Discriminating between dispersion and lyoprotection effects in biocatalysis in organic media

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Abstract: The increment of activity and solubility in 1,4-dioxane of lipase B from *Candida antarctica*, lipase from *Pseudomonas cepacia*, and subtilisin, were investigated as a function of the methoxypoly(ethylene glycol)–protein (PEG–protein) ratio employed during lyophilization. Both activity and solubility markedly increased as the PEG–protein ratio was increased. The increment of activity at low PEG–protein ratios, however, was much higher than that of solubility. These data suggest that the PEG-induced activation effect is due mainly to a lyoprotection effect rather than to relaxation of diffusional limitations.

Key words: hydrolases, activity, dispersion, lyoprotection, circular dichroism.

Résumé : On a étudié l'augmentation de l'activité et de la solubilité de la lipase B de la *Candida antartica*, de la lipase de *pseudomonas cepacia* et de la subtilisine dans le 1,4-dioxane en fonction du rapport méthoxypoly(éthylèneglycol)/protéine (PEG/protéine) utilisé lors de la lyophilisation. L'activité ainsi que la solubilité augmentent toutes les deux de façon remarquable avec une augmentation du rapport PEG/protéine. Toutefois, l'augmentation de l'activité à des rapports PEG/protéine faibles est beaucoup plus grande que celle de la solubilité. Ces données suggèrent que l'effet d'activation induite par le PEG résulte principalement d'un effet de lypoprotection que d'une relaxation des limitations diffusionnelles.

Mots clés : hydrolases, activité, dispersion, lyoprotection, dichroïsme circulaire.

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Introduction

Biocatalysis in non-aqueous media has been widely studied and employed, as shown by its numerous applications in the areas of synthesis, food, and even analysis (1–3). Although this methodology has numerous advantages, its main drawback is the lower catalytic efficiency of enzymes in organic solvents than in aqueous solutions. This phenomenon can be ascribed to different causes, including restricted protein flexibility, low stabilization of the transition state of the enzyme–substrate intermediate, partial-enzyme denaturation by lyophilization, non-optimal acid–base conditions in the surrounding of the enzyme molecule, or poor catalyst dispersion (4). Several methods, such as the dispersion of the biocatalyst by immobilization on solid supports (5–9), the use of lyoprotectants (10, 11) or protein dissolution in the organic media by ion-pair forming surfactants (12), amphipatic lipids (13), or poly(ethylene glycol) (PEG) (14), were employed to increase enzymatic activity.

Although PEG was found to markedly improve the enzymatic activity of some hydrolytic enzymes in organic solvents, it has not been determined if the activation is due to lyoprotection or to biocatalyst dispersion–dissolution (15, 16). Here, we try to shed light on this issue by correlating the increment of activity and solubility of two lipases and subtilisin, in 1,4-dioxane, at different PEG–protein ratios.

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Dedicated to Professor J. Bryan Jones on the occasion of his 65th birthday.

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Experimental

Materials

Lipase B from *Candida antarctica* (CALB) was purified, as reported by Secundo et al. (16), from a crude preparation (experimental product SP 525) kindly provided by Novo Nordisk. Lipase from *Pseudomonas cepacia* (lipase PC) was purified, as reported by Secundo et al. (15), from a commercial crude preparation of lipase PS (Amano). Subtilisin and PEG (MW 5000) were purchased from Sigma and 1,4-dioxane of spectrophotometric grade from Aldrich. All other reagents and compounds were of analytical grade.

Preparation of enzyme samples

Purified CALB (0.2 mg) and lipase PC (0.2 mg) were dissolved in potassium phosphate buffer (1 mL, 5 mM, pH 7), divided into 20-μg portions, and added to PEG (0.05–5 mg) dissolved in water (0.4 mL) (PEG–protein ratio (w/w) 2.5–250); final volume 0.5 mL. Subtilisin (3 mg) was

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dissolved in potassium phosphate buffer (1 mL, 5 mM, pH 7), divided into 0.3-mg portions, and added to PEG (2.5–125 mg) dissolved in water (0.4 mL) (PEG–protein ratio (w/w) 8–417); final volume 0.5 mL. The samples were then frozen and lyophilized. Each sample was prepared in double to allow for the measurement of activity and to record the circular dichroism spectrum. Control samples were analogously prepared, but without PEG. The protein content was determined by Biorad Protein Assay.

Circular dichroism spectra

Far-UV circular dichroism (CD) spectra were recorded with a Jasco 500 A spectropolarimeter over the range of 200–250 nm for the sample dissolved in water and 210–250 nm for the samples dissolved in dioxane. (Below 210 nm, the high absorbance of dioxane causes a low signal-to-noise ratio that prevents the measurements.) All spectra were recorded at 25°C. The optical path was 0.5 cm in the case of lipases and 0.1 cm in the case of subtilisin. The PEG–enzyme samples were added to 1 mL of 1,4-dioxane (or water), vigorously shaken, and immediately used for CD analysis. All spectra were baseline corrected.

