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# On the activity loss of hydrolases in organic solvents I. Rapid loss of activity of a variety of enzymes and formulations in a range of organic solvents

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**Abstract**

Dehydrated enzyme powders have been used extensively as suspensions in organic solvents to catalyze synthetic reactions. Prolonged enzyme activity is necessary to make such applications commercially successful. However, it has recently become evident that the stability and thus activity of many enzymes is compromised in organic solvents. Herein we explore the stability of various hydrolases (i.e., lipases from *Mucor meihei* and *Candida rugosa*, α-chymotrypsin, subtilisin Carlsberg, and pig-liver esterase) and various formulations (lyophilized powder, cross-linked enzyme crystals, poly(ethylene glycol)-enzyme conjugates) in different organic solvents. The results show a roughly exponential activity decrease for all enzymes and formulations studied after exposure to organic solvents. Inactivation was observed independent of the enzyme, formulation details, and the solvent. In addition, no relationship was found between the magnitude of inactivation and the value of initial activity. Thus, quite active formulations lost their activity as quickly as less active formulations. The estimated half-times ($t_{1/2}$) for all enzymes and preparations ranged from 1.8 h for subtilisin C. co-lyophilized with methyl-β-cyclodextrin to 61.6 h for the most stable poly(ethylene glycol)-α-chymotrypsin preparation. The data here presented indicates that the inactivation is likely not related to changes in enzyme structure and dynamics.


*Keywords:* α-Chymotrypsin; Enzyme stability; Lipase from *Mucor meihei*; Lipase from *Candida rugosa*; Pig-liver esterase; Poly(ethylene glycol)–protein conjugates; Subtilisin Carlsberg

## 1. Introduction

Enzymes have been successfully employed to catalyze a number of transformations and chiral resolutions of biological and industrial importance in organic solvents [1–9]. It is well documented that these non-natural media offer some advantages over the natural-aqueous environment, such as: prevention of autolysis (in case of proteases), and increased thermostability due to reduced structural mobility [10,11]. In fact, it has been shown that enzymes can perform catalysis in organic solvents at temperatures far above those temperatures that denature enzymes in aqueous systems [8]. Despite this, enzyme inactivation in organic solvents has been reported. For example, Fagain has recently reviewed bioreactor stability, shelf life and operational stability of a variety of enzymes suspended in neat organic solvents and aqueous-organic solvent mixtures [12]. However, the mechanism of enzyme inactivation in organic solvents is still unclear. Recent studies on various formulations of subtilisin Carlsberg have shed some light into this problem, showing that the enzyme loses most of its initial activity after only a few hours of exposure to organic solvents [13,14] and that the inactivation was independent of the reaction temperature, the solvent, and enzyme hydration [14]. It was suggested that the enzyme inactivation

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probably does not involve structural changes because cross-linked enzyme crystals (CLEC), which have been shown to be structurally defined in organic solvents [10], also lost most of their initial activity after a few hours of incubation. Furthermore, FT-IR spectroscopic investigations employing subtilisin Carlsberg did not show secondary structural changes upon inactivation in organic solvents [15].

It has been demonstrated that enzyme activity in an organic solvent depends, among other factors, on the structural integrity of the catalyst and its dynamics [16]. Basically, the more "native-like" structural and dynamic properties are in the organic solvent, the more active the catalyst. If enzyme stability were to depend on enzyme dynamics in an organic solvent one would in general expect that those formulations with the highest initial activity – being the most dynamic and structural intact ones – should suffer the most drastic decrease in activity. On the other hand, structurally impaired lyophilized powders [11,17–19] or cross-linked enzyme crystals displaying huge structural rigidity should be the most stable formulations in organic solvents. In this work we systematically investigated if the loss of activity in neat organic solvents is a common phenomenon of different enzymes and whether or not there is any relationship between the initial activity and the inactivation of the catalysts. The results obtained show that inactivation did not significantly depend on the initial activity, formulation, enzyme, or solvent. It is unlikely that the inactivation observed is caused by structural or dynamic changes, but that a local active site effect is responsible for the observed inactivation, an interpretation consistent with some literature data reviewed in the introduction.

