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Enzymatic production of (3*S*,4*R*)-(−)-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-carboxylic acid using a commercial preparation of lipase A from *Candida antarctica*: the role of a contaminant esterase

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Abstract—The enantioselective hydrolysis of (3*RS*,4*RS*)-*trans*-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate (±)-**2** was effected using a commercial preparation of lipase from *C. antarctica A* (CAL-A). We found that the hydrolytic activity of the lipase (immobilized on a number of very different supports) with this substrate was negligible. However, a contaminant esterase with Mw of 52 KDa from this commercial preparation exhibited much higher activity with (±)-**2**. This enzyme was purified and immobilized on PEI-coated support and the resulting enzyme preparation was highly enantioselective in the hydrolysis of (±)-**2** (E >100), hydrolyzing only the (3*S*,4*R*)-(−)-**3**, which is a useful intermediate for the synthesis of pharmaceutically important (−)-paroxetine. Optimization of the reaction system was performed using a racemic mixture with a substrate concentration of 50 mM. This enzyme preparation was used in three reaction cycles and maintained its catalytic properties.

1. Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are perhaps the most frequently used enzymes in organic chemistry because they couple a wide substrate specificity to high regio- and enantioselectivity.[1–5] However, most commercial preparations of lipases are crude and may contain some contaminant proteins (in some cases with catalytic activities, e.g. esterases and proteases), which in many cases are actually responsible for the activity against the studied compounds. This may become a critical factor when the activity is assayed against 'poor' substrates, where the catalytic activity is very low.

Bearing in mind the difficulties of conventional procedures for the full purification of lipases from these crude preparations, we propose herein a simple and general methodology that allows the separation of lipases from other contaminant proteins in a single step, as a general procedure to identify the enzyme responsible for the observed activity. This is based on the affinity of microbial lipases to adsorb on hydrophobic surfaces by interfacial activation.[6,7] Thus, by incubating the crude enzyme preparation under low ionic strength conditions in the presence of hydrophobic supports, lipases may be easily immobilized, purified and hyperactivated, while other proteins (e.g. esterases) remain in the supernatant liquid.[8–10] This general strategy may simplify the studies in organic chemistry, because it can allow researchers to determine whether the lipase is the actual catalyst responsible for the outcome of the reaction under study, and enables the use of other techniques to improve the performance of the biocatalyst (e.g. preparing high loading biocatalyst or using protein engineering to improve the performance of an enzyme in a particular reaction).

The work reported herein involves the application of this technique to one of the most popular commercial lipase preparations, that of lipase from *C. antarctica* (*fraction A*) (CAL-A).[11,12] As an example, the kinetic resolution of (3*RS*,4*RS*)-*trans*-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate (±)-**2**, an interesting

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precursor for paroxetine through stereoselective hydrolytic reaction was studied.

Paroxetine is a drug that modulates the physiological actions of 5-hydroxy-tryptamine [5-HT], being potentially useful in the treatment of a variety of human diseases including depression, obsessive compulsive disorder and panic disorder.[13] This compound is an enantiomerically pure (−)-*trans*-3,4-disubstituted piperidine. Interest in the preparation of this compound from the pharmaceutical industry is shown by the increasing number of strategies developed for its preparation as a single enantiomer. Among the reported methods are the selective recrystallization of diastereomeric salts,[14] chiral auxiliary-assisted syntheses,[15–17] biocatalytic resolutions,[18,19] and the asymmetrization of a prochiral diester intermediate.[20]

