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Effect of the immobilization protocol on the properties of lipase B from *Candida antarctica* in organic media: Enantiospecifc production of atenolol acetate

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ABSTRACT

In this work, we intend to check the effect of the immobilization protocol on the performance of lipase B from *Candida antarctica* (CalB) in organic medium. To this purpose, CalB has been immobilized on Eupergit C (EC) under different conditions and on EC partially modified with ethylenediamine (EDA), iminodiacetic acid (IDA) or metal chelate (IDA-Cu^{2+}) and used for kinetic resolution of (R/S) 4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]benzeneacetamide (*rac*-atenolol). Enantiomeric resolution of atenolol was carried out by a transesterification reaction using vinyl acetate as acylant agent and an organic solvent as reaction medium. After a preliminary optimization of the reaction to obtain satisfactory yields, toluene was selected as the optimal solvent and the performances of the different CalB biocatalysts were compared. The R enantiomer was preferred in all cases but their performances were substantially different, with high differences in reaction rates, reaction yields in this kinetically controlled synthesis (EC-CalB gave a conversion of 76% while EC-IDA-Cu^{2+}-CalB gave just a 27%), and enantiospecificities (EC-CalB gave an *E* value of 65 while EC-IDA-Cu^{2+}-CalB gave a value of 13). Replacing toluene with hexane caused a decrease in enzyme activity, reaction yields and enantiospecificity of the reaction. It was remarkable that some preparations were much more sensitive to this solvent change than others. Considering that the activity decreased by less than 10% per reaction cycle, these differences are likely associated with the differences in the enzyme catalytic properties caused by the different immobilization protocols and not by inactivation of immobilized enzyme preparations during the reaction. These results confirmed that use of different immobilization protocols may be a powerful tool for altering enzyme properties when used in organic media.

1. Introduction

Lipases (EC 3.1.1.3) are highly versatile enzymes exhibiting excellent biochemical properties such as enantio- and regioselectivity and specificity in both aqueous and non-aqueous media [1–3]. Most of these enzymes may have two different conformations: a closed form and an open one. In homogeneous aqueous medium, this equilibrium is displaced towards the closed, inactive form, because an alpha-helicoidal polypeptide chain ("lid") covers its active site hindering its interaction with the medium [4–6]. However, in the presence of hydrophobic interfaces – droplets of oils [7,8], hydrophobic proteins [9], other open lipase molecules [10], or on the surface of hydrophobic supports [11] – the open-lid form is stabilized by its adsorption via the hydrophobic areas around the active center. This may cause structural changes and permits that the active center may be distorted without losing its catalytic function. Small changes in reaction media (change in reaction conditions, use of different organic solvents, addition of ionic liquids, etc.) or enzyme structure by enzyme engineering (chemical modifications, random or site directed mutagenesis, enzyme immobilization, etc.) can cause dramatic changes in the conformational equilibrium of a lipase and in the shape of its active center and, therefore, alter lipase biochemical properties (activity, selectivity or specificity) [12–16].

Several commercial applications of lipases are founded on their enantiospecificity for biosynthesis of esters, acids, amines and optically pure alcohols by kinetic resolution of racemic mixtures [1,2]. Likewise, in recent years the pharmaceutical industry has sought new routes for synthesis of enantiomerically pure chiral compounds because it has been well established that biological activity often has a close relationship with chirality [17]. There are many examples in the literature showing different biological activities – even an opposite one – between enantiomers of one compound [17,18]. Among these are aminoalcohols, such as propranolol and atenolol, where only their S-enantiomers exhibit antagonism of

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beta-adrenergic receptors while the other isomer only produces undesired side-effects [18].

There are few reports dealing with enantioselective lipase-catalyzed resolution of (R/S)-(4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]benzeneacetamide) (atenolol) [19–21] because of the poor activity and enantiospecificity usually displayed by lipases for this substrate, a secondary alcohol. Strategies such as protein engineering or molecular directed evolution have been proposed to improve enzyme specificity *versus* similar secondary alcohol [22–24].

