temperatures (0, 15, and 25°C). The stability drastically decreases when the temperature is raised from 0 to 25°C. After 5 h at 0, 15, and 25°C, the enzyme residual activity was 90, 55, and 20%, respectively. The curves are clearly multiphasic.

In order to improve the stability of the enzyme at this pH, these studies were repeated in the presence of substrates (or related compounds) of the lipase. No protecting effects were observed with butyric acid or butanol (*see* also below).

Immobilization–Multiinteraction of Lipase on Agarose–Aldehyde Gels

The time course of immobilization and further multiinteraction between lipase and agarose–aldehyde gels at pH 10.0 at either 15°C or 25°C, is presented in Fig. 2. In both cases, lipase immobilizes very quickly on

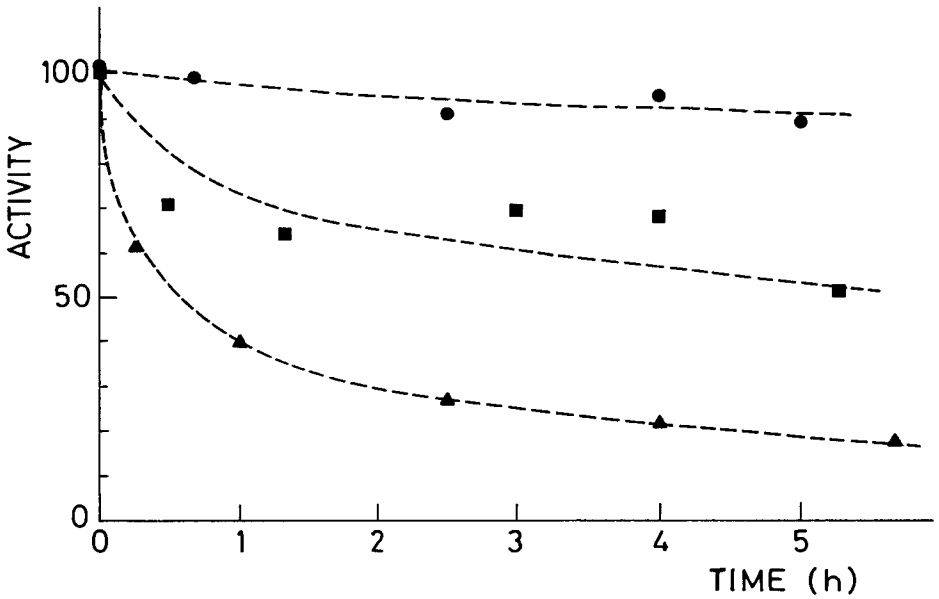

Fig. 1. Residual activity of soluble lipase vs time in 0.1*M* bicarbonate buffer, pH 10.0, at different temperatures: (●) O°C; (■) 15°C; (▲) 25°C.

this highly activated support. The activity in the supernatant disappears much faster than the activity of the soluble enzyme in the experiments of Fig. 1. The activity of the whole suspension decreases also very quickly as the enzyme immobilization proceeds, but a plateau of activity is reached, corresponding approximately to 30% of the activity of the starting soluble enzyme. This plateau remains practically constant when immobilization–multiinteraction is performed at 15°C and decreases very slowly when the temperature is 25°C. This means that insoluble derivatives with fair retention of activity can be prepared at 15 or 25°C. On the other hand, borate buffer is not a good reaction media at 25°C (*see* inset), since activity of the suspension decreased rapidly and approached zero after 3 h.

The decrease in catalytic activity of the immobilized lipase is produced during the first period of the contacting process, in which one covalent bond between support and enzyme is formed. After this, the immobilized enzyme results are much more stable at the alkaline pH than its soluble counterpart. As the contact time increases, multiple bonds are favored, and this multiinteraction seems not to effect the catalytic activity of the immobilized enzyme.