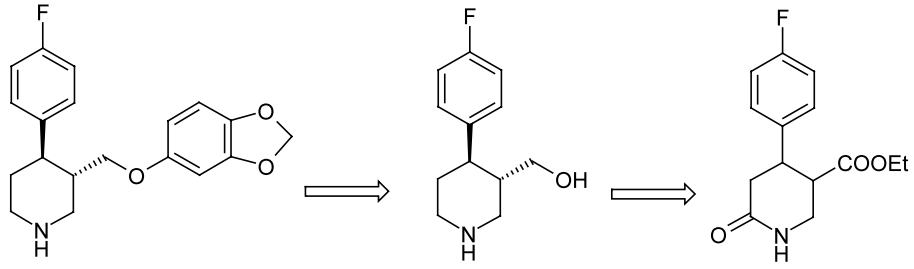
The retrosynthetic analysis shown in Scheme 1 demonstrates the role of (±)-**2** as intermediate in the synthesis of (−)-paroxetine.[21] Gotor et al.[22] have demonstrated that crude CAL-A has the interesting potential to perform the stereoselective hydrolysis of different derivatives of substrate (±)-**1** with high enantiomeric excess, exhibiting an enantioselectivity opposite to that shown by lipase from *C. antarctica B*.[22,23] However, they did not observe any catalytic activity when using a piperidinone substrate structurally similar to (±)-**2**.

2. Results and discussion

2.1. Activity of different fractions of the commercial preparation of CAL-A against substrate (±)-2

We first evaluated the enzymatic activity of different immobilized preparations of CAL-A (glyoxyl, glutaraldehyde, PEI, octyl-agarose) in the hydrolysis of substrate (±)-**2** (Table 1). Activities against substrate (±)-**2** were very similar in all cases except in the case of the octyl-agarose CAL-A preparation, where the activity was almost negligible (decreasing by a 100-fold factor).

To check the reasons for this extremely low activity against substrate (±)-**2** detected for the interfacially adsorbed preparation, we analyzed the composition of the commercial enzyme extract. Fig. 1 shows that there are three main proteins in the commercial preparation of CAL-A. As expected, the major protein adsorbed on octyl agarose was the lipase with a molecular weight of 45 KDa (such adsorption at low ionic strength in hydrophobic supports has been reported as a rapid and simple method for the purification of microbial lipases[9]). We released the enzyme from the octyl support using Triton X-100 and immobilized this released enzyme (pure lipase) on other supports (glyoxyl, glu-



(3*S*,4*R*)-(-)-**Paroxetine** (-)-**1** (±)-**2**

Scheme 1. Retrosynthetic analysis of (−)-paroxetine.

Table 1. Enzymatic activity of different immobilized preparations of CAL-A-catalyzed hydrolysis of (±)-**2** at 45°C in a mixture of 25 mM sodium phosphate at pH 7 to a concentration of substrate of 2 mM. The enzymatic load of the immobilized preparations was 6 mg protein/g support

Immobilized preparation	Time (h)	Conversion (%)	Enzyme activity
Non-purified			
Octyl	160	12	0.000125
Glyoxyl	3.3	20	0.01
Glutaraldehyde	62	15	0.04
PEI	56	18	0.032
Purified enzyme			
Glyoxyl	111	20	0.0003
Glutaraldehyde	50	18	0.0006
PEI	58	18	0.00052
Supernatant after octyl-agarose adsorption			
Glyoxyl	2.33	14	0.01
Glutaraldehyde	0.5	12	0.038
PEI	1	17	0.030

