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Highly enantioselective biocatalysts by coating immobilized lipases with polyethyleneimine

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ABSTRACT

Polyethyleneimine (PEI) modification on immobilized lipases greatly enhanced their enantioselectivity in the kinetic resolution of (±)-2-hydroxy-phenylacetic acid methyl ester. The enantiomeric ratio (*E*) of CNBr-agarose-CRL rose from 8 (without coating) to 20 (ee = 90%) after PEI coating in the hydrolysis at pH 5. In the case of CAL-B, the coating highly improved the enantioselectivity of the immobilized lipase from *E* = 1.5 (without coating) to *E* > 100 (ee > 99%). Moreover, this coating strategy improved the stability of the biocatalyst at high temperatures and in the presence of high co-solvent concentrations.

1. Introduction

The development of new biocatalysts with high selectivity, activity and stability for the application on different kinds of chemical reactions is of great interest for the implementation of enzymes on industrial processes.

Many different strategies have been developed to improve the catalytic properties of enzymes, such as rational protein engineering, directed evolution [1–3], immobilization methods [4,5] or chemical modification of specific amino acids on the protein [6].

Particularly, the immobilization approach was a very efficient and interesting strategy in tuning the catalytic properties of lipases [7], in part because of the peculiar catalytic mechanism of these enzymes [8]. Also some chemical modifications using polyethylene glycol or activated dextrans have given good results [9,10].

Polyethylenimine (PEI) is a polymer with high density of ionized tertiary, secondary, and primary amino groups. This fact may permit the strong ionic exchange of the polymer on any area of a protein surface containing anionic groups. Thus, this polymer has been used to stabilize proteins in solution by preventing oxidation, aggregation, and so on [11–13]. Also, supports coated with PEI were used to strongly adsorb many different proteins at neutral pH value [14] and stabilize multimeric enzymes by preventing subunit dissociation [15].

Furthermore, the PEI-coating strategy was used to stabilize immobilized lipases in anhydrous organic solvents [16], or immobilized penicillin G acylase in high co-solvent aqueous media [17].

This coating of all negative areas on the protein surface could introduce a hydrophilic environment around the active site and perhaps modify the electrostatic interactions and therefore the shape of the active site (Fig. 1).

Herein an efficient methodology to greatly improve the enantioselectivity of lipase from *Candida antarctica B* (CAL-B) and *Candida rugose* (CRL) has been developed by coating the enzymes in immobilized form with polyethyleneimine (Fig. 1). The kinetic resolution of (±)-2-hydroxy-phenylacetic acid methyl ester **1**, a particular interesting building block in the synthesis of different drugs [18–20] was used as model reaction.

2. Materials and methods

2.1. Materials

C. antarctica lipase (fraction B) (CAL-B) was purchased from Novozymes. *C. rugose* lipase, high molecular weight branched PEI (HMwPEI, Mw = 25,000, PDI = 2.5, molar ratio of primary/secondary/tertiary amine = approx. 1/1.2/0.76), p-nitrophenylbutyrate (pNPB) and (±)-α-hydroxy-phenylacetic acid methyl ester [(±)-**1**] were purchased from Sigma Chem Co. Lipases were purified and immobilized on octyl-agarose, glyoxyl-agarose and CNBr-agarose as previously described [21,22]. Biocatalysts were prepared with a loading of 1 mg lipase per gram support.

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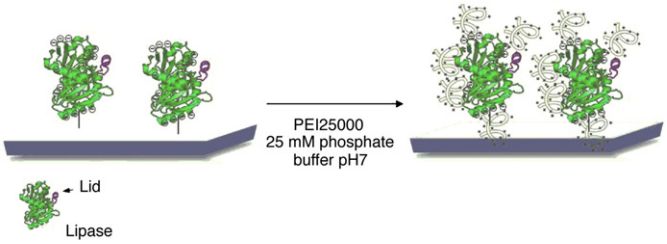


Fig. 1. PEI coating of immobilized lipases.

2.2. Enzymatic activity assay

The activities of the soluble lipase, supernatant and enzyme suspension were analyzed spectrophotometrically measuring the increment in absorbance at 348 nm produced by the release of *p*-nitrophenol (pNP) ($\in = 5.150\ M^{-1}\,cm^{-1}$) in the hydrolysis of 0.4 mM *p*NPB in 25 mM sodium phosphate at pH 7 and 25 °C. To initialize the reaction, 0.05–0.2 mL of lipase solution or suspension was added to 2.5 mL of substrate solution in magnetic stirring. Enzymatic activity is given as μmol of hydrolyzed pNPB per minute per mg of enzyme (IU) under the conditions described above.