## 2. Materials and methods

### 2.1. Chemicals

The enzymes, subtilisin Carlsberg and subtilisin (EC 3.4.21.14) covalently modified with $PEG_{5000}$ (polyethylene glycol), pig-liver esterase, α-chymotrypsin and the lipases from *Candida rugosa* and *Mucor meihei* were purchased as lyophilized powders from Sigma–Aldrich. The cross-linked enzyme crystals (CLECs) were purchased from Altus Biochemicals. Succinimidyl activated $PEG_{5000}$ and $PEG_{20,000}$ (mPEG–succinimidyl propionate "mPEG–SPA") were purchased from NEKTAR. The solvents were all purchased from Aldrich in the anhydrous form (water content below 0.005%), and were dried prior to their use as recommended [20]. *Sec*-phenethyl alcohol was purchased from Aldrich and vinyl butyrate was purchased from TCI.

### 2.2. Enzyme preparation and kinetic measurements

Lyophilized formulations of subtilisin Carlsberg and α-chymotrypsin were obtained by rapidly freezing solutions of 5 mg/mL in 20 mM potassium phosphate buffer (pH 7.8) in liquid nitrogen followed by drying for 24 h using a Savant freeze-drying system. Pig-liver esterase and the two lipases were prepared in the same manner but the aqueous buffer was adjusted to pH 8.0 and 7.0 respectively. Co-lyophilizates were prepared by co-dissolving the desired amount of additive (in a 1:6 (g/g) ratio of enzyme to additive) with the enzyme in buffer prior to freeze-drying. CLECs were used as received. Covalent modification of α-chymotrypsin with $PEG_{5000}$ was carried out as described by Al-Azzam et al. [21]. Covalent modification of subtilisin with $PEG_{20,000}$ was performed by dissolving the desired amount of enzyme in 20 mM sodium borate buffer (pH 9.0) followed by adding activated PEG to achieve a 1:3 enzyme:PEG weight ratio. The mixture was allowed to react for 15 min under slow stirring at room temperature. The reaction mixture was then transferred to the refrigerator for 3 h followed by quenching by decreasing the pH to 5.5 with a dilute solution of HCl. The solution was then dialyzed (50 kDa $M_W$ cut-off) in a 1 L beaker filled with nanopure water. The water was periodically changed during a 24 h period. The solution was then lyophilized for 48 h. Next, the powder was dissolved in a sodium phosphate buffer to adjust its pH (to 7.8), separated in vials and lyophilized for a second time in individual vials for 48 h. The concentration of subtilisin was obtained from calibration curves by measuring the UV absorbance at 280 nm.

All kinetics experiments employed the transesterification reaction between *sec*-phenethyl alcohol and vinyl butyrate. The formation of the ester product was followed by gas chromatography [22]. The GC instruments (HP 6850 and HP 6890, fitted with Chirasil CB columns, FID detectors and He as carrier gas) were calibrated with the chiral ester products of the reactions. The product peak areas and retention times were the same in the presence or absence of the substrates. The substrates (70 mM alcohol and 200 mM vinyl butyrate) and the solvent (1.0 mL) were dried over molecular sieves prior to their use. The water content was less than 0.01% as determined by colorimetric Karl Fisher titration. All kinetic experiments were terminated before 10% of the product had been formed. The enzyme enantioselectivity ($V_R[S]/V_S[R]$) was determined by measuring the initial rates of the reactions for both enantiomers [23] and is equal to the ratio $[k_{cat}/K_M]_R/[k_{cat}/K_M]_S$ [24]. Half times were estimated by fitting the data to the following equation: $y = a\exp(-kt) + C$, and the $t_{1/2}$ values were estimated from the following equation: $A = A_0\exp(-kt)$. Therefore, they represent the half-life of the enzymes before they reached a constant–non-cero-residual activity [14].

### 2.3. Enzyme stability in organic solvents

Enzyme stability was determined by measuring the enzyme activity (initial velocity) as a function of the incubation time after storage in organic solvents. The enzyme formulations (10 mg of lyophilized enzyme powders or 1 mg of CLEC, PEG–enzyme conjugates, and co-lyophilizates) were suspended in 1.0 mL of the chosen organic solvent

under $N_2$ and were incubated at 45 °C under constant shaking (300 rpm). To measure the enzyme activity after different incubation times, the substrates were added to initiate the reaction and the product formation followed as previously described [14].