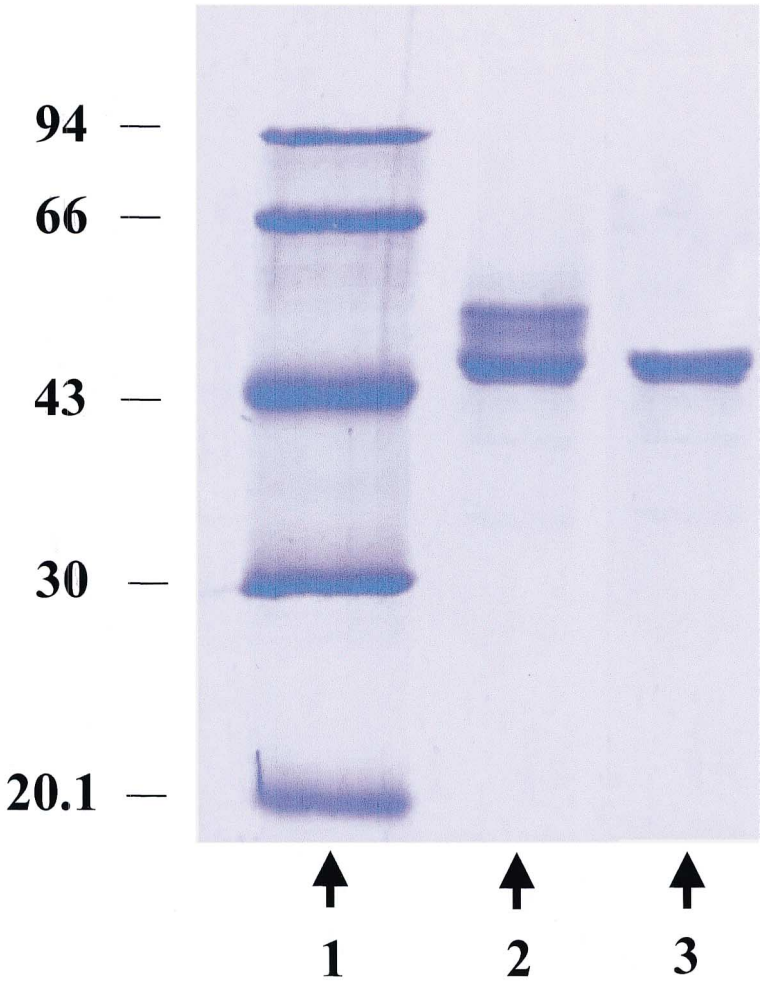


Figure 1. SDS-PAGE gel of different CAL-A preparations. Lane 1—molecular weight markers. Lane 2—commercial CAL-A preparation. Lane 3—proteins adsorbed on octyl preparation.

taraldehyde and PEI supports). The activities of these immobilized preparations were very low compared to that obtained when the immobilized crude commercial preparation. To check the possible effects of detergent on the enzymatic activity, some crude enzyme was mixed with detergent and immobilized on PEI. The resulting activity was similar to the conventionally dissolved lipase.

Thus, it appeared that the enzyme responsible for the activity against substrate (±)-**2** was not CAL-A. To confirm this possibility, we immobilized the supernatant liquid obtained after adsorbing the crude preparation on octyl-agarose (i.e. where most of the CAL-A lipase has been eliminated). This supernatant presented <10% of the activity of the preparation against ethyl butyrate or <5% of the activity against olive oil. However, this supernatant presented the highest activity against substrate (±)-**2**. The activities achieved with these immobilized preparations were very similar to that achieved with the crude preparation. Thus, we concluded that CAL-A lipase was not responsible for the activity against substrate (±)-**2** and that this activity was a consequence of one of the other contaminant proteins.

In this way, we have tried to find a protocol that allows the preparation of the pure enzyme having activity against (±)-**2**. The commercial preparation was first exposed to octyl-agarose that mainly adsorbs the CAL-A lipase. To eliminate traces of CAL-A that remained in the supernatant liquid, we exposed the previous supernatant to octadecyl-Sepabeads (a more hydrophobic support).[10] This treatment fully eliminated both the contaminant CAL-A and the protein with a molecular weight of 49 KDa from the supernatant, together with a small percentage of the protein with molecular weight of 52 KDa. However, most of this protein (52 KDa) could be obtained in pure form in the remaining supernatant after the second adsorption (Fig. 2). After that, the enzymatic activity of the immobilized octadecyl preparation was checked in the hydrolysis of substrate (±)-**2** and was again found to be very low. Desorption of the proteins adsorbed on octadecyl-Sepabeads and immobilization on other supports also failed to provide better results.

