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# Lipase activity and conformation in neat organic solvents

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**Abstract**

The methods commonly employed to improve the catalytic efficiency and to determine the conformation of lipases in organic solvents are briefly examined and discussed. Special attention is dedicated to the properties of different formulations of lipase from *Pseudomonas cepacia* (lipase PC) and lipase B from *Candida antarctica* (CALB). Non-covalent lipases/PEG complexes are particulary interesting because they are easy to prepare, very active and soluble in suitable organic solvents. This last property makes it possible to carry out conformational studies of lipases by circular dichroism and intrinsic protein fluorescence. Moreover, correlations between enzyme activity and solubility in organic solvents, shed light on the effects of diffusional limitations and lipase conformational changes on the catalytic efficiency of enzymes in organic solvents.


*Keywords:* Lipases; Activity; Conformation; Organic solvents

## 1. Introduction

Enzymes in organic solvents have been largely studied and employed in the areas of synthesis, food and even analysis [1–4]. The use of organic solvents is especially advantageous to transform substrates that are unstable or poorly soluble in water. Furthermore, at low water activity many side-reactions that are water dependent can be prevented, including the denaturation of enzymes which, in organic media, show higher thermal stability. In absence of water, the synthesis by hydrolases (mainly lipases and proteases) of ester and amide bonds can be favored over hydrolysis. By varying the organic solvent it is also possible to control the substrate specificity and the regio- and enantioselectivity of a given enzyme [5]. However, although enzymes in organic media show numerous advantages, their catalytic efficiency is, in most cases, orders of magnitude lower than in aqueous systems. This behavior can be ascribed to different causes such as diffusional limitations, high saturating substrate concentrations, restricted protein flexibility, low stabilization of the enzyme–substrate intermediate and even partial enzyme denaturation by lyophilization which becomes irreversible in anhydrous solvents [6].

This paper aims to briefly review selected studies carried out to improve the activity of enzymes in pure organic solvents and, where possible, to correlate the activity with their conformation. Particular emphasis will be dedicated to the studies that have been done with lipase from *Pseudomonas cepacia* (lipase PC) and lipase B from *Candida antarctica* (CALB). These studies may shed light on the effects that are responsible for the lower activity of enzymes in organic solvents, in particular on diffusional limitations and conformational changes of the enzyme.

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### *1.1. Hydrolase formulations and activity in organic solvents*

Biocatalysis in organic solvents generally refers to those systems in which the biocatalyst is suspended in neat organic solvents. Such systems are different from the biphasic ones or from reverse micelles, in which the hydrophobic substrates are partitioned in the organic phase whereas the enzyme is dissolved in bulk water (biphasic systems) or in micropools of water (reverse micelles). When suspended in dry organic solvents only a minimal amount of water (less than 5% v/v), that acts as "lubricant", is necessary to maintain the dynamical properties of the enzyme [1,7]. In their pioneering studies, Zaks and Klibanov [7,8] utilized, for catalysis in organic solvents, lyophilized enzyme powders coming from buffered solutions where the pH was adjusted to the biocatalyst optimal value. Since then, there has been an evolution in the formulation of biocatalysts that has led to the preparation of numerous catalysts with high catalytic efficiency and stability in organic solvents.

An easy and fast method is to immobilize enzymes by adsorption on porous supports. To this end, numerous inorganic materials (Celite, silica, aluminas, zirconia, controlled-pore glasses) and polymers (polyamides, polypropylene, polyacrylates) [9–12], with different porosity and bead size, have been used. In this procedure, particular attention has to be dedicated to the optimization of the enzyme loading (weight of enzyme/weight of support) [10,13,14]. In fact, a low enzyme loading can be deleterious for biocatalyst stability because of the strong interactions with the matrix [15,16]. On the contrary, a high loading leads to multi layers of enzyme molecules, which decreases the accessibility of the substrate to the individual enzyme active site and, as a consequence, the specific activity of the biocatalyst. Besides adsorption, the entrapment of enzymes in sol–gel materials represents another immobilization procedure particulaly interesting for lipases. Reetz and coworkers have prepared lipases entrapped in different sol-gels, where the nature of the silane monomers and the water/silane stoichiometry were optimized producing immobilized lipases with excellent specific activity and stability [17]. The increased activity of enzymes observed with adsorption and entrapment procedures has been ascribed to reduced diffusional limitations. In fact, enzyme molecules adsorbed on a surface or entrapped in a rigid tridimensional net of sol–gel materials are more accessible than those present in a particle of lyophilized powder. In the last case, the substrate has to diffuse through a solid phase to reach the active sites of the enzyme molecules present in the deeper layers of the aggregate [18]. Concerning the catalytic constants of adsorbed enzyme, studies carried out by us evidenced that, at the same water activity value ($a_w$), the apparent $V_{max}$ of lipase PC immobilized on Celite was up to 10 times higher than that of the crude powder. On the contrary, the apparent $K_M$ for the nucleophile was not significantly different for the two forms and increased similarly as a function of the $a_w$ value (water competes with the alcohol for the acyl enzyme) [10].