2.3. Purification of CAL-B

The enzyme was purified from commercial crude extract by interfacial adsorption as previously described [21]. 1 mL of commercial solution (12 mg protein) was added to 19 mL of 5 mM phosphate buffer pH 7 and 1 g of octyl-agarose was added and the reaction was maintained for 3 h. After that, the suspension was filtered by vacuum and the solid was washed several times with distilled water. More than 95% of the enzyme was immobilized. This was used as immobilized preparation for selectivity studies.

For the preparation of the covalent immobilized catalysts, the lipase was desorbed from the support adding 10 mL of a solution of 25 mM phosphate buffer pH 7 with 1% Triton X-100 (v/v) to 1 g of octyl-CAL-B. SDS-PAGE gel of this preparation reveals just one protein band. A solution of 0.12 mg lipase purified/mL was obtained.

2.4. Covalent immobilization of CAL-B

A solution of 0.12 mg/mL of 25 mM phosphate buffer pH 7 with 1% Triton X-100 of purified lipase was used in each case. The immobilization was followed by the enzymatic assay described previously.

Immobilization on CNBr-activated agarose: The immobilization of CAL-B on CNBr-activated support was performed for 15 min at 4 °C to reduce the possibilities of getting a multipoint covalent attachment between the enzyme and the support. 10 mL of lipase solution was added to 1 g of support for 1 h. The enzyme-support immobilization was ended by incubating the support with 1 M ethanolamine at pH 8 for 2 h. Finally, the immobilized preparation was washed with abundant water, to eliminate the detergent. The immobilization yield was >95%.

Immobilization on glyoxyl-agarose: The pH of lipase solution (10 mL) was adjusted at pH 10.1 and the solution was added to 1 g of support and the reaction was maintained for 24 h. When the immobilization was finished — analyzed by activity assay described before — 20 mg of $NaBH_4$ was added and after 30 min the immobilized preparation was abundantly washed with distilled water. The immobilization yield was >95%.

2.5. Polymer coating of immobilized enzymes

1 g of polyethyleneimine was dissolved in 20 mL of 25 mM sodium phosphate adjusted to pH 8.0. Then, 1 g of immobilized lipase preparation (1 mg lipase/g support) was added to this solution. The suspension was gently stirred for 1 h and the modified enzyme preparations were washed with distilled water (10 × 50 mL) (Scheme 1). The immobilized preparations were filtered under vacuum and stored at 4 °C.

2.6. Enzymatic hydrolysis of (±)-1

The hydrolysis of **1** was performed by adding 0.04 g of catalyst to 10 mL of 5 mM substrate in 10 mM buffer solution (sodium acetate at pH 5 or sodium phosphate at pH 7) at 25 °C. During the reaction, the pH value was maintained constant by automatic titration using a pH-stat Mettler Toledo DL50 graphic. The degree of hydrolysis was analyzed by reverse-phase HPLC (Spectra Physic SP 100 coupled to an UV detector Spectra Physic SP 8450) on a Kromasil C18 column (15 × 0.4 cm) supplied by Analisis Vinicos (Spain). At least, triplicates of each assay were made. The elution was performed with a mobile phase of acetonitrile (30%, v/v) and 10 mM ammonium phosphate (70%, v/v) at pH 2.95. The flow rate was 1 mL/min. The elution was monitored by recording the absorbance at 254 nm.

2.7. Determination of enantiomeric excess

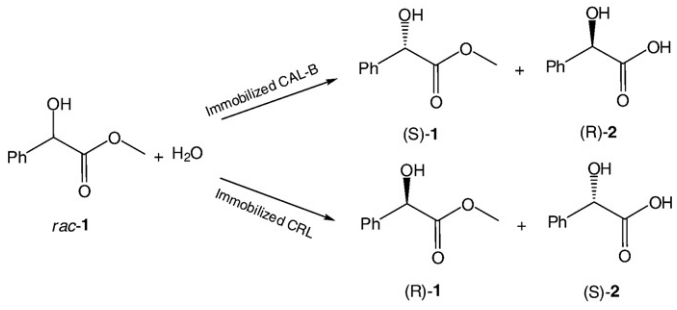
The enantiomeric excess (ee) of the produced acid (**2**) was analyzed by Chiral Reverse Phase HPLC. The column was a Chiracel OD-R and the mobile phase was an isocratic solution of (5%, v/v) acetonitrile and (95%, v/v) 0.5 M $NaClO_4/HClO_4$ at pH 2.3 and the analyses were performed at a flow of 0.5 ml/min by recording the absorbance at 225 nm.