Measurements of catalytic activity

The transesterification between 1-octanol and vinyl acetate to give 1-octyl acetate and acetaldehyde was used as model reaction for the three enzymes considered. In all cases, 1 mL of 1,4-dioxane containing 1-octanol (0.19 M) and vinyl acetate (1.1 M) was added to the different enzyme samples. All the reagents, enzyme samples, and 1,4-dioxane were equilibrated against molecular sieves ($a_w < 0.1$) before being used. The reaction mixtures were shaken at 200 rpm and 25°C. At scheduled times, aliquots were withdrawn from the reaction mixtures and the conversion determined by GLC. (HP-1 Crosslinked Methyl Silicone Gum, 25 m, 0.32 mm ID, Hewlett-Packard; conditions: oven temperature from 35°C (initial time 10 min) to 160°C with a heating rate of 15°C min^{-1}, H_2 as carrier gas.) The retention times for vinyl acetate, acetic acid, 1-octanol, and 1-octyl acetate were 1.8, 2.2, 11.5, and 13.0 min, respectively. Using these reaction conditions, the catalytic activity for CALB, lipase PC, and subtilisin was, respectively, 2.20, 0.16, and 0.028 μmol min^{-1} for the samples without PEG, whereas it was 6.75, 0.68, and 0.098 μmol min^{-1} for the samples at the highest PEG–protein ratio tested (250 for lipases and 417 for subtilisin).

The possibility that PEG could affect the enzymatic activity by competing as a nucleophile with octanol seems very unlikely because, as shown in Fig. 2, the activity increased as a function of PEG concentration or, when the plateau value was reached, remained constant.

Results and discussion

Figure 1 shows that CALB, at a PEG–protein ratio higher than 125 (w/w), is fully solubilized in 1,4-dioxane, as shown by the almost complete overlap between the spectra recorded in the organic solvent and in water. In addition, the similarity between the spectra outside the range typical for a protein, in our case from 350 to 250 nm (data not shown), should rule out the presence of scattering phenomena usually caused by sizeable aggregates or very fine protein dispersions (17). Moreover, it is worth noting that the enzyme only showed a CD signal in dioxane when it was previously lyophilized from an aqueous solution containing PEG. When the enzyme was suspended in dioxane, it did not give a CD signal in the 210–350 nm range, even when PEG was subsequently added (up to 5 mg mL^{-1}) to the protein suspension. This result, which is in agreement with previous observations on other proteins (14), demonstrates that CALB is soluble in 1,4-dioxane and its secondary structure is the same as in water. On the other hand, the spectra obtained for CALB at PEG–protein ratios lower than 125 showed a proportionally lower signal, which can be attributed to a decrease in the solubility of the protein (14). Analogous behavior was observed for lipase PC and subtilisin (spectra not shown).

Fig. 1. Circular dichroism spectra, in water and 1,4-dioxane, of CALB colyophilized with PEG. PEG–protein ratio of 250 for the spectrum recorded in water (*g*) and 2.5 (*a*); 5 (*b*); 25 (*c*); 50 (*d*); 125 (*e*); and 250 (*f*) for those recorded in 1,4-dioxane.

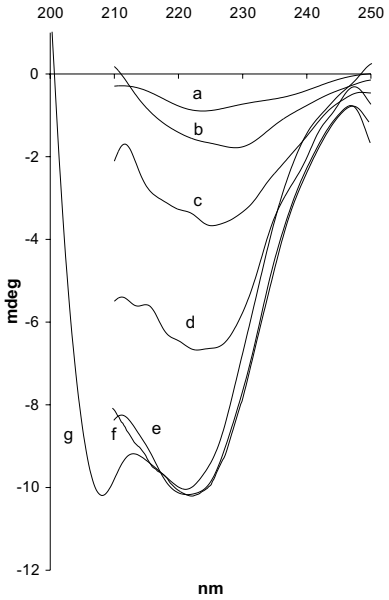
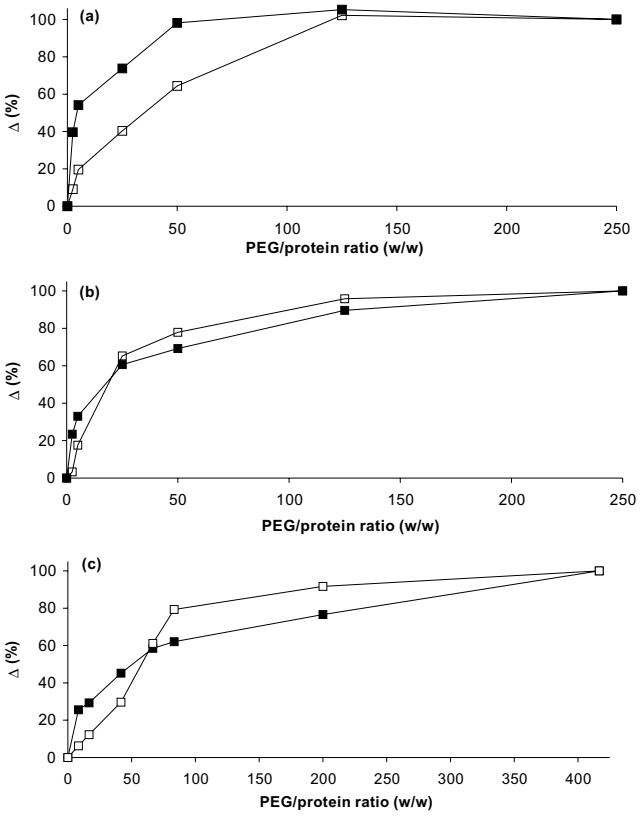