The enzyme of Mw 52 kDa was adsorbed on PEI-agarose support. This support was chosen because of the possibility of reusing the support after enzyme

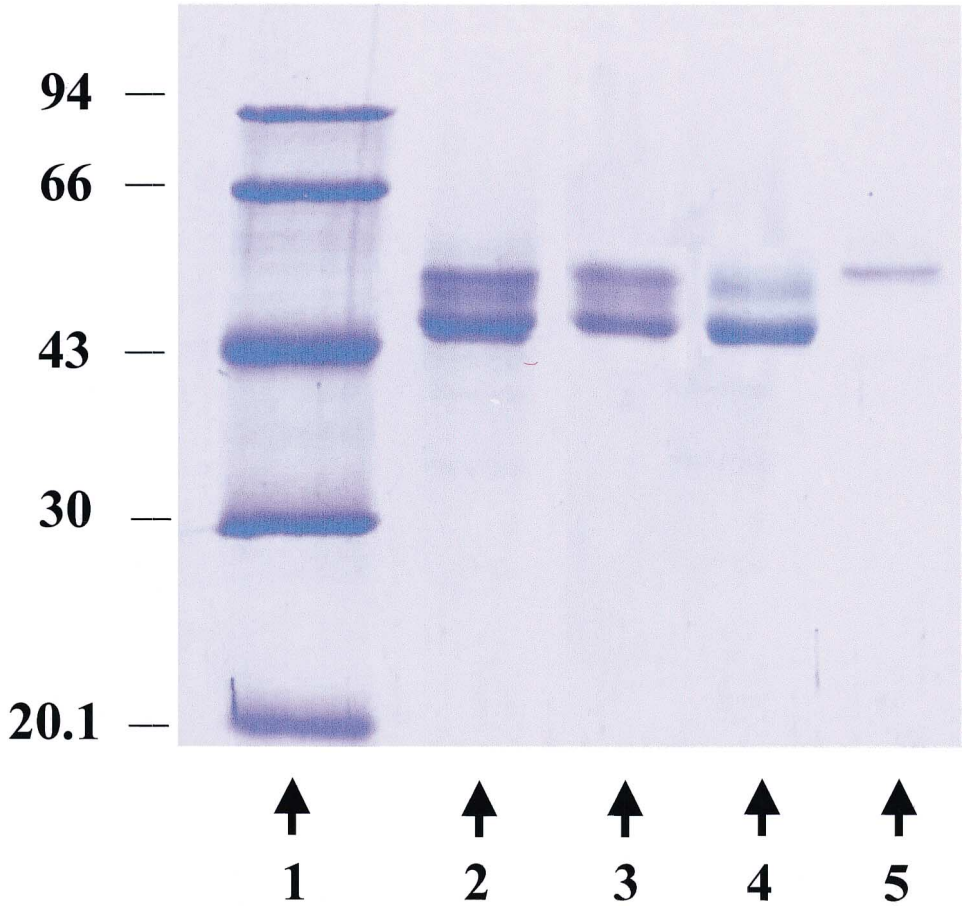


Figure 2. SDS-PAGE gel of different fractions in the CAL-A commercial preparation. Lane 1—molecular weight markers. Lane 2—commercial CAL-A preparation. Lane 3—supernatant after adsorption on octyl preparation. Lane 4—first supernatant adsorbed on octadecyl-Sepabeads. Lane 5—supernatant after octadecyl immobilization.

Table 2. Hydrolysis reaction substrate (±)-**2** at 50 mM with 25% of co-solvent at 45°C and pH 7 catalyzed by CE-PEI immobilized preparation

Co-solvent	Enzymatic activity
2-Methoxyethyl ether (diglyme)	5.5
Cellosolve	1.16
Dioxane	1.77
Acetonitrile (ACN)	0.09

inactivation and the very simple immobilization protocols.[24] This immobilized preparation presented activity against substrate (±)-**2** to a similar extent to using immobilized crude commercial preparation.

Therefore, the contaminant esterase (CE) present in the commercial preparation of CAL-A seems to be responsible for most of the enzymatic activity against (±)-**2**. We suggest that this contaminant is an esterase and not a lipase because it was adsorbed only to a very low extent on hydrophobic supports.[9,10]

In order to optimize the activity of the immobilized preparation, we assayed the maximum loading of the enzyme on our PEI coated support and it was possible to immobilize approximately 65 mg of pure CE. This immobilized preparation showed activity in the hydrolysis of substrate (±)-**2** using the substrate at a concentration of 2 mM in aqueous media of around 4 μmol h^{-1} g^{-1} (10-fold higher than the same immobilized preparation using crude enzyme). To determine the stability of the PEI-CE preparation, it was studied under different conditions: thus, it was incubated at 45°C in aqueous buffer and in the presence of 10–30% of several co-solvents (acetonitrile, dioxane, diglyme) analyzing the effect of the co-solvent nature and concentration on the enzyme stability. In all cases, the enzyme preparation maintained 100% of its initial activity after 2 weeks of incubation.