Here we propose a simple but efficient way to improve lipase properties in the resolution of (R/S)-atenolol; the preparation of a small library of immobilized lipase biocatalysts. They have been prepared using different immobilization protocols, and perhaps they can present different orientations regarding the support surface [25]. By this methodology, it is possible to slightly distort different areas of the lipase structure or to reduce global protein flexibility and thus alter the catalytic properties of the enzyme [25]. Moreover, there are previous reports stating that substrate structure [26–28], solvent composition of the reaction medium [29–31], thermodynamic activity of water (a_w) [30,32,33] or temperature [34,35] significantly influence lipase enantiospecificity.

Lipase B from *Candida antarctica* (CalB), one of the most widely used lipases, was selected for this study [36]. This is a lipase with esterase activity, due to the fact that CalB presents a small lid that does not fully seclude the active center from the medium [37,38]. Even with this small lid, CalB maintains its capacity to become adsorbed by the hydrophobic areas around the active center on hydrophobic surfaces and act on the interface oil–water [4–6], although this adsorption did not produce an increment on enzyme activity. The enzyme has been used in many different reactions [36,39,40]. The enzyme structure is well known, it presents a molecular weight of 33 kDa [38,41] and an isoelectric point of 6.0 [41]. The properties of this enzyme have previously been modulated via different immobilization protocols, mainly in aqueous media [25]. This may be possible due to some mobility of the lid like area recently studied [42].

Eupergit C (EC), an epoxy support [43,44], has been employed to prepare lipase derivatives with different orientations. Epoxy supports may react with many different groups of the proteins surface (amino, hydroxyl, thiol), but they do so very slowly [45]. In fact, proteins become immobilized on epoxy supports via a two-step mechanism. First the enzyme is physically adsorbed on the support; second, covalent linkages between adsorbed enzyme and epoxy groups on the support occur [46–48]. EC is fairly hydrophobic and standard immobilization usually is performed using 1 M of sodium phosphate to produce hydrophobic adsorption of the proteins. Based on this two-step immobilization mechanism, heterofunctional epoxy supports have been designed [44,49]. These supports are epoxy supports where a small percentage of the epoxy groups have been modified to present carboxylic, amino or metal chelate groups. Thus, the enzyme is firstly physically adsorbed via different mechanisms, even using a very similar support. After this physical adsorption, further covalent attachment between the enzyme and the epoxy groups placed on the support may be obtained.

2. Materials and methods

2.1. Materials

Lipase from *C. antarctica B* (CalB) was obtained from Novozymes (Parana, Brazil). Racemic and pure isomers of (R,S)-atenolol-HCl were purchased from Sigma–Aldrich (St. Louis, USA). Eupergit C was donated by Rohm Pharma (Darstamdt, Germany). All reagents and solvents were of analytical grade.

2.2. Methods

2.2.1. Lipase purification

Lipase was purified by chromatography based on the adsorption of the lipase on octyl agarose beads at low ionic strength [50–52]. 2 ml of commercial CalB (50 mg/ml having an activity of 28 U/mg) was diluted in 98 ml of 5 mM sodium phosphate pH 7 and 20 g of octyl agarose beads was added. After 2 h, the adsorbed enzyme was filtered and washed with distilled water. Then, the enzyme was resuspended in 100 ml of 5 mM sodium phosphate pH 7 containing 1% (v/v) Triton X-100 to desorb the enzyme [50–52]. The final enzyme solution presented an activity of 39 U/ml (very likely due to the elimination of some inhibitors during the purification). For further use, the enzyme was diluted 10 folds in 5 mM sodium phosphate pH 7 to dilute the detergent.

2.2.2. Determination of enzyme activity of lipase from Candida antarctica B

This assay was performed by measuring the increase in absorbance at 348 nm produced by the released *p*-nitrophenol in the hydrolysis of 0.4 mM *p*-nitrophenylpropionate (*p*-NPP) in 25 mM sodium phosphate buffer at pH 7.0 and 25 °C. To start the reaction, 0.05 ml of lipase solution or suspension was added to 2.5 ml of substrate solution. One international unit of activity (U) was defined as the amount of enzyme that hydrolyzes 1 μmol of *p*-NPP per minute under the conditions described previously.