2.8. Calculation of E value

The enantiomeric ratio (*E*) was defined as the ratio between the percentage of hydrolyzed R and S isomers (from racemic mixture) at hydrolysis degrees between 10 and 20%, where the reaction kinetic is in first order. R- and S isomers were used as standard enantiomerically pure products. Also the *E* value was calculated from the enantiomeric excess of the release acid (ee_p) and the conversion degree (*c*) using the equation $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$ described by Chen et al. [22].

2.9. Inactivation of CNBr-CAL-B preparations against T and co-solvent

0.5 g of biocatalyst was dissolved in 5 mL of 25 mM sodium phosphate buffer (with 0% or 60% (v/v) acetonitrile), incubated at 25 °C or 50 °C. The remaining activity at different times was measured by the assay described above using pNPB as substrate.



Scheme 1. Kinetic resolution of (±)-**1** by different lipase immobilized preparations.

Table 1
Effect of the PEI-coated on CAL-B preparations in the hydrolysis of (±)-**1** at pH 7 and 25 °C.

Immobilized preparation	Activity[a]	c[b] (%)	ee[c] (%)	E ratio
CNBr-CAL-B	1.00	20	70	6.8
CNBr-CAL-B-PEI	1.20	12	73	7
Glyoxyl-CAL-B	0.036	14	88	18
Glyoxyl-CAL-B-PEI	0.20	19	88	19
Octyl-CAL-B	5.56	20	61	4.2
Octyl-CAL-B-PEI	12.32	13	61	4.2

[a] Activity in μmol g_{cat}^{-1} min^{-1}.
[b] c = conversion.
[c] ee_p = enantiomeric excess of product (*R*-**2**).

3. Results and discussion

The PEI modification on three different immobilized preparations of CAL-B was applied and the activity and enantioselectivity of these biocatalysts were evaluated in the hydrolysis of **1** at pH 7 (Table 1, Scheme 1).

The activity of the enzyme was improved in all cases, 5 fold for glyoxyl-CAL-B (enzyme immobilized by multipoint covalent attachment), 2 fold for octyl-CAL-B (enzyme immobilized by hydrophobic interactions) and 1.2 fold increment for CNBr-CAL-B (enzyme immobilized by one-point covalent attachment). However, the enantioselectivity of the enzyme in these experimental conditions was not altered after coating (Table 1). The modified biocatalysts were evaluated at pH 5 considering the effects on the lipases selectivity by changes on the experimental conditions [23]. The activity of CNBr-CAL-B and octyl-CAL-B slightly decreased after coating whereas a huge increment in the activity value was observed for glyoxyl-CAL-B (44 fold) (Table 2). The enantioselectivity slightly increased at these conditions for glyoxyl-CAL-B and octyl-CAL-B after coating whereas a remarkable enhancement was observed for CAL-B immobilized on CNBr-agarose; PEI coating improved the enantiomeric ratio from 1.5 (without coating) to more than 100 (ee>99% of *R*-**2**).

In view of this enormous effect on the enantioselectivity, the PEI-coating effect was studied on the stability of CNBr-CAL-B at high temperature or co-solvent concentration (Fig. 2).

When the CAL-B immobilized preparations were incubated at 50 °C and pH 5, the coating immobilized enzyme maintained more than 70% activity after 24 h (Fig. 2A).

High co-solvent concentration is a relevant parameter for a possible industrial application of this strategy, especially using quite hydrophobic substrates. The stability of these biocatalysts was studied at pH 5 in the presence of 60% acetonitrile (Fig. 2B). The PEI-CNBr-CAL-B preparation preserved more than 90% of catalytic activity after 30 h of incubation.

In order to expand the scope of the methodology, the effect of PEI coating was also studied on CRL — another very useful enzyme in biotransformations — immobilized on CNBr-agarose (Table 3).