A different approach to improve the activity of enzymes in organic solvent is based on the use of excipients [19–25] or salts [26] added to the aqueous enzyme solution before lyophilization. These additives can improve the activity by different mechanisms. Additives such as competitive inhibitors or substrate analogues (removed after lyophilization by anhydrous extraction) may cause a so-called "imprinting" effect [19,20]. This means that the conformational changes of the enzyme active site, induced by interactions with these ligands, are retained in organic solvents because of the enhanced enzyme rigidity. A lyoprotective effect is obtained by means of additive such as sugars (sucrose, sorbitol, etc.) [21], poly(ethylene glycol) [22,23] and crown ethers [24,25], which should prevent conformational changes of the protein during the lyophilization process.

A possible explanation for the activation effects observed with subtilisin Carlsberg and lipase from *Mucor javanicus* lyophilized with potassium chloride (98% w/w KCl, 1% phosphate buffer, 1% enzyme) is the increase of enzyme active site polarity that stabilizes the transition state. The polarity of the active site should be increased by the high polarity of salt ions and/or by the water retained in the immediate environment of the catalytic active site [27].

Another way to obtain enzymes highly stable in organic solvents is that based on cross-linked enzyme crystals (CLECs), where the cross linking among the enzyme molecules of the crystal is done by glutaraldheyde. A variation of this method is that proposed

by Sheldon and coworkers, who have cross-linked enzyme aggregates (CLEAs) instead of crystals [28].

The effectiveness of various procedures mentioned in this paper to improve the catalytic efficiency of enzymes in organic solvents, has been investigated and compared by us using lipase PC (Fig. 1) and CALB (Fig. 2). The study has been carried out in different organic solvents and at different $a_w$ values; the alcoholysis of vinyl butyrate (lipase PC) or vinyl acetate (CALB) with 1-octanol was used as model reaction, and both the transesterification and hydrolytic initial rates were determined. The results reveal that some formulations of lipases, in particular lipase PC entrapped in sol–gel (Sol-Gel-AK-PC),