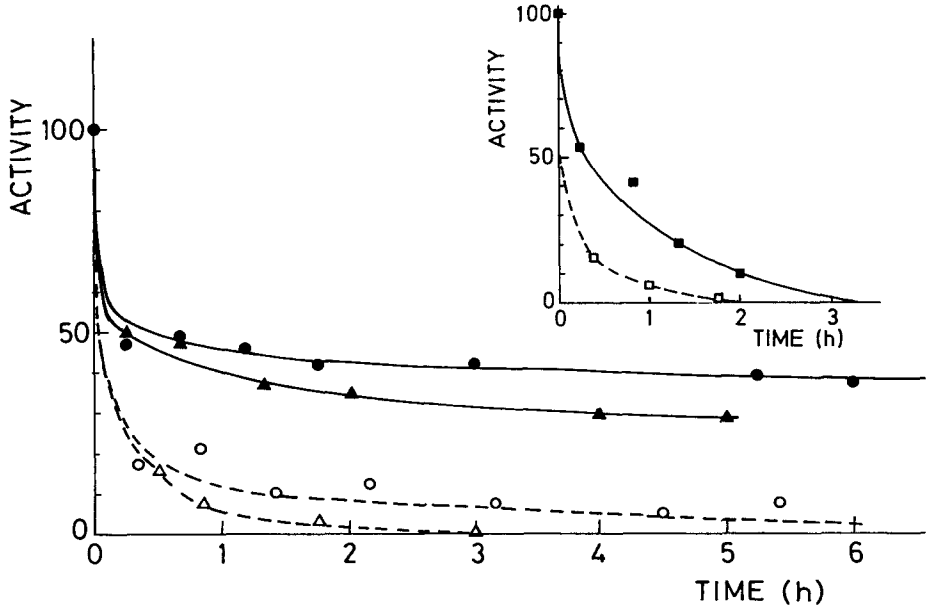

Fig. 2. Time course of the immobilization reaction. Closed symbols, activity in the whole suspension; open symbols, id. in the supernatant. Main figure: In 0.1*M* bicarbonate buffer, pH 10.0; circles, 15°C; triangles, 25°C. inset: In 0.1*M* borate buffer, pH 10.0; 25°C.

Properties of Agarose–Lipase Derivatives

We have prepared different lipase-agarose derivates by using different combinations of the two variables tested in this paper (temperature, and contact time between enzyme and activated support). The experimental conditions used for the preparation of the different derivatives are given in Table 1.

Activity

The catalytic efficiency (defined as the ratio of the specific activity of the insolubilized enzyme to that of the soluble one) can be calculated by the ratio A_{imm}/A_o, where A_o is the activity before starting the immobilization, and A_{imm} the activity of the immobilized enzyme. A_{imm} has been evaluated by A_{sus}-A_{sup}, when A_{sus} and A_{sup} are, respectively, the activity measured in the suspension and in the supernatant. In the case of higher temperatures and/or contact times, the catalytic efficiency values had to

Table 1
Properties of the Insolubilized Derivatives

Derivative[a]	Enzyme immobilized, %	Catalytic efficiency, %
IL-1-0	89	15
IL-1-15	95	21
IL-1-25	95	32
IL-5-0	89	16
IL-5-25	96	24
IL-24-15	100	30
ILB[b]-2-25		10
ILB[b]-5-0		32

[a]The first figure in this notation refers to the contact time (h); the second, to the temperature (°C).
[b]These derivatives have been obtained in borate buffer instead of bicarbonate.

be corrected for the enzyme inactivation. However, since the enzyme immobilizes much faster than inactivates and becomes drastically stabilized after the first stages of the immobilization/multiinteraction process, and since 90–100% of the starting enzyme was immobilized in all experiments, the corrections were small. The catalytic efficiency (Table 1) is not very different (15–32%) for all derivatives. Therefore, this loss of catalytic activity seems to be not very dependent on the different experimental conditions in which derivatives were prepared and, hence, on the intensity of the enzyme support multiinteraction in this range of experimental conditions. At short contact times, the efficiency increases with temperature. At 25°C, the efficiency diminishes with time.