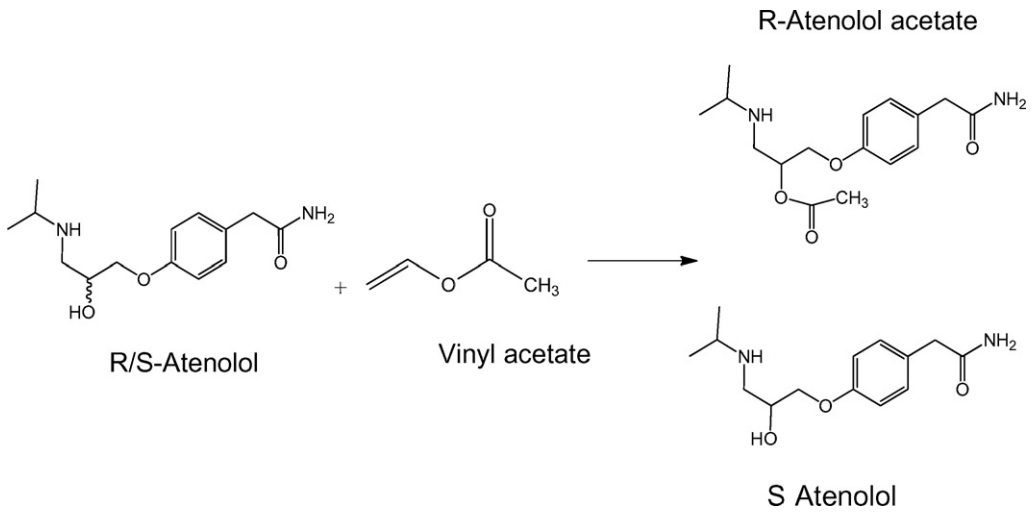
2.2.3. Preparation of epoxy supports for lipase immobilization

Epoxy groups from commercial Eupergit C (EC) were partially modified with different chemical compounds according to Mateo et al. [49] with some modifications. Optimization of the supports was carried out by studying the total immobilization rate and the covalent immobilization rate of CalB. Epoxy-modified supports for lipase immobilizations were the following:

(1) Aminated support (EC-EDA): A sample of 10 g of wet EC support was incubated for different times (from 0.5 to 24 h) in 60 ml of 0.2 M ethylenediamine (EDA) at pH 8.5 under gentle stirring. After that, the support was washed with distilled water and stored at 4 °C. The degree of modification was determined by titration with 10 mM NaOH of the amino groups introduced in the support.
(2) Iminodiacetic acid support (EC-IDA): A sample of 10 g of wet EC support was incubated for different times (from 0.5 to 24 h) in 18 ml of 2 M iminodiacetic acid (IDA) dissolved in 0.1 M borate buffer at pH 9 and 25 °C under very gentle stirring. The support was then washed with distilled water and stored at 4 °C. The degree of modification was determined by titration with 10 mM NaOH of the carboxylic groups introduced in the support.
(3) Copper-IDA support (EC-IDA-Cu^{2+}): A 10 g portion of IDA-Eupergit C was incubated in 60 ml of 0.2 M $CuSO_4$ solution with very gentle stirring. After 2 h, the support was washed with distilled water. This treatment should totally involve IDA groups in the support. The modification degree of the EC-IDA-Cu^{2+} supports was measured by detecting copper atoms by atomic absorption spectroscopy.

2.2.4. Immobilization of CalB on different Eupergit C supports

A 1 g sample of support was suspended in 10 ml of enzyme solution (containing 39 U of enzyme) in sodium phosphate pH 7 (at a concentration of 5 mM for, EC-EDA, EC-IDA and EC-IDA-Cu^{2+} [49,53] and a concentration of 1 M [54] for EC supports) containing 0.1% Triton X-100 at 25 °C. Moreover, 10 ml of crude CalB diluted in 5 mM sodium phosphate at pH 7 (having 28 U/ml) was directly offered to 1 g of EC. Periodically, samples of the supernatants were withdrawn and their enzyme activities were analyzed. After immo-



Scheme 1. Scheme of the reaction.