Figure 2 compares the increment of activity and solubility of the three enzymes as a function of the PEG–protein ratio. From zero to the highest ratio tested, not only did the activity increase 3.1-, 4.3-, and 3.5-fold for CALB (*a*); lipase PC (*b*); and subtilisin (*c*), respectively, but the enzymes were also fully solubilized. Nevertheless, it is evident that for all the enzymes the initial increment of activity was much steeper than that of solubility. Table 1 reports the increments of activity and solubility for CALB, lipase PC, and subtilisin at low PEG–protein ratios. It can be seen that the increment of activity was for all the enzymes much higher than the increment of solubility. For example, passing from 0 to a PEG–protein ratio of 2.5 for the lipases or 8 for protease, the increment of activity was 4.4-, 7.1-, and 4.1-fold higher than that of solubility for CALB, lipase PC, and subtilisin, respectively.

These data prove that the activating effect of PEG is not directly correlated to enzyme solubility. As a consequence, the activity increase should be due to a lyoprotective effect

Fig. 2. Increment of activity (■) and solubility (□) of CALB (*a*); lipase PC (*b*); and subtilisin (*c*) as a function of the PEG–protein ratio. The increment value (Δ) for activity and solubility is defined as $\Delta = (x - x_o)/(x_{max} - x_o)$, where x is the initial reaction rate or, for solubility, the CD signal at 220 nm obtained at a given PEG–protein ratio; x_o and x_{max} are the values obtained in the absence of PEG and at the highest PEG–protein ratio tested, respectively. For solubility, x_o was considered 0. (There was no CD signal for all the enzymes tested.) Each point was the average of three measurements.



of PEG on the secondary structure of the enzyme (18, 19), rather than to the relaxation of diffusional limitations obtained through the solubilization of the enzyme in the organic medium. It is worth pointing out that the data reported here are in agreement with our previous findings (16), which, through the method proposed by Wescott and Klibanov (20) and Bedell et al. (21) (a method that correlates the catalytic activity with the relative percentage of active and chemically inactivated enzyme present in a given amount of enzyme protein), proved that the activating effect of PEG on CALB is not due to the relaxation of diffusional limitations. Furthermore, we have previously found that different formulations of lipase from *Pseudomonas cepacia* (crude powder, Celite adsorbed, or PEG modified) have very similar K_M values (22). This result also suggests the absence of diffusion limitations. Concerning this point, however, it should be emphasized that a recent study by Rees and Halling (23) demonstrated that both of these approaches (K_M determination and use of mixtures of active and inactivated enzyme) were unable to explain the severe "solid-phase" mass-transfer limitations that occur in lyophilized enzymes in organic media. In conclusion, the present work indicates that the lyoprotectant effect of PEG plays a major role in improving biocatalyst performance in organic solvents. Furthermore, the comparison of the effects produced by different concentrations of a given additive on the activity and solubility of an enzyme in organic solvents appears to be a simple and general approach to distinguish between the lyoprotective and dispersive action of the additive itself.

Table 1. Increments of activity and solubility for CALB, lipase PC, and subtilisin at low PEG–protein values.

Enzyme	PEG–protein ratio	Increment of activity (%)[a]	Increment of solubility (%)[a]
CALB	2.5	39.6	9.1
CALB	5	54.2	19.6
Lipase PC	2.5	23.5	3.3
Lipase PC	5	33.0	17.6
Subtilisin	8	25.6	6.3
Subtilisin	17	29.3	12.3

[a]For the definition of increment see the legend of Fig. 2.

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