On the other hand, the activity of the immobilized derivatives did not change when borohydride was added after the desired contact time. Apparently, the mild conditions in which reduction was carried out did not exert any deleterious effect on the structure of immobilized lipase.

Thermal Stability

The storage stability of soluble and insolubilized lipase at 50°C in 0.1*M* phosphate buffer, pH 7.2, 0.1*M* NaCl, has been studied. The plots of residual activity vs storage time adjust quite well to first-order kinetics (results not shown), and allow to calculate the half-lives of inactivation (*see* Table 2). In general, "stabilization" increases with temperature and contact time. The best derivative obtained, prepared at 25°C, and 5 h of multiinteraction enzyme support, was 142-fold more stable than the soluble enzyme. Hence, although activity of the derivatives seems to be not very sensitive to the intensity of the process of enzyme-support multiinteractions, stability increases as the intensity of this process does.

Table 2
Storage Stability of Lipase at 50°C and pH 7.2

Enzyme	Half-life, h	Stabilization factor
Soluble	0.5	1
IL-1-0	25	50
IL-1-25	60	120
IL-5-25	71	142
ILB-2-25	35	70

Effect of Organic Solvents on Activity and Stability

It is anticipated that the multipoint binding between enzyme and support, responsible for the enhanced thermal stability of the insoluble derivatives, also will provide a better stability against the deleterious effects of organic compounds. Because of the use of tributirin as a model triacylglycerol for evaluating lipasic activity, possible substrates or inhibitors (i.e., butanol and butyric acid) have been investigated. In addition, other common solvents have been tested.

The influence of butanol on the esterasic activity for native lipase and for derivatives (IL-1-15 and IL-1-25, is shown in Fig. 3. The activity is enhanced at low concentrations of alcohol, reaches a maximum—at 1.25% in the three cases—and then decreases. However, the enhancement of activity becomes more pronounced as the "rigidity" (thermal stability) of lipase increases. Thus, maximal activities of 151% (with respect to the absence of butanol) are reached for soluble enzyme, 380% for derivative IL-1-25 (120-fold stabilized), and 190% for IL-1-15 (of intermediate stabilization). Thermally stabilized agarose-lipase derivates result more resistant than soluble lipase to the harmful effect of high concentrations of butanol. Thus, at 7.5%, the soluble enzyme retains only 20% of activity, whereas the more stable derivative (IL-1-25) retains 115%.

Low concentrations of butyric acid produce an enhancement of catalytic activity in soluble lipase, but not in the immobilized/stabilized derivatives (Fig. 4). However, at higher concentrations, the native enzyme suffers an important loss of catalytic activity: 50% of residual activity is reached at 7% butyric acid, a concentration at which the insoluble derivatives remain fully active. Now, as in the case of butanol, immobilized/estabilized lipase is more resistant than soluble enzyme to the deleterious effects of butyric acid.

When the study was extended to other organic media, such as ethanol, acetonitrile, and dioxan, no increase of activity was observed in any case. High concentrations of organic solvents yield loss of catalytic activity,