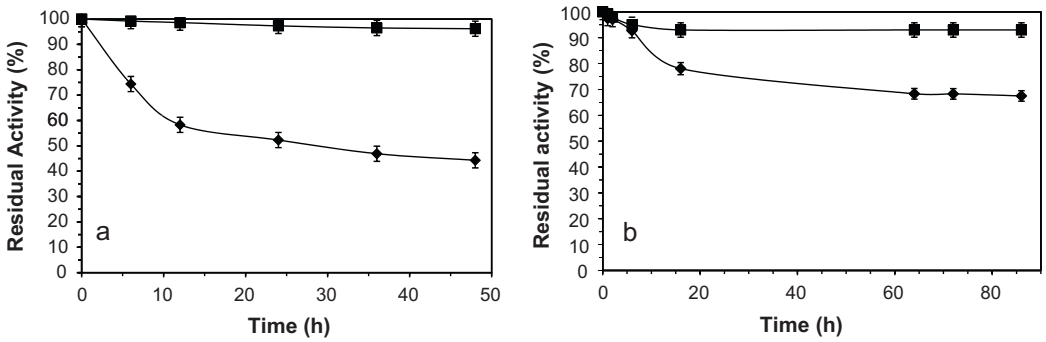


Fig. 1. Immobilization courses of CalB on EC. (a) Immobilization of purified CalB on Eupergit C in 1 M sodium phosphate at pH 7 as described in Section 2. (b) No purified CalB immobilized at 5 mM sodium phosphate on EC. Suspension (■) and supernatant (♦).

bilization, to completely block the epoxy groups, 1 g of support or enzyme-support derivative was incubated in 5 ml of 50 mM sodium phosphate containing 1 M of glycine–HCl at pH 8.5 for 24 h at 20 °C [55]. The immobilized preparations of CalB were then washed with distilled water and stored at 4 °C.

2.2.5. Enzymatic synthesis of atenolol acetate

The scheme of the reaction is shown in Scheme 1. Esters of (R/S)-atenolol were synthesized in 10 ml screw-top vials. In a typical experiment, atenolol (concentration range was 3.3–16.6 mM) and 33.3 mM vinyl acetate was dissolved in 3 ml toluene or hexane. The water activity values of the reaction components were adjusted before the reaction to $a_w = 0.15$ by pre-equilibration in the presence of saturated salt solution $MgCl_2$ (a_w 0.15) during 48 h [56,57]. The water activity of the pre-equilibrated system was measured by a Karl Fischer titrator. The reaction was initiated by the addition of immobilized lipase preparation. The reaction mixture was continuously shaken at 200 rpm and 25 °C.

2.2.6. HPLC analysis

Atenolol acetate was quantified by HPLC (Agilent 1100) using a silica gel C-18 column (Zorbax C-18, Agilent technologies). The mobile phase was composed of acetonitrile and 25 mM potassium phosphate buffer at pH 6 (70:30). The samples were eluted at a flow of 1.0 ml/min and quantified with a diode array detector (Hewlett Packard Model: G1315B) set at 230 nm. Enantiomers were resolved using a chiral column (25 cm, ES-OVM, Agilent technologies, USA) and the enantiomeric excess (ee) values of substrate and product were determined. As mobile phase, methanol and 25 mM potassium phosphate buffer at pH 6 (30:70, v/v) at a flow 1.0 ml/min was used. The enantiomeric ratios (E) were calculated from the ee values and yields using the equation reported by Chen et al. [58].

3. Results and discussion

3.1. Immobilization of CalB on Eupergit C (EC)

Fig. 1a shows the immobilization course of purified CalB on EC at high ionic strength (i.e. under standard conditions [46–48]). The enzyme immobilization proceeded slowly, likely due to the 0.1% Triton X-100 reducing hydrophobic adsorption between the enzyme molecules and the slightly hydrophobic surface of the support. After 48 h, around 50% of CalB was immobilized, whereas the enzyme suspension maintained a 90% of the activity initially offered to the support. Addition of an aqueous solution of 1% Triton X-100 in distilled water to the immobilized enzyme suspension after 48 h of immobilization released 25% of the immobilized enzyme from the support. This was due to the percentage of enzyme molecules just hydrophobically adsorbed on the support. After washing the support with distilled water, 80% of the immobilized enzyme activity was recovered (Table 1). This preparation was named standard EC-CalB.