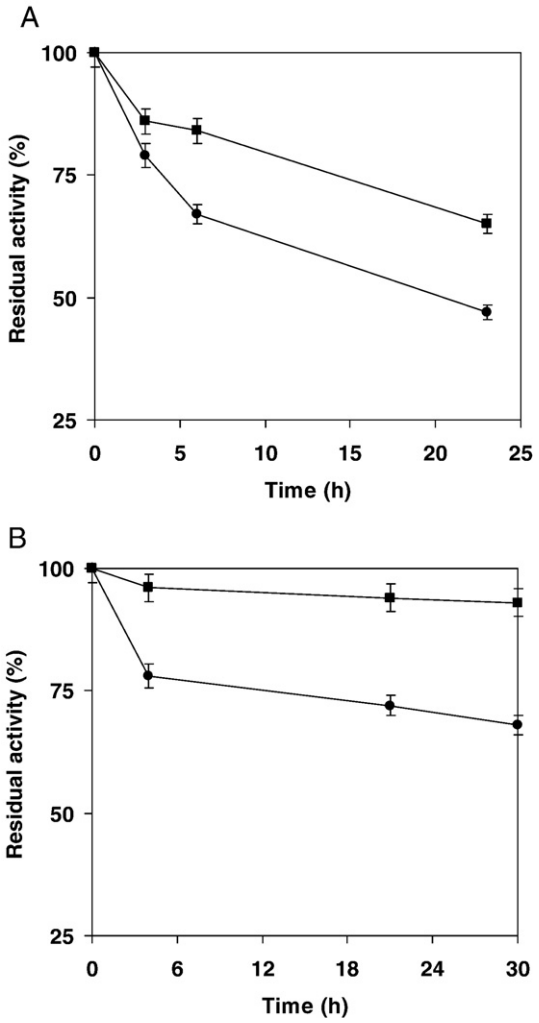


Fig. 2. Stability of PEIylation on CNBr-CAL-B immobilized preparation. A. Incubation at 50 °C and pH 5. B. Incubation at 25 °C, pH 5 in the presence of 60% acetonitrile (v/v). CNBr-CAL-B (circles) and PEI-CNBr-CAL-B (squares).

The modification of immobilized CRL with PEI caused an improvement on the activity of 2 fold also at pH 7 and 5. However, in the same way that with CAL-B, the enantioselectivity was not affected by coating when the biocatalyst was used at pH 7, although the enantiomeric ratio (E) of the immobilized lipase rose from 8 (without coating) to 21 (ee = 90% of S-**2**) at pH 5.

In conclusion, physical modifications of lipase surfaces on solid-phase by polyethyleneimine seem to be a simple and effective tool to modify the lipase enantioselectivity. This methodology permitted to modulate the selectivity of two known enzymes (CAL-B and CRL) specially immobilized on CNBr-agarose in the hydrolysis of (±)-**1**. Thus, the CNBr-CAL-B preparation after the PEI modification maintained the catalytic activity, improved the stability at high T and co-

Table 2
Effect of the PEI-coated on CAL-B preparations in the hydrolysis of (±)-**1** at pH 5 and 25 °C.

Coating	Activity[a]	c[b] (%)	ee[c] (%)	E ratio
CNBr-CAL-B	0.80	12	19	1.5
CNBr-CAL-B-PEI	0.72	12	>99	>100
Glyoxyl-CAL-B	0.014	9	85	13
Glyoxyl-CAL-B-PEI	0.625	22	86	17
Octyl-CAL-B	5.66	13	79	8.0
Octyl-CAL-B-PEI	4.78	19	80	9.0

[a] Activity in μmol g_{cat}^{-1} min^{-1}.
[b] c = conversion.
[c] ee_p = enantiomeric excess of product (*R*-**2**).

Table 3
Effect of the PEI-coated on CNBr-CRL preparation in the hydrolysis of (±)-**1** at 25 °C and 10 mM phosphate buffer.

Biocatalyst	pH	Activity[a]	c[b] (%)	ee[c] (%)	E ratio
CNBr-CRL	7	17	22	68	6.4
CNBr-CRL-PEI	7	43	15	74	7.7
CNBr-CRL	5	10	15	75	8
CNBr-CRL-PEI	5	20	10	90	21

[a] Activity in nmol g_{cat}^{-1} min^{-1}.
[b] c = conversion.
[c] ee_p = enantiomeric excess of product (S-**2**).

solvent concentration, and greatly enhanced the enantioselectivity from $E = 1.5$ to $E > 100$.

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