2.2. Optimization of the reaction conditions: high substrate concentration

To perform an industrial reaction, it is necessary to use a higher substrate concentration than the 2 mM used above (e.g. 50 mM). To achieve this goal it was necessary to add an organic co-solvent. The solubility of substrate (±)-**2** in several co-solvents was analyzed in a previous work,[23] where the best results were obtained using dioxane or acetonitrile. However, we have to take into account the inhibitory effect produced by the

co-solvent on the PEI-CE preparation. Table 2 shows the enzymatic activity of this immobilized preparation with 25% of several co-solvents having fully soluble substrate. The higher enzymatic activity for the immobilized preparation was found when diglyme was used as co-solvent (even by a fourfold factor). These results suggest that diglyme should be the co-solvent used in the hydrolysis reaction of substrate (±)-**2** since dioxane and acetonitrile has a very high inhibitory effect on the enzyme activity of PEI-CE.

The optimum temperature to solubilize substrate (±)-**2** was determined to be 45°C in the previous work.[23] Therefore, the hydrolytic resolution of (±)-**2** at 50 mM was performed in 25% diglyme at 45°C and pH 7, using the maximum enzyme loading in the preparation of the PEI-CE. Table 3 shows that this immobilized preparation presented a very high enantioselectivity (E >100) towards the hydrolysis of the 3*S*,4*R* diastereoisomer, obtaining enantiomerically pure (3*R*,4*S*)-(+)-**2** and (3*S*,4*R*)-(−)-**3** at 50% conversion.

For the applicability of this reaction in an industrial process, it is necessary to know the capacity of the immobilized preparation when it is re-used in several reaction cycles. The PEI-CE was used in three cycles, catalyzing the stereoselective hydrolysis of (±)-**2** at a concentration of 50 mM under the optimum conditions presented above. The catalytic properties remained unchanged through the successive cycles. A conversion of 50% was achieved after 72 h, obtaining pure (3*S*,4*R*)-(−)-**3** (ee >99%) from each reaction cycle.

3. Conclusion

The results shown in this paper exemplify how a contaminant enzyme from a commercial preparation of lipases may be responsible for a given catalytic activity. Simple methodologies, such as interfacial activation adsorption of lipases on hydrophobic supports, may be used to determine whether a lipase or a contaminant esterase is responsible for the observed activity of a certain preparation.

Thus, in this case it is possible to resolve (±)-*trans*-4-(4′-fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate (±)-**2** using an immobilized contaminant esterase contained in the commercial CAL-A preparation when CAL-A showed almost no activity with this compound.

Table 3. Enantioselective hydrolysis of substrate (±)-**2** at 50 mM and 25% of diglyme catalyzed by CE-PEI at pH 7 and 45°C

Time (h)	Conversion (%)	Preferred enantiomer	ee_s	E
8	14	3*S*,4*R*	16	>100
72	50		99	>100

Thus, the PEI-CE was used to obtain (3*S*,4*R*)-(−)-**3** with high enantioselectivity (E >100), which is an intermediate (with the correct configuration) in the synthesis of (−)-Paroxetine. Moreover, this immobilized preparation could be used in three cycles of reaction, with no change in its catalytic properties.