Attempts to immobilize pure enzyme preparations on EC at low ionic strengths were unsuccessful (less than 5% immobilization after 48 h) because Triton X-100 prevented any adsorption of the enzyme on the support, thus avoiding reaction between epoxy groups and enzyme molecules that remained in solution [44,46–49] (results not shown). However, immobilization of non-purified CalB

Table 1
Immobilization of CalB on different EC supports.

Immobilized biocatalyst[a]	Immobilization yield[b] (%)	Recovered activity (%)	Activity[c] (U/g)
EC-CalB	50	80 ± 2	15.4 ± 0.3
LIS-EC-CalB	27.3	52 ± 2	5.6 ± 0.3
EC-EDA-CalB	44.2	71.6 ± 3	10.5 ± 0.2
EC-IDA-CalB	19	66 ± 2	6.7 ± 0.3
EC-IDA-Cu^{2+}-CalB	41	76 ± 1	13.2 ± 0.2

[a] Using optimal modification degree of epoxy groups from EC supports.
[b] Immobilization yield: percentage of enzyme that is immobilized on the support.
[c] Activity on *p*-NPP: percentage of activity exhibited by the immobilized enzyme when compared to the activity of the soluble form.

at low ionic strength in the absence of detergents achieved significant enzyme immobilization yields (Fig. 1b). Around 30% of CalB could be immobilized after 48 h. This result contrasts with the immobilization of other enzymes under similar conditions, where immobilization yields tend to be negligible [49,53]. This unique property of CalB might be due to its tendency, like other lipases, to become adsorbed on hydrophobic surfaces via the areas surrounding the active center [51]. After 48 h of immobilization, total enzyme activity of the suspension decreased by around 10% which, considering an immobilization yield of 30%, implies an immobilized enzyme activity decrease of around 35%. On the other hand, incubation of this immobilized preparation with aqueous solutions of 1% Triton X-100 at low ionic strength released around 60% of the immobilized enzyme, suggesting that most of the immobilized enzyme molecules had not covalently reacted with the support. Thus, after blocking remaining epoxy groups with glycine and washing of immobilized CalB preparations with distilled water, 50% of the expected activity was observed (Table 1). This preparation was called LIS-EC-CalB.

These results suggested that enzyme immobilization at high ionic strengths and in the presence of detergents and the immobilization at low ionic strength in the absence of detergents followed different mechanisms. These differences might be explained by the immobilization of CalB on the support by different areas of the protein as observed by the different degrees of enzyme desorption after 48 h when the immobilized enzyme preparations were incubated under desorption conditions. Moreover, enzyme activity recovery was also quite different in both cases, also suggesting the involvement of different protein zones in the reaction with the support.

At low ionic strength and in the absence of detergents, CalB would be expected to be immobilized involving the hydrophobic areas around its active center on the fairly thin, hydrophobic polymeric fibers of Eupergit C. Nevertheless, in this area of the surface of CalB, there are not lysine groups [37,38] available for reaction with epoxy groups of the support, which could explain the low degree of immobilization. On the other hand, at high ionic strength, adsorption of CalB via a similar mechanism did not occur; perhaps the high ionic strength makes the exposition of this hydrophobic area around its active site difficult. That way, the enzyme likely interacts with the support at other surface areas where there are more groups able to react with the support.

Next, a preliminary study of the production of atenolol acetate with the EC-CalB preparation was performed; looking for enzyme reaction conditions that could give high reaction yields where the enantiospecificity of the different immobilized CalB preparations could be properly compared.

3.2. Effect of different variables on the synthesis of atenolol acetate catalyzed by EC-CalB

Fig. 2 shows that reaction rates of enzymatic atenolol acetate production using toluene as solvent were 20% higher than with hexane. Consequently, reaction optimization was performed using toluene.