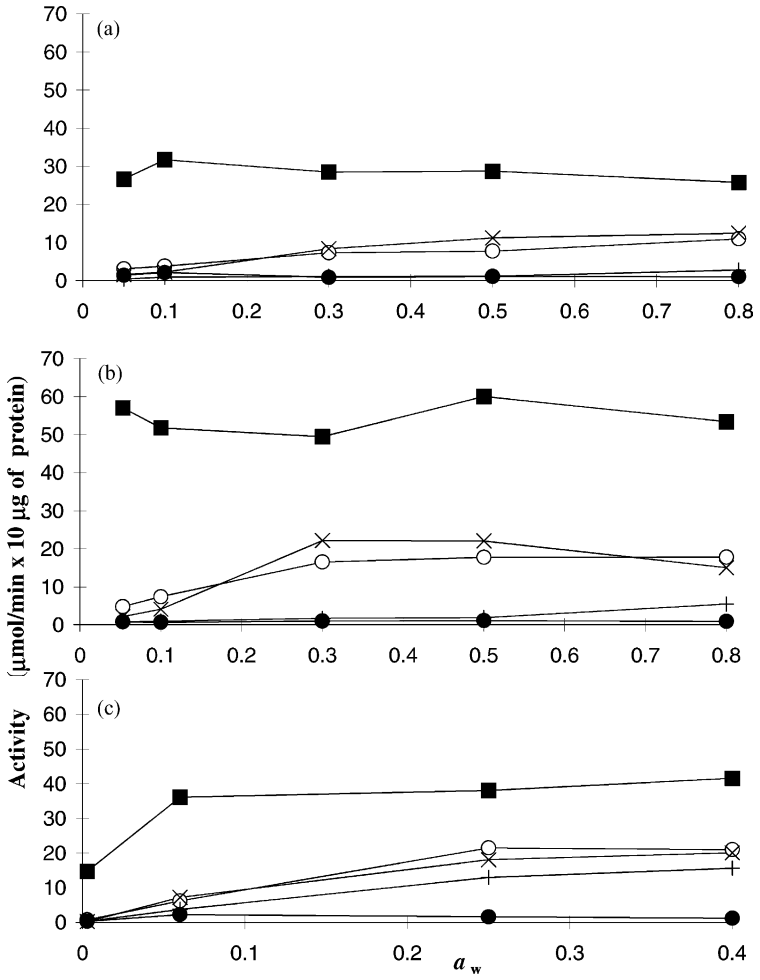


Fig. 1. Total activity (transesterification plus hydrolysis) of Sol-Gel-AK-PC (■), PEG + PC (○), PEG-PC (×), crude PC (+) and CLEC-PC (●) in benzene (a), carbon tetrachloride (b) and 1,4-dioxane (c). Previous to mixing, benzene (or carbon tetrachloride), vinyl ester, enzyme and substrate were separately adjusted to the desired water activity ($a_w$) in sealed containers for at least 2 days, at 25 °C, with the vapour phase of saturated salt solutions of known water activity [47]: LiCl ($a_w$ 0.11), $MgCl_2$ ($a_w$ 0.33), $Mg(NO_3)_2{\cdot}6H_2O$ ($a_w$ 0.53) and KCl ($a_w$ 0.84). For $a_w$ <0.1, the samples were equilibrated against molecular sieves [48]. In the case of 1,4-dioxane, the enzyme, the vinyl ester and the substrate were equilibrated against molecular sieves and added to dioxane containing a water concentration of 0.028 M, 0.55, 2.75 or 5.5 M which correspond to $a_w$ values, respectively 0.003, 0.06, 0.25 and 0.4 [49]. (Due to the high amount of water needed to affect the $a_w$ value in 1,4-dioxane we disregarded the contribution of the water present in the enzyme, vinyl ester and substrate.) For further experimental details see [22].