## 3. Results and discussion

We first employed different formulations (CLECs, lyophilized from buffer, co-lyophilized with methyl-β-cyclodextrin, and subtilisin–PEG conjugates) of the serine protease subtilisin Carlsberg, a frequently employed enzyme in non-aqueous enzymology, in the commonly used solvents THF, acetonitrile, and 1,4-dioxane [25]. The various formulations employed showed vastly different initial velocities $V_S$ (Table 1). Four days of incubation in the solvents caused a drop in activity for all formulations of at least 67%. Some variation was found after incubation of different formulations in the solvent THF. The least active and presumably least dynamic formulation, lyophilized subtilisin, showed the least activity loss in THF (with a $t_{1/2}$ of 20.5 h). A substantial drop in enantioselectivity was observed for formulations with initially good enantioselectivity values.

The data allow exclusion of substrate diffusional limitations, which might have been caused by swelling of the powder particles or formation of protein aggregates, for being responsible for the inactivation. Specifically, in contrast to the other formulations, the subtilisin–$PEG_{20,000}$ conjugate was completely soluble in the solvent but the inactivation was comparable to other samples. Interestingly enough, the kinetics of the two different subtilisin–PEG conjugates in the two different solvents were very similar (Fig. 1) even though the physical state of the sample (suspension versus solution) and the initial activities were quite different. Inspection of the fully soluble enzyme in 1,4-dioxane (this preparation was found to be most soluble in this solvents) after the incubation period did not reveal the presence of any cloudiness or solid deposits excluding aggregation as being the cause of enzyme inactivation. Two additional important points are worthwhile mentioning. First, the subtilisin–$PEG_{20,000}$ conjugate is, to the best of our knowledge, the most active subtilisin preparation ever reported in a non-aqueous system. Furthermore, even after 4 days of incubation and nearly 90% of activity loss, this formulation still had a much higher activity than any other preparation initially.

Next, we proceeded to investigate the inactivation for various other enzymes to establish the generality of the phenomenon. Four different enzymes were employed, namely lipases from *M. meihei* and *C. rugosa*, α-chymotrypsin, and pig-liver esterase.

### 3.1. Lipase from C. rugosa

Two preparations of this enzyme (the CLECs and the lyophilized powder) were studied in the two solvents in which

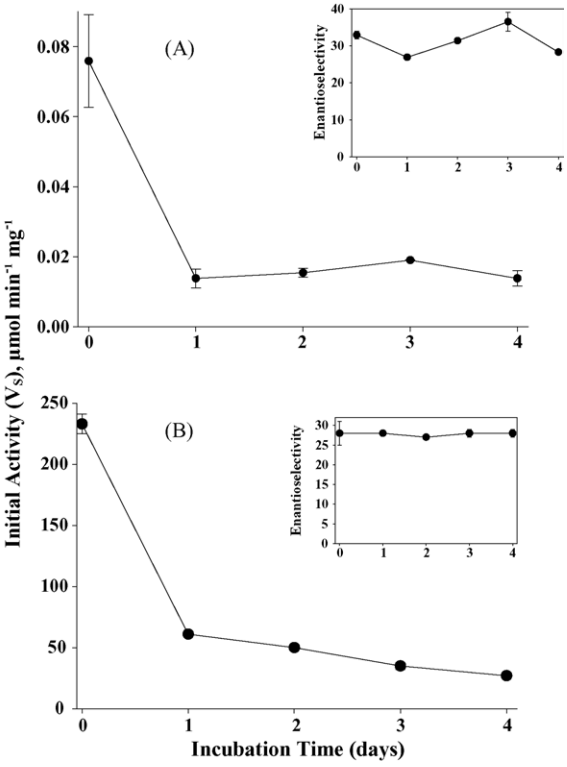


Fig. 1. Plot of the initial activities of (*S*) enantiomer formation catalyzed by subtilisin–PEG conjugates for the transesterification reaction between *sec*-phenethyl alcohol and vinyl butyrate vs. the incubation time. (A) Subtilisin–$PEG_{5000}$ in THF, and (B) subtilisin–$PEG_{20,000}$ in 1,4-dioxane.

they were most active, i.e., cylohexane and octane. As before, regardless of preparation and solvent a drastic drop in activity was observed in the first 24 h while enantioselectivity was not affected markedly (Fig. 2, Table 2). The initial activity of the lyophilized powder in octane was higher than in cyclohexane, but in the former it lost 99.2% of its original activity after the incubation period. Similarly, the relative active CLEC preparation lost 96 and 96.3% of its initial activity in these two solvents.