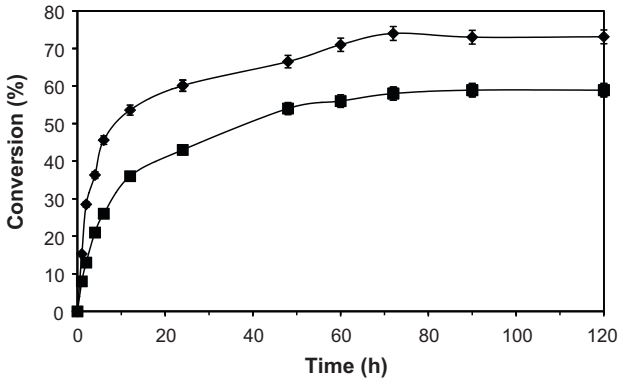


Fig. 2. Effect of organic solvent nature on atenolol acetate synthesis. Reaction conditions were: atenolol (16.6 mM), vinyl acetate (33.3 mM), EC-CALB (90 mg of biocatalyst/ml of reaction mixture) and toluene or hexane (to a total volume of 3 ml). Experiments were carried out as described in Section 2.2. Toluene (♦) and hexane (■).

Maximum yields in this transesterification reactions may be quite over the thermodynamic equilibrium and they are the result of 3 reactions; the synthesis of the target product and the hydrolysis of the activated acyl donor and product [59,60]. Thus, maximum yields are transient and depend on the kinetic properties of the enzyme. Activated acyl donor and nucleophile concentrations are important determinants of the yields obtained in transesterification reactions such as this [59,60]. We tried to obtain high enough maximum yields to be able to perform an appropriate comparison between the different biocatalysts used in this study. At higher activated acyl donor concentrations, higher amounts of acyl-enzymes are formed, therefore higher yields can be achieved. In this kinetically controlled enzyme reaction, a competition between substrate atenolol and water by the acyl-enzyme is produced [59,60]. Thus, a higher nucleophile concentration, it should be possible to get both higher reaction rates and yields at least until enzyme saturation by the nucleophile.

Fig. 3 shows that increase of atenolol concentration from 3.3 to 16.6 mM improved both reaction rates (from 0.016 to 0.066 μmol/min) and yields (from 50% to more than 70%). The almost linear increase of both reaction rates and yields as atenolol concentration was increased suggests that enzyme affinity for this compound is not very high. Reaction yields at 16.6 mM atenolol were well-suited for further studies of kinetic resolution of atenolol by CalB.

Increasing vinyl acetate concentration from 16.6 to 33.3 mM (Fig. 4) improved both reaction rates (from 0.032 to 0.066 μmol/min) and yields (from 45% to 70%). Thus, in the range concentrations used it was possible to obtain a first order increase on reaction rates, which suggests that EC-CalB affinity for vinyl acetate is also poor. However, both reaction yields and

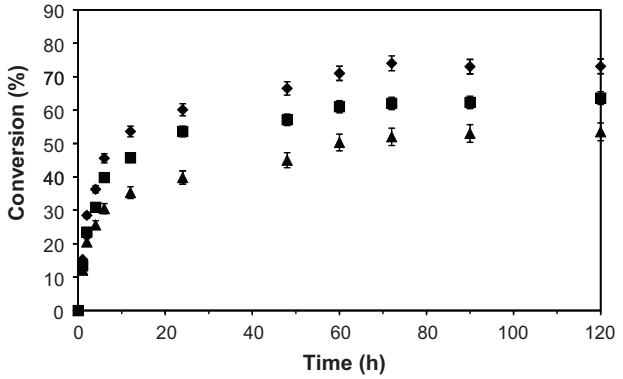


Fig. 3. Effect of atenolol concentration on the rates of transesterification of (R/S)-atenolol. The concentration of vinyl acetate was 33.3 mM, the biocatalyst used was EC-CalB (90 mg of biocatalyst/ml of reaction mixture) and the solvent was toluene (to a total volume of 3 ml). Experiments were performed as described in Section 2.2. [Atenolol] (▲) 3.3 mM, (■) 10 mM, and (♦) 16.6 mM.