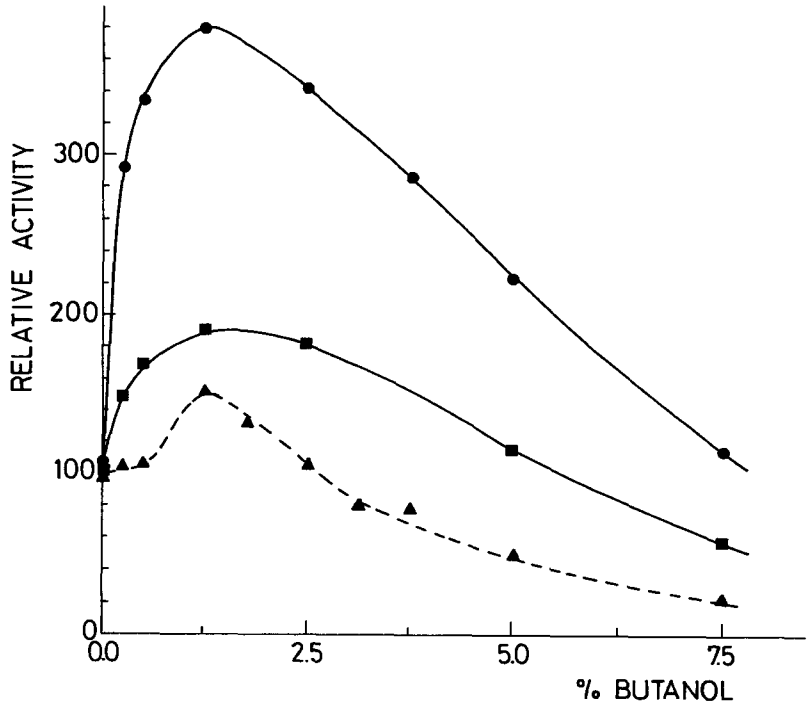

Fig. 3. Activity of lipase as a function of percent butanol (v/v) in the reaction mixture for the standard assay. (▲) soluble enzyme; (■) derivative IL-1-15; (●) derivative IL-1-25.

more intense in the soluble enzyme than in agarose-lipase derivatives (Table 3).

Finally, the influence of the optimal butanol concentration (1.25%) in the stability of our enzyme preparations, was checked. At 50°C and pH 7.2, the native enzyme is equally stable with or without butanol, but derivative IL-5-25, in the presence of butanol, has a stabilization factor of about half (75-fold). Furthermore, butanol does not exert any stabilizing effect on soluble lipase at the alkaline pHs required for immobilization on agarose(aldehyde) gels; on the contrary, the stability in 0.1*M* bicarbonate buffer, pH 10.0, containing 1.25% butanol, decreases an order of magnitude (data not shown).

Correlation Between Esterasic and Lipasic Activity

The effect of immobilization on lipasic (measured with tributirin) and esterasic (determined with pNPB) activities in our preparations, was compared. Although soluble lipase had a ratio $A_{pNPB}/A_{tributirin}$ of 90 (au), this