### 3.2. Lipase from M. meihei

The lyophilized powder of this enzyme was not very active initially but it retained 50% of its original activity after 14 and 34 h of incubation in octane and cyclohexane, respectively (Fig. 3, Table 2). After 4 d of incubation it lost 52.3 and 83.3% of its original activity in these solvents (Table 2). The high enantioselectivity observed for this enzyme in these two solvents persisted during the incubation period (Fig. 3), and actually this was the most enantioselective enzyme employed in these studies.

### 3.3. Pig-liver esterase

The enzyme was tested in octane (one of the solvents in which this enzyme was found to be most active) and it behaved in a similar manner as the other enzymes; it lost

Table 1
Activity data for various preparations of subtilisins prior to and after 4 days of incubation in the respective solvent

| | Subtilisin Carlsberg | | | | | |
|---|---|---|---|---|---|---|
| | Co-lyophilized (MβCD)[a] | CLEC[a] | | Lyophilized powder[a] | $PEG_{5000}$ | $PEG_{20,000}$ |
| | THF | THF | ACN | THF | THF | 1,4-Dioxane |
| Initial activity before incubation, at day 0 (μmol/min mg) | 1.35 ± 0.05 | 0.59 ± 0.05 | 0.115 ± 0.01 | 0.015 ± 0.03 | 0.08 ± 0.01 | 233 ± 8 |
| Initial activity after 4-days of incubation (μmol/min mg) | 0.1 | 0.06 | 0.02 | 0.005 | 0.012 | 25 |
| % Activity loss after incubation | 92.6 | 89.8 | 82.6 | 66.7 | 85 | 89.3 |
| Estimated half times ($t_{1/2}$) (h) | 1.8 | 20.8 | 8.5 | 20.5 | I.D.[b] | 10.7 |
| Enantioselectivity at day 0 | 59 | 59 | 5.5 | 31 | 33 ± 1 | 28 ± 3 |
| Enantioselectivity at day 4 | 40 | 45 | 5.5 | 29 | 29 ± 2 | 28 ± 1 |

Initial velocity data are given for (*S*) enantiomer formation.
[a] Data taken from Susimar Gonzalez Martinez et al. [14].
[b] Insufficient data points.

about 75% of its initial activity during the incubation period (Table 3, Fig. 4).

### 3.4. α-Chymotrypsin

Chymotrypsin covalently modified with $PEG_{5000}$ and lyophilized α-chymotrypsin behaved similar than the other enzymes in octane (Fig. 5A and B). However, the $PEG_{5000}$-modified preparation remained highly active for several days, retaining most of its original activity after the 6th day of incubation (losing only 43% of its initial high activity, with a $t_{1/2}$ of 61.6 h, Table 3 and Fig. 5A). This was the most stable preparation studied so far, and it was the second most active enzyme as well (after $PEG_{20,000}$–subtilisin).

Overall the data reveal some interesting points. First of all, in general, for most of the enzymes here studied the