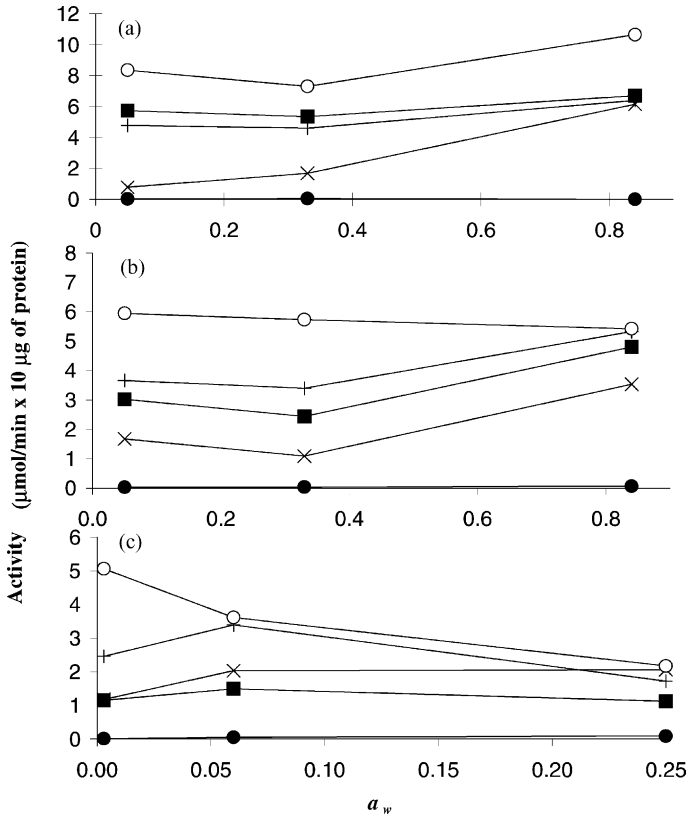


Fig. 2. Total activity of CALB + PEG (○), Novozym 435 (■), CALB + OA (+), purified CALB (×) and crude CALB (●) in toluene (a), carbon tetrachloride (b) and dioxane (c). Water activity was adjusted as reported in Fig. 1. For further experimental details see [23].

lipase PC covalently linked to methoxypoly(ethylene glycol) (PC–PEG) or simply co-lyophilized with PEG (PC + PEG) or, in the case of CALB, the lipase co-lyophilized with PEG (CALB + PEG) showed very high total activity values (transesterification plus hydrolytic activity) in organic solvents. In fact, these lipase formulations have a specific activity in organic media of the same order of magnitude of that showed in aqueous buffer (Tables 1 and 2).

A comparison of the ratios of transesterification over hydrolytic activity in organic solvents is shown in Table 3. We have found that lipase PC crystallized and cross-linked (CLEC-PC) and PC + PEG have a transesterification/hydrolysis ratio which is up to three times higher than that obtained with crude PC. An analogous result has been reported by Adlerkreutz [9] who has found that, at the same $a_w$, a higher alcoholysis/hydrolysis ratio is obtained with α-chymotrypsin immobilized on the polyamide support Accurel PA6 compared to that obtained with the same enzyme immobilized on Celite. These data prove that the choice of the support is important not only for the improvement of the total catalytic efficiency of the enzyme, but also for the final yield obtained in ester synthesis.

### *1.2. Conformational studies of enzymes in organic solvents*

Although organic solvents might resemble the hydrophobic microenvironment present in living organisms (e.g. biological membranes), undoubtedly they are not natural media for enzymes. Thus, it is logical to consider conformational changes of the enzyme molecule as possible cause for the lower catalytic efficiency observed in organic solvents compared to that in aqueous solutions. To clarify this point, several

Table 1
Transesterification and total activity in carbon tetrachloride over hydrolytic activity in aqueous buffer of lipase PC[a]

| Enzyme form | Transesterification activity in organic solvent[b]/hydrolytic activity in aqueous buffer[c] | Total activity in organic solvent[d]/hydrolytic activity in aqueous buffer[c] |
|---|---|---|
| Sol-Gel-AK-PC[e] | 0.83[f] | 1.3[g] |
| PC + PEG[h] | 0.26[g] | 0.39[i] |
| PC–PEG[j] | 0.32[g] | 0.48[k] |
| CLEC-PC[l] | 0.02[g] | 0.02[g] |
| Crude PC[m] | 0.04[i] | 0.12[i] |

[a] The activities in organic solvent and aqueous buffer were referred to the same amount of lipase protein.
[b] Transesterification activity was measured at 25 °C in organic solvent using octanol (0.19 M) as nucleophile and vinyl butyrate (0.79 M) as acyl donor.
[c] The hydrolytic activity in potassium phosphate buffer (0.05 M, pH 7) was determined at 25 °C using tributyrin as substrate (13.3 μl/ml).
[d] Total activity is the transesterification plus the hydrolytic activity occurring in organic solvent. The hydrolytic activity in organic solvent was estimated measuring the amount of butyric acid formed in parallel with the transesterification reaction.
[e] Lipase PC entrapped in sol–gel.
[f] Transesterification and total activity measured at $a_w$ 0.11.
[g] Transesterification and total activity measured at 0.53.
[h] Lyophilized with PEG.
[i] Transesterification and total activity measured at 0.84.
[j] Covalently linked to PEG.
[k] Transesterification and total activity measured at 0.33.
[l] Crystallized and cross-linked.
[m] As commercialized by Amano.