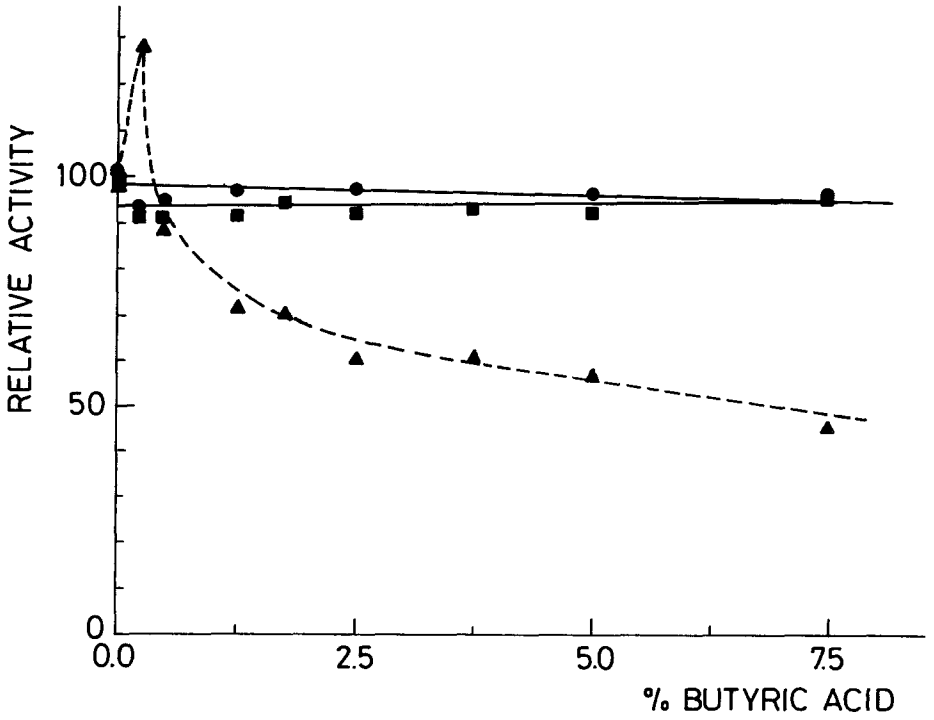

Fig. 4. Activity of lipase as a function of percent butyric acid in the standard assay mixture. (▲) soluble enzyme; (●) derivative IL-1-15; (■) derivative IL-1-25.

Table 3
Activity of Lipase in the Presence of Organic Solvents

Organic solvent	Enzyme	% solvent yielding 50% activity
Ethanol	Soluble	12.5
Ethanol	IL-1-25	21.2
Butanol	Soluble	5.0
Butanol	IL-1-15	7.5
Butanol	IL-1-25	—[a]
Acetonitrile	Soluble	11.2
Acetonitrile	IL-1-25	15.0
Dioxan	IL-1-25	22.0

[a] At 7.5% butanol, this derivative still maintains 115% activity (at higher concentrations, butanol is inmiscible).

figure decreased upon immobilization (i.e., the corresponding value for derivative IL-1-25 was 50).

DISCUSSION

In the insolubilization of enzymes on a support, it is important that the activation method employed can yield an activated matrix with a broad range of activation degrees: a support with very low activation would yield enzyme insolubilized through a single covalent bond, whereas with the most activated ones the protein will be bound through multiple linkages. In this study, we have selected a highly activated matrix: 18 aldehyde group/10 nm^2 (this is the cross-sectional area of a small protein of dimensions $3 \times 3 \times 4$ nm and mol wt 16,800 (*15*)). Since the lipase from *C. rugosa* is a big glycoprotein of mol wt about 120,000 (*16,17*), with this well activated support the enzyme could theoretically be bound through many linkages.

Kimura et al. (*18*) have previously studied the immobilization of this lipase with photo-crosslinkable prepolymers and also by insolubilization on glass. The multibonded insoluble derivatives in the present work have been prepared by varying contact time and temperature—and also by varying the nature of the buffer—present very different properties. The insolubilization of lipase on this highly activated support is very rapid during the first period—characterized by a sharp decrease in activity (Fig. 2). During subsequent periods, more bonds between matrix and protein are formed yet the enzymatic activity does not change substantially. After reduction with borohydride, the activity of the various derivatives was practically identical to that measured during the immobilization (ranging from 15 to 32% of the activity of the soluble enzyme that had been immobilized). Rigid derivatives, i.e., those in which the interaction between enzyme and support has been favored by longer time or higher temperature, have similar or higher activity, and higher stability. It appears that the loss of catalytic activity is produced during the first stage of the immobilization-multiinteraction process. After that, the possible increase of the enzyme-support multiinteraction (the derivatives become more stable) does not seem to affect the catalytic activity. In previous studies conducted in our laboratory with trypsin (*9*) and penicillin G acylase (*10*)—enzymes that in their native state show a higher stability than this lipase —it was found that very intense enzyme-support multipoint attachments preserved 100% of the catalytic activity of the soluble enzymes. In this case, the catalytic action of the enzyme might involve a certain flexibility of the protein molecule around the active site. Thus, even a small degree of "rigidity" may induce a significant decrease in catalytic activity. A further increase of the enzyme-support multipoint attachment improves the rigidity of the overall tridimensional structure, but does not exert additional concrete effects in the region of the active center.