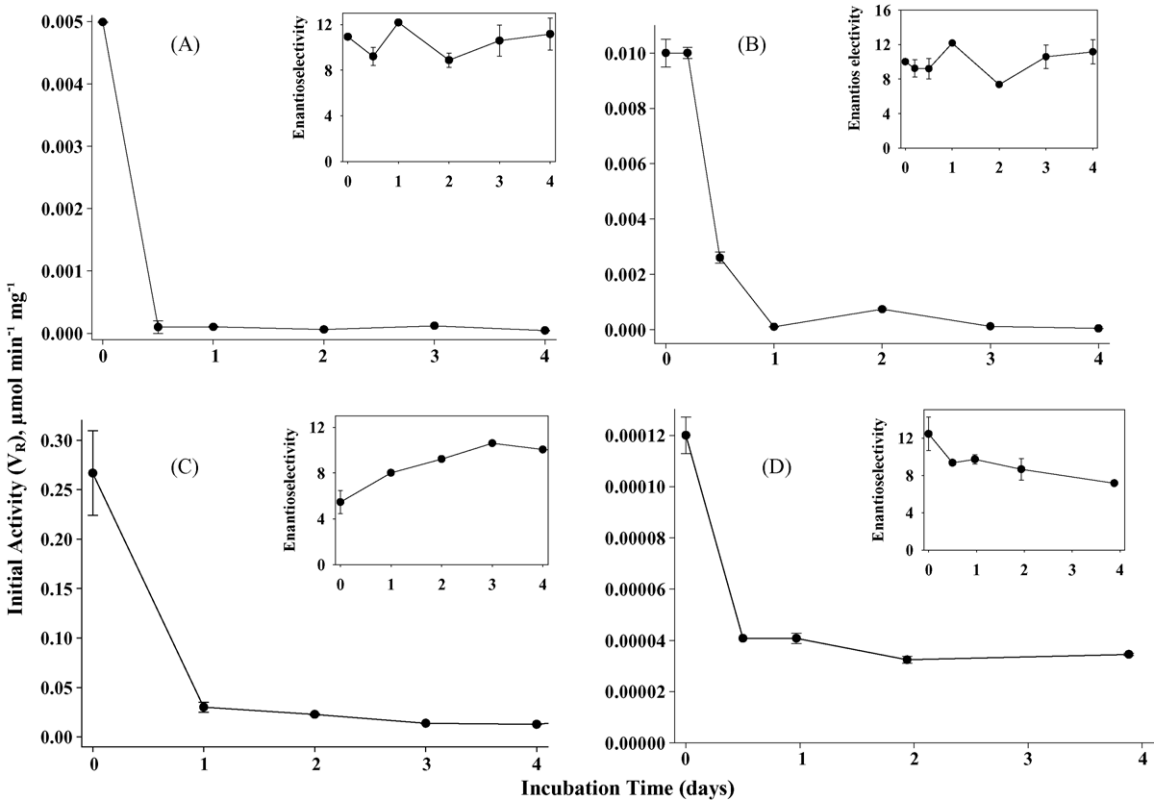


Fig. 2. Plot of the initial activities of (*R*) enantiomer formation catalyzed with lipase from *C. rugosa* for the transesterification reaction between *sec*-phenethyl alcohol and vinyl butyrate vs. the incubation time. (A) Lyophilized powder in octane, (B) CLECs in octane, (C) CLECs in cyclohexane, and (D) lyophilized powder in cyclohexane.

Table 2
Activity data for various preparations of lipase from *C. rugosa* and *M. meihei* prior to and after 4 days of incubation in the respective solvent

| | Lipase from *C. rugosa* | | | | Lipase from *M. meihei* | |
|---|---|---|---|---|---|---|
| | CLEC | | Lyophilized powder | | Lyophilized powder | |
| | Octane | Cyclohexane | Octane | Cyclohexane | Octane | Cyclohexane |
| Initial activity before incubation, at day 0 (μmol/min mg) | 0.01 | 0.27 | $5.0 \times 10^{-3}$ | $1.2 \times 10^{-4}$ | 0.044 | 0.042 |
| Initial activity after 4-days of incubation (μmol/min mg) | $4.0 \times 10^{-4}$ | 0.01 | $4.0 \times 10^{-5}$ | $3.5 \times 10^{-5}$ | 0.021 | 0.007 |
| % Activity lost after incubation | 96 | 96.3 | 99.2 | 70.8 | 52.3 | 83.3 |
| Estimated half times ($t_{1/2}$) (h) | 4.2 | I.D.[a] | I.D. | I.D. | 14 | 34 |
| Enantioselectivity at day 0 | $10 \pm 1$ | $5.5 \pm 0.2$ | $11 \pm 1$ | $12 \pm 2$ | >100 | >100 |
| Residual enantioselectivity | $11 \pm 2$ | $9 \pm 1$ | $11 \pm 2$ | $8 \pm 1$ | >100 | >100 |

Initial velocity data are given for (*R*) enantiomer formation.
[a] Insufficient data points.

decrease of activity observed under all conditions is never complete but rather approaches a certain value at which no further inactivation is observed, and the activity remains constant for the remaining 4 days of incubation. However, for subtilisin–$PEG_{20,000}$, *M. meihei* in cyclohexane, pig-liver esterase and $PEG_{5000}$–α-chymotripsin, a longer incubation period is required to decisively establish that these enzymes too, approach a certain value of inactivation. Most of the decrease in activity occurred quite rapidly within 24 h while the stability in the remaining days was much better. This observation suggests that structural changes might not be the cause for the loss of activity, since it is difficult to speculate on any reason why this phenomenon (of solvent-induced structural changes) would stop after the first day of incubation or so. This interpretation is in agreement with FTIR data obtained by Santos et al. on subtilisin [15]. Inactivation was not paralleled by structural changes. Furthermore, inactivation was also observed for CLECs for which the cross-linking prevents large structural alterations [26,27], and their structure has been shown to be almost native in various organic solvents [10].