Table 2
Transesterification and total activity in toluene over hydrolytic activity in aqueous buffer of CALB[a]

| Enzyme form | Transesterification activity in organic solvent[b]/hydrolytic activity in aqueous buffer[c] | Total activity in organic solvent[d]/hydrolytic activity in aqueous buffer[c] |
|---|---|---|
| CALB + PEG[e] | 0.51[f] | 0.70[g] |
| Novozym 435[h] | 0.29[f] | 0.44[g] |
| CALB + OA[i] | 0.26[f] | 0.42[g] |
| Purified CALB[j] | 0.16[g] | 0.40[g] |
| Crude CALB[k] | 0.003[l] | 0.004[l] |

[a] The activities in organic solvent and aqueous buffer were referred to the same amount of lipase protein.
[b] Transesterification activity was measured at 25 °C in organic solvent using octanol (0.19 M) as nucleophile and vinyl acetate (1.1 M) as acyl donor.
[c] The hydrolytic activity in potassium phosphate buffer (0.05 M, pH 7) was determined at 25 °C using vinyl acetate as substrate (100 μl/ml).
[d] Total activity is the transesterification plus the hydrolytic activity occurring in organic solvent. The hydrolytic activity in organic solvent was estimated measuring the amount of acetic acid formed in parallel with the transesterification reaction.
[e] CALB lyophilized with PEG.
[f] Transesterification and total activity measured at $a_w$ <0.1.
[g] Transesterification and total activity measured at 0.84.
[h] As commercialized by Novo-Nordisk.
[i] Lyophilized with oleic acid.
[j] Purified from crude CALB.
[k] As commercialized by Novo-Nordisk (experimental product SP 525).
[l] Transesterification and total activity measured at 0.33.

Table 3
Ratios of transesterification over hydrolytic activity for the various lipase PC forms in different organic solvents at $a_w$ <0.1[a,b]

| Solvent | PC + PEG | PC–PEG | Crude PC | CLEC-PC | Sol-Gel-AK-PC |
|---|---|---|---|---|---|
| Carbon tetrachloride | 4.4 | 3.9 | 1.5 | 8.4 | 2.0 |
| Benzene | 9.9 | 5.0 | 2.6 | 8.7 | 2.9 |
| 1,4-Dioxane | 9.7 | 4.1 | 2.0 | 7.6 | 9.3 |

[a] For abbreviations see legend in Table 1.
[b] Transesterification activity was measured at 25 °C in organic solvents using octanol (0.19 M) as nucleophile and vinyl butyrate (0.79 M) as acyl donor. The hydrolytic activity in organic solvents was estimated measuring the amount of butyric acid formed in parallel with the transesterification reaction.

studies have been carried out to analyze the structure of proteins in pure organic solvents. The X-ray crystal structure of cross-linked enzyme crystals of subtilisin in acetonitrile, as well of γ-chymotrypsin in hexane, has demonstrated that the three-dimensional structure of these enzymes in organic solvents does not show significant differences compared to that observed in aqueous solutions [29,30]. Desai and Klibanov [31], using high-resolution NMR spectroscopy and monitoring the hydrogen isotope exchange of bovine pancreatic trypsin inhibitor, found that acetonitrile, tetrathydrofuran, ethyl acetate and butanol do not cause significant conformational changes. Although the native conformation is not thermodynamically favored in non-aqueous media, the enzyme does not undergo unfolding likely because of kinetic barriers [7]. In fact, the absence of water causes a decrease of protein flexibility. In hydrosoluble organic solvents-water mixtures, or in water immiscible solvents at high $a_w$ values, proteins become more flexible but less stable [1,32,33].

If the conformation of protein powders does not seem to be affected by organic solvents, the lyophilization process utilized to prepare dry enzymes for catalysis in organic media can modify their secondary structure. The FT-IR data obtained with lipase PC and CALB powders show that the lyophilization process causes a marked change in the secondary structure of both enzymes (Table 4) [34]. In particular, the α-helix content decreases, whereas the β-sheets content increases. These data are in agreement with those obtained by Griebenow and Klibanov [35] who have found that lyophilization induces similar variations of the secondary structure of several other proteins. However, when a protein, e.g. bovine pancreatic trypsin inhibitor, is co-lyophilized with sorbitol, the β-sheets structure and, at a lesser extent, the α-helix content are similar to that of the protein dissolved in aqueous solution. We too have found a very high similarity between the secondary structure of lipase PC lyophilized with PEG and that of the same enzyme dissolved in aqueous buffer (Table 4). Santos et al. [25] have ascribed the increase of activity observed in organic solvents for subtilisin Carlsberg lyophilized with crown ethers (18-crown-6 or 15-crown-5), to the fact that the enzyme has a more native-like structure in these conditions than when lyophilized from buffer alone. It should be emphasized that protein conformational modifications induced by lyophilization are generally reversible when the powder is redissolved in water, whereas they are irreversible when it is suspended in organic media, especially at low water activity.