4. Experimental

4.1. General

The commercial extract of *C. antarctica* (*fraction A*) (CAL-A) (Novozym 868) was from Novo Nordisk (Denmark). Octyl-agarose 4BCL and were purchased from Pharmacia Biotech (Uppsala, Sweden). Octadecyl-Sepabeads was generously donated by Resindion Srl (Mitsubishi Chem. Corp.) (Milan, Italy). Glyoxyl-agarose 6BCL, 10BCL were kindly donated by the company Hispanagar SA (Burgos, Spain). Glutaraldehyde, Triton X-100, *p*-nitrophenyl propionate (*p*-NPP), polyethyleneimine (PEI) of molecular weight 25000 were from Sigma. Glyoxyl-agarose,[25] glutaraldehyde-agarose[26] and PEI-agarose[24] were prepared as previously described. (3*R*,4*S*)-, (3*S*,4*R*)- and (3*RS*,4*SR*)-*trans*-4-(4′-Fluorophenyl)-6-*oxo*-piperidin-3-ethyl carboxylate [(+)-, (−)- and (±)-**2**] were kindly donated by Vita Invest S.A. (Barcelona, Spain). Other reagents and solvents used were of analytical or HPLC grade.

4.2. Fractionation of crude CAL-A preparation

CAL-A crude preparation was exposed to octyl-agarose and octadecyl-Sepabeads in sodium phosphate buffer (5 mM, pH 7) at 25°C. This protocol has been shown to selectively adsorb lipases via interfacial activation mechanism. To desorbe the lipase from the support, 1% of Triton X-100 was employed. The desorbed and non-adsorbed proteins were stored at 4°C after their further immobilization.

4.3. Immobilization of different fractions of the proteins contained in CAL-A commercial preparation on different supports

Different immobilized preparations from crude and the different fractions of the purified enzyme were prepared following the procedures described below.

(i) Interfacial adsorption on hydrophobic supports, octyl-agarose[9] and octadecyl-Sepabeads.[10] Moreover, this protocol was used to purify the enzyme by adsorption chromatography.
(ii) Ionically adsorbed lipase on solid supports coated with PEI[24] (ionic microenvironment surrounding large areas of the protein). The immobilizations were carried out in sodium phosphate buffer (5 mM, pH 7) at 25°C. After immobilization, the immobilized preparations were washed with distilled water.
(iii) Multipoint covalent immobilization on glyoxyl-agarose beads (through areas with the highest density

of lysine (Lys) groups).[27] The immobilizations were carried out in aqueous sodium bicarbonate solution (25 mM, pH 10) at 25°C. To end the multipoint covalent attachment, sodium borohydride was added to a concentration of 1 mg/mL. After 30 min the immobilized preparation was washed with an excess of distilled water.

(iv) Covalent immobilization on glutaraldehyde-agarose beads.[26] The immobilizations were carried out in sodium phosphate buffer (25 mM, pH 7) at 25°C. To reduce the reactive groups, a volume of sodium bicarbonate (100 mM, pH 10) containing sodium borohydride (2 mg/mL) was added. After 30 min the immobilized preparation was washed with an excess of distilled water.

In all cases, the activity of suspensions and supernatants was assayed using the *p*-NPP assay as described below. Enzyme load was 0.5 mL crude preparation/g support in order to prevent diffusion problems and in all cases more than 95% of the esterase activity became immobilized on all different supports used. Protein concentration was determined by the Bradford method.[28] The calibration curve was obtained with bovine serum albumin (BSA) for determining protein concentrations in the range of 0.1–1.5 mg/mL.

4.4. Hydrolysis of *p*-nitrophenylpropionate (*p*-NPP)

This assay was performed by measuring the increase in the absorbance at 348 nm produced by the release of *p*-nitrophenol in the hydrolysis of *p*-NPP (0.4 mM) in sodium phosphate buffer (25 mM, pH 7) at 25°C. To initialize the reaction, lipase solution (0.05 mL) or suspension was added to substrate solution (2.5 mL). One international unit of *p*-NPP activity was defined as the amount of enzyme that is necessary to hydrolyze 1 μmol of *p*-NPP per minute (IU) under the conditions described above.

4.5. Enzymatic hydrolysis of (±)-2

Determination of the activity of different immobilized preparations from CAL-A on the hydrolysis reaction were performed by adding a sample of the preparation (1 g) to a mixture of phosphate sodium buffer (5 mL, 10 mM, pH 7) and 45°C to a substrate concentration of 2 mM (Fig. 3).