The insoluble lipase prepared by our methodology is very stable at a fairly high temperature (50°C) and neutral pH (for example, derivative IL-5-25 was 142-fold more stable than native lipase). Another interesting effect of this immobilization is the decrease observed in the ratio of esterasic and lipasic activities (from 90 to 50). Changes in specificity and/or selectivity of enzymes upon insolubilization have been previously found (*19*).

The very rigid derivatives obtained by this procedure resisted well the deleterious action of several water-miscible solvents. This stabilization may be important in order to exploit, is selected reactions, the relaxation of enzyme specificity that is observed in the presence of organic solvents (*20*). In addition, butanol exerted as important promoting effect on the initial esterasic—but not the lipasic—activity of the soluble as well as on the insolubilized lipase. This enhancement of the activity was found to be more pronounced in derivatives where the process of multiinteraction between enzyme and support was more intensive.

ACKNOWLEDGMENTS

We thank V. M. Fernández for helpful comments and critical reading of the manuscript. This research has been supported by a postdoctoral Fellowship from the Spanish MEC (to CO) and by a grant (BT85-43) from the CAICYT.

REFERENCES

1. Borgstrom, B. and Brockmam, H. L., eds. (1984), *Lipases*, Elsevier, Amsterdam.
2. Macrae, A. R. and Hammond, R. C. (1985), *Biotechnology and Genetic Engineering Reviews* **3,** 193.
3. Zaks, A. and Klibanov, A. M. (1985), *Proc. Natl. Acad. Sci. USA* **82,** 3192.
4. Margolin, A. L. and Klibanov, A. M. (1987), *J. Amer. Chem. Soc.* **109,** 3802.
5. Desnuelle, P. (1972), *The Enzymes*, 3rd ed., vol. 7, Boyer, P. D., ed., Academic, New York, pp. 575-616.
6. Benzonana, G. and Esposito, S. (1971), *Biochim. Biophys. Acta* **231,** 15.
7. Klibanov, A. M. (1982), *Adv. Appl. Microbiol.* **29,** 1.
8. Guisán, J. M. (1988), *Enzyme Mic. Technol.* **10,** 375.
9. Blanco, R. M. and Guisán, J. M. (1988), *Enzyme Mic. Technol.* **10,** 227.
10. Guisán, J. M., Blanco, R. M., and Alvaro, G. (1987), *Proc. 4th European Congress on Biotechnology*, vol. 2, Neijssel, O. M., van der Meer, R. R. and Luyben, K. Ch. A. M. eds., Elsevier, Amsterdam, pp. 97-100.
11. Blanco, R. M., Calvete, J. J., and Guisán, J. M. (1988), *Enzyme Mic. Technol.*, in press.
12. Guisán, J. M., Serrano, J., Melo, F. V., and Ballesteros, A. (1987), *Appl. Biochem. Biotechnol.* **14,** 49.
13. Blanco, R. M. and Guisán, J. M. (1988), *Enzyme Mic. Technol.*, in press.

14. Morrison, R. J. and Boyd, R. N. (1973), *Organic Chemistry,* Allyn and Bacon, Boston.
15. Anfinsen, C. B., Cuatrecasas, P., and Taniuchi, H. (1971), *The Enzymes,* vol. 4, Boyer, P. D., ed., Academic, New York, pp. 177–204.
16. Tomizuka, N., Ota, Y., and Yamada, K. (1966), *Agr. Biol. Chem.* **30,** 576.
17. Tomizuka, N., Ota, Y., and Yamada, K. (1966), *Agr. Biol. Chem.* **30,** 1090.
18. Kimura, Y., Tanaka, A., Sonomoto, K., Nihira, T., and Fukui, S. (1983), *Eur. J. Appl. Microbiol. Biotechnol.* **17,** 107.
19. Guisán, J. M. and Ballesteros, A. (1981), *Enzyme Mic. Technol.* **3,** 313.
20. Nasri, M. and Thomas, D. (1986), *Nucl. Acids. Res.* **14,** 811.