We have found that PEG not only preserves protein conformation during the lyophilization process (lyoprotective effect), but can also dissolve (up to 0.2 mg/ml) enzymes such as lipase PC, CALB and subtilisin in several organic media [36,37]. Non-covalent enzyme + PEG complexes are prepared by mixing the enzyme solution with a PEG solution to obtain the desiderated PEG/protein ratio; then the resulting

Table 4
Secondary structure percentages of lipases determined by FT-IR

| Proteins | α-Helix (%) | β-Sheets (%) |
|---|---|---|
| CALB | | |
| Aq. Sol. | 33 ± 1 | 15 ± 1 |
| Lyophilized | 12 ± 1 | 37 ± 1 |
| Lipase PC | | |
| Aq. sol. | 33 ± 2 | 18 ± 1 |
| Lyophilized | 22 ± 2 | 28 ± 1 |
| Colyophilized with PEG | 31 ± 2 | 19 ± 1 |

solution is quickly frozen and lyophilized. This approach is much simpler than that previously reported by Inada and co-workers [38] and by us [39] in which PEG with a molecular mass of 5000 Da was covalently linked to enzymes. For comparison reasons, enzyme + PEG was prepared with PEG having the same molecular mass. It is worth pointing out that some solvents such as dimethylsulfoxide, formamide, 2,2,2-trifluoroethanol or 2,2,2-trichloroethanol can dissolve proteins but they also cause their unfolding [40–42].

By means of fluorescence and circular dichroism (CD) spectroscopy, we have investigated the conformation of lipases + PEG complexes in dioxane [34]. Far UV-CD spectra have shown that lipase PC has the same secondary structure in dioxane and in aqueous buffer (Fig. 3). Moreover, the CD signal intensity at 220 nm as function of the PEG/lipase ratio (w/w) has demonstrated that complete dissolution of the enzyme can be obtained at a ratio higher than 150 (Fig. 4). The comparison of the intrinsic fluorescence data (Table 5) of PC + PEG and *N*-acetyl-tryptophan ethyl ester in dry dioxane and in water suggests that the enzyme preserves a native conformation in the organic medium. In fact, for PC + PEG neither increase of intrinsic fluorescence intensity nor shift of the $\lambda_{max}$ of the emission (329 nm) take place in dioxane. By contrast, when the enzyme is thermally denatured, its fluorescence signal in the organic solvent shows a significant red shift of the $\lambda_{max}$ as well as a marked increase of intensity (Table 5).

In a similar conformational study carried out with subtilisin covalently linked to PEG we have proved that the enzyme is soluble, active and highly stable in dioxane. Instead, PEG-subtilisin undergoes conformational changes and inactivation in acetonitrile [43]. It should be noted that circular dichroism and fluorescence investigations cannot be carried out in solvents more hydrophobic than dioxane because they either do not dissolve the enzyme + PEG complexes (e.g. hexane) or they are not spectrophotometrically transparent in the range of interest (e.g. chlorinated or aromatic solvents). Dordick and co-workers [44] have carried out CD and EPR spectroscopic analyses of subtilisin solubilized in organic solvents by ion pairing with anionic surfactants (AOT). They have found that in octane the solubilized enzyme has a stability that is up to three orders of magnitude higher than that observed in water, while when dissolved in THF it has a very low catalytic efficiency and stability. Kwon et al. [45] have found, by FT-IR amide *I* analysis, that the secondary structure of subtilisin is not affected by solubilization in octane or benzene by ion pairing with AOT or by covalent modification with PEG, and that the catalytic activity is higher than that of the suspended enzyme. Analogously, Okahata et al. [46] have shown that lipases improve their catalytic activity in organic solvents after being solubilized by coating with lipid monolayers.