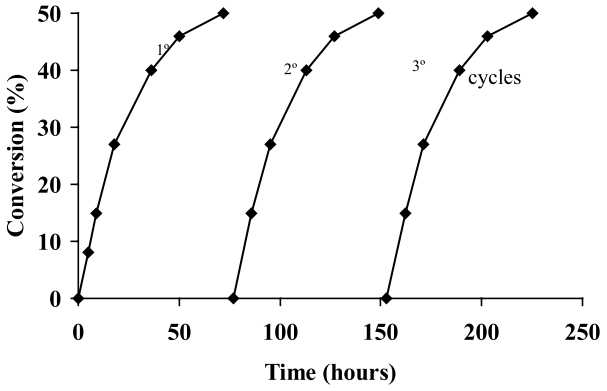


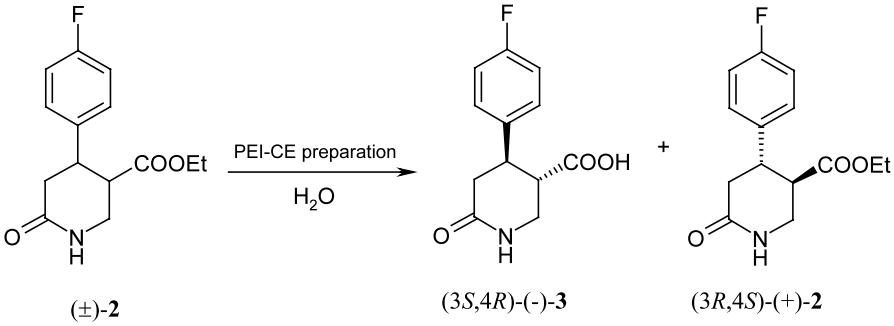
Figure 3. Different reaction courses of enzymatic hydrolysis of (±)-**2** catalyzed by PEI-CE. Experiments were performed using a substrate concentration of 50 mM with 25% diglyme at pH 7 and 45°C. Each cycle was 72 h.

Determination of the activity of CE-PEI preparation of CAL-A on the hydrolysis reaction was performed by adding a sample of the preparation (1 g) to a mixture of sodium phosphate buffer at (10 mL, 10 mM, pH 7) at 45°C with 25% of different co-solvents to a concentration of 50 mM (Scheme 2).

Finally, the activity reaction was performed in several cycles by adding 4 g of immobilized preparation to a solution of sodium phosphate buffer (20 mL, 10 mM, pH 7) at 45°C with 25% of diglyme to a substrate concentration of 50 mM.

In all cases, the pH value was kept constant during the reaction by automatic titration using a Mettler Toledo DL50 graphic pH-stat. The enzymatic activity was defined as μmol of substrate hydrolyzed per hour per g of support.

The degree of hydrolysis was quantified by reverse–phase HPLC (Spectra Physic SP 100) coupled with an UV detector (Spectra Physic SP 8450) on a Kromasil C18 (25×0.4 cm) column supplied by Analisis Vinicos (Spain). The elution was isocratic with a mobile phase of acetonitrile (30%) and 10 mM ammonium phosphate buffer (70%) at pH 3.0 and a flow rate of 1 mL/min.



Scheme 2. CE-catalyzed hydrolysis of (±)-**2**.

The elution was monitored by recording the absorbance at 270 nm. The retention time of the acid was 4.67 min while the ester appeared at 19 min. Each assay was carried out at least in triplicate.

4.6. Determination of enantiomeric excess and *E* value

At different degrees of conversion, the enantiomeric excess (ee_s) of the remaining ester was analyzed by Chiral Phase HPLC. The column was a Chiral-AGP (100×4.0 mm), the mobile phase was 10 mM ammonium phosphate buffer at pH 7.00. The analyses were performed at a flow rate of 0.5 mL/min by recording the absorbance at 210 nm. The retention time of the (3*R*,4*S*)-(+)-**2** was 14.73 min and the (3*S*,4*R*)-(−)-**2** appeared at 18.57 min identified using the pure enantiomers.

Enantiomeric ratio is expressed as an *E* value calculated from the enantiomeric excess (ee_s) of the remaining ester and the conversion degree (c) according to the previously reported method of Chen et al.[29]

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