The data also seems to be incompatible with water stripping and subsequent reduced flexibility being responsible for the activity drop. This phenomenon occurs primarily in water-miscible organic solvents such as THF and acetonitrile while inactivation is also observed in very apolar solvents in which water stripping cannot occur. In general, the data does not seem to indicate that the inactivation is being caused by large structural and dynamic changes of the catalyst because regardless of enzyme, formulation, and solvent substantial

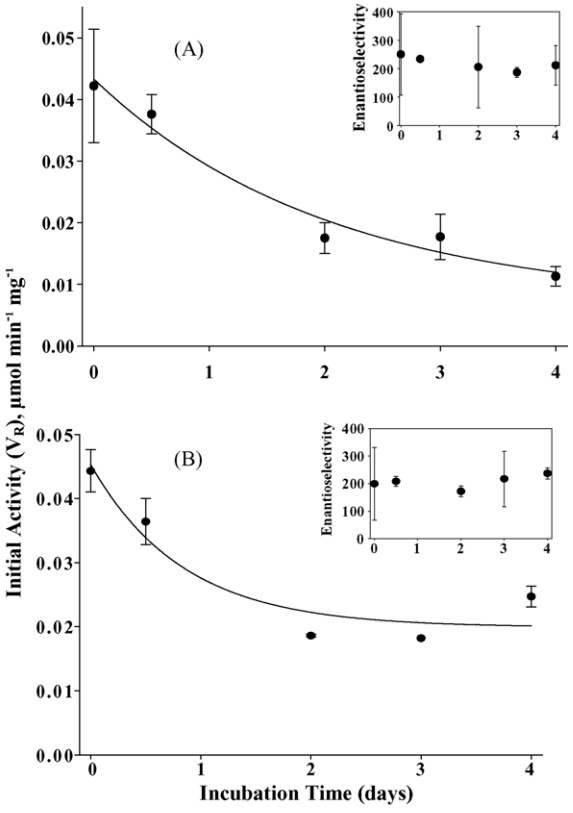


Fig. 3. Plot of the initial activities of (*R*) enantiomer formation catalyzed with lipase from *M. meihei* for the transesterification reaction between *sec*-phenethyl alcohol and vinyl butyrate vs. the incubation time. Lyophilized powder in (A) cyclohexane and (B) octane.

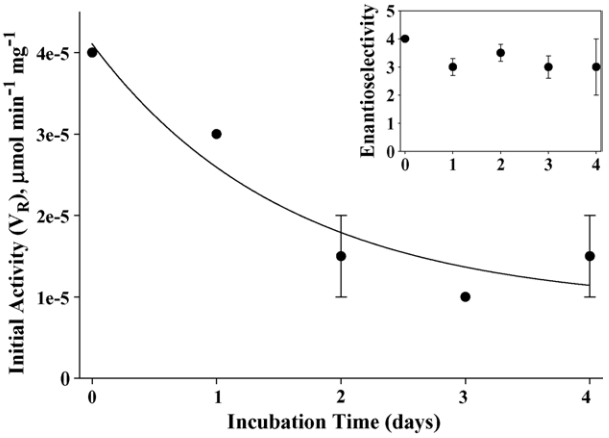


Fig. 4. Plot of the initial activity of (*R*) enantiomer formation catalyzed with pig-liver esterase for the transesterification reaction between *sec*-phenethyl alcohol and vinyl butyrate in octane vs. incubation time. Initial activity of the most reactive "*R*" enantiomer vs. the incubation time.