### *1.3. On the issue of diffusional limitations and lyoprotection effects*

Dissolution of enzymes in organic solvents by the methods described above has been successfully

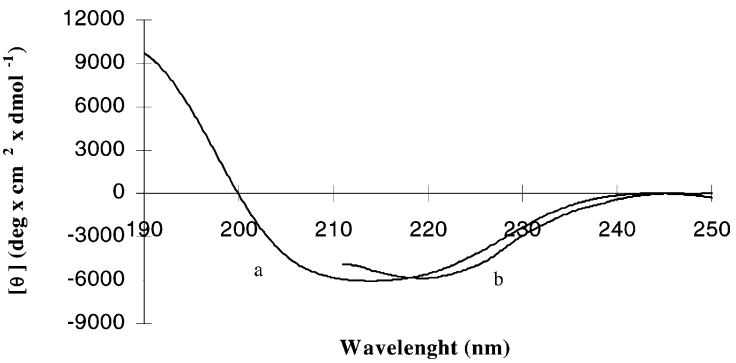


Fig. 3. Far-UV-CD spectra of lipase PC in water (a) and of lipase PC + PEG (b) in dioxane. The lipase PC + PEG sample (0.02 mg of lipase lyophilized with 5 mg of PEG) was dissolved in 1 ml of dioxane and analyzed. The spectrum in water was recorded using a solution of lipase PC having the same concentration (0.02 mg/ml) of the lipase PC + PEG sample. For further experimental details see [36].

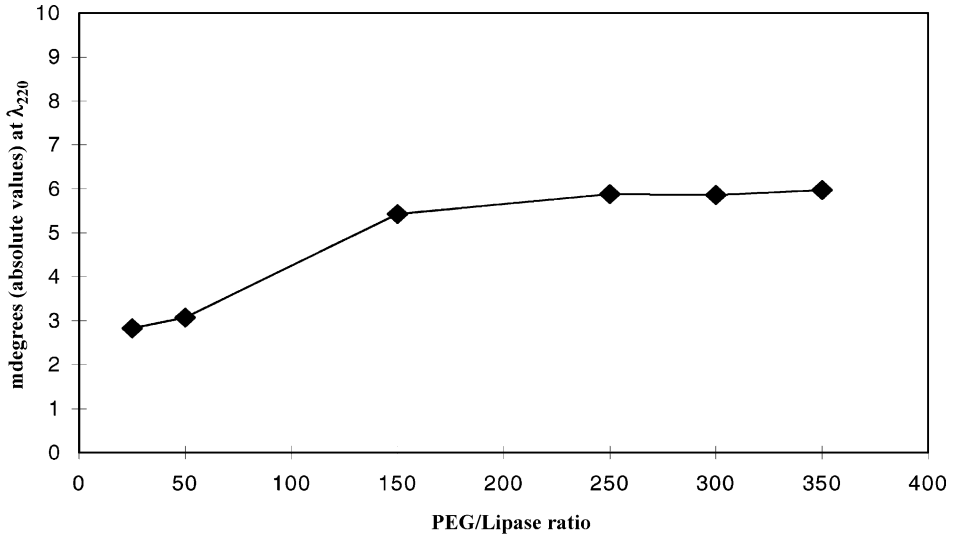


Fig. 4. Intensity of CD signal at 220 nm of lipase PC + PEG as a function of PEG/lipase ratio (w/w). A fixed amount of enzyme (0.02 mg) was dissolved in 0.5 ml of 5 mM potassium phosphate buffer, pH 7, containing 0.5–7 mg of PEG and lyophilized. The samples were then added with 1 ml of dioxane and analyzed. For details see reference [36].

employed to carry out conformational studies of hydrolases in non-aqueous media and, at the same time, to increase the catalytic activity. Enzymes dissolved in organic solvents have, in general, catalytic activities of the same order of magnitude of those displayed in water. Thus, the lower efficiency of other enzyme formulations (e.g. enzyme powders, CLECs, etc.) might be mainly ascribed to substrate diffusional limitations. However, since the methods employed to dissolve enzymes in organic solvents make use of additives (forming complexes or covalent links with the enzyme) and lyophilization steps, it can be argued that lyoprotection might also be responsible for the higher activity observed.