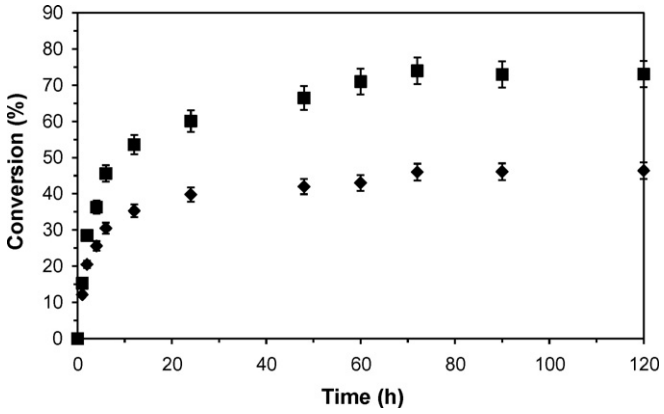


Fig. 4. Effect of vinyl acetate concentration on the rates of transesterification of (R/S)-atenolol. The concentration of atenolol was 16.6 mM, the biocatalyst was EC-CALB (90 mg of biocatalyst/ml of reaction mixture) and the solvent was toluene (to total volume of 3 ml). Experiments were performed as described in Section 2.2. Vinyl acetate: (♦) 16.6 mM; (■) 33.3 mM.

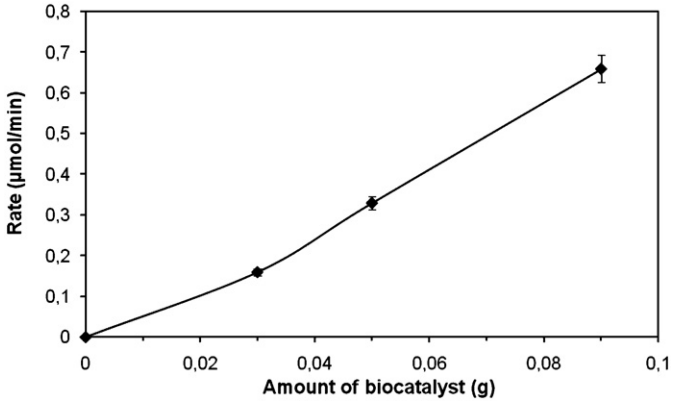


Fig. 5. Effect of biocatalyst loading on the reaction rates on the transesterification of vinyl acetate and (R/S)-atenolol. 16.6 mM atenolol and 33.3 mM vinyl acetate in toluene (to a total volume of 3 ml) were used. EC-CalB was used as biocatalyst. Experiments were performed as described in Section 2.2.

reaction rates were good enough under these conditions for our purposes.

As expected, increasing the amount of biocatalyst produced an increase in the reaction rates in a linear fashion (Fig. 5). This result suggests that the stirring during enzyme reactions was sufficient so as to avoid external diffusion limitations and that the support did not significantly affect the results. Thus, the comparison between the different biocatalysts was performed using 16.6 mM *rac*-atenolol, 33 mM vinyl acetate and 90 mg of biocatalyst/ml in the reaction mixture, because in these reaction conditions were obtained high enough yields in the production atenolol acetate to perform the studies.

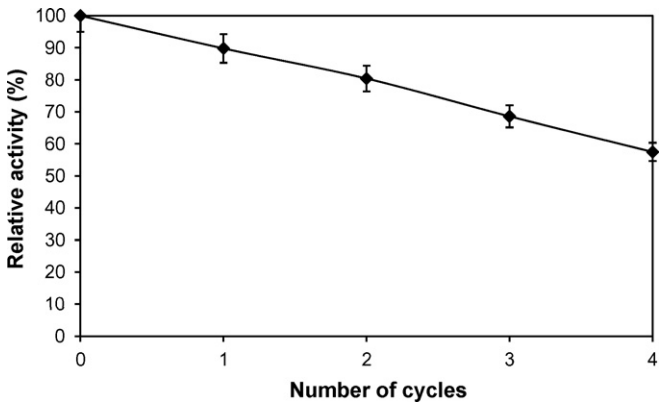


Fig. 6. Operation stability of the EC-CalB in catalyzing (R/S)-atenolol transesterification with vinyl acetate in toluene. Reaction conditions: atenolol (16.6 mM), vinyl acetate (