Table 3
Activity data for various preparations of pig-liver esterase and α-chymotrypsin prior to and after 4 days of incubation in octane

| | α-Chymotrypsin | | Pig-liver esterase |
|---|---|---|---|
| | $PEG_{5000}$ | Lyophilized powder | Lyophilized powder |
| Initial activity before incubation, at day 0 (μmol/min mg) | 9.0 | $0.019 \pm 0.003$ | $4.0 \times 10^{-5} \pm 5 \times 10^{-6}$ |
| Initial activity after 4-days of incubation (μmol/min mg) | 5.1 | $0.0081 \pm 0.0004$ | $1.0 \times 10^{-5}$ |
| % Activity lost after incubation | 43 | 57.4 | 75 |
| Estimated half times ($t_{1/2}$) (h) | 61.6 | 9.5 | 26 |
| Enantioselectivity at day 0 | 7.5 | $2.9 \pm 0.5$ | $4 \pm 0.2$ |
| Residual enantioselectivity | 6.0 | $4.8 \pm 0.5$ | $3 \pm 1$ |

Initial velocity data are given for (*S*) enantiomer formation for α-chymotrypsin and (*R*) enantiomer formation in case of pig-liver esterase.

inactivation was observed under all conditions. However, the data does seem to point to a local event at the active site being involved in the inactivation. On the other hand, even structural perturbations of active site residues seem to be irreconcilable with the observations because enantioselectivity was mostly unaffected by the inactivation. However, one should point out that constant enantioselectivity could also be explained with a mechanism by which a portion of the enzyme molecules become inactivated while others remain unaffected and active.

Experiments geared at investigating local changes of the active site, for example changes in the protonation state of the active site residues are currently ongoing. Preliminary results of co-lyophilized and lyophilized subtilisin C. in THF show that the enzyme pH memory is completely lost after the incubation period (unpublished results). These data support that local active site related events are likely involved in enzyme inactivation in organic solvents.

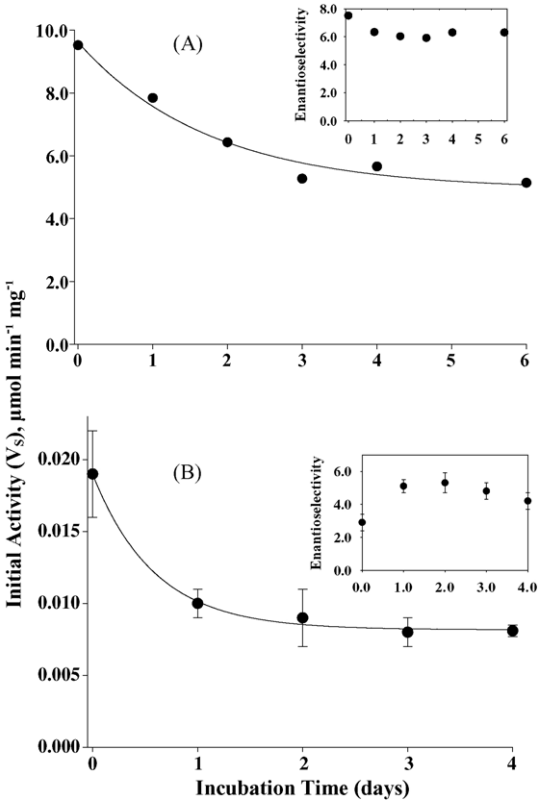


Fig. 5. Plot of the initial activities of (*S*) enantiomer formation catalyzed by α-chymotrypsin for the transesterification reaction between *sec*-phenethyl alcohol and vinyl butyrate vs. the incubation time. (A) Conjugate with $PEG_{5000}$ and (B) lyophilized powder.

From the point of view of applications in organic synthesis it is important to point out that the enzyme–PEG conjugates were by far the best formulations of all the different formulations studied. Even in cases when substantial activity drops were observed, the remaining activity was still superior after the incubation when compared with the initial activities of the other formulations, and as can be seen from the estimated $t_{1/2}$ shown on all tables, the PEG-modified enzymes remain highly catalytically active for several hours. A more detailed study on the activity of such enzyme–PEG conjugates is currently on its way.

## 4. Conclusions

The enzymes studied showed similar low storage stability on organic solvents. It appears that there is only limited influence of the solvent, the mode of enzyme preparation, and the nature of the enzyme itself. It appears that enzymes in general become inactivated in pure organic solvents by a common mechanism: a roughly exponential decay up to a constant residual activity, which, for most enzymes, seems to persist for the duration of the incubation experiments. The most plausible explanations, compatible with our results, are a breakdown of the catalytic machinery (disruption of the acid–base catalytic properties of the active-site imidazole, for example) or a change in the protonation state of active site residues.

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