To distinguish if the activating effect of an additive colyophilized with an enzyme is due to lyoprotection or decrease of diffusional limitations, we have measured the increase of catalytic activity and protein solubility as a function of the PEG/protein ratio (Fig. 5) [37]. The data obtained show that the increase of CALB activity as function of the PEG/protein ratio is steeper than that of protein solubilization. This would suggest that lyoprotection is more important than relief of diffusional limitations because the

Table 5
Fluorescence of lipase PC, PC + PEG, N-Ac-L-Tyr-OEt and N-Ac-L-Trp-OEt in water, 1,4-dioxane and 1,4-dioxane/water mixtures

| Solvent | PC + PEG | | PC | | N-Ac-L-Trp-OEt | | N-Ac-L-Tyr-OEt | |
|---|---|---|---|---|---|---|---|---|
| | $\lambda_{max}$ (nm) | $I$ | $\lambda_{max}$ (nm) | $I$ | $\lambda_{max}$ (nm) | $I$ | $\lambda_{max}$ (nm) | $I$ |
| Water | 329 | 65 | 329 | 70 | 353 | 22 | 307 | 25 |
| 1,4-Dioxane/water ($a_w = 0.75$) | 329 | 65 | | | | | | |
| 1,4-Dioxane/water ($a_w = 0.25$) | 329 | 52 | | | | | | |
| 1,4-Dioxane/water ($a_w = 0.25$)[a] | 338 | 120 | | | 338 | 100 | | |
| 1,4-Dioxane | 329 | 50 | 329[b] | 5[b] | 333 | 144 | 307 | 120 |

[a] After thermal denaturation at 100 °C for 10 min.
[b] The same value was obtained also when 5 mg PEG were added to the sample.

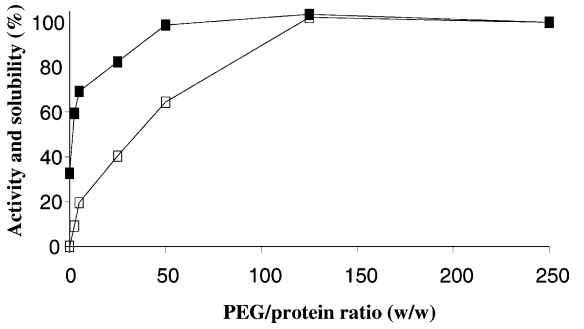


Fig. 5. Activity (■) and solubility (□) of CALB as a function of the PEG/protein ratio. The activity value was taken as 100 at the PEG/protein ratios of 250. The percentage of solubilized enzyme was calculated from the ratio between the CD signal at 220 nm obtained for a given PEG/protein ratio and that obtained at the highest PEG/protein ratio tested. It should emphasized that the signal obtained at the highest PEG/protein ratio was the same in 1,4-dioxane and water. Each point was the average of three measurements.

highest activity is obtained before all of the protein is solubilized (Fig. 5).

## 2. Conclusions

Several methods, such as dispersion of the enzyme on solid supports, use of additives, CLECs and CLEAs, enzyme dissolution in organic media by ion-pairing or by covalent or non-covalent complexation with PEG, have been employed to improve biocatalyst efficiency in organic solvents. In particular, we have focused on lipases + PEG formulations since they are very simple to prepare and show high catalytic activity. In fact, the transesterification activity of lipase PC + PEG and CALB + PEG is, respectively, about 30 and 51% of the hydrolytic activity displayed by these enzymes in aqueous buffer. Moreover, since lipases co-lyophilized with PEG are soluble in dioxane, we could investigate their conformation by CD and fluorescence proving that these enzymes maintain their native structure even when dissolved in water-miscible solvents. Through a study correlating enzyme activity and solubility in 1,4-dioxane as a function of the PEG/protein ratio, it has also been possible to attribute the PEG-induced activation to lyoprotection rather than to relaxation of diffusional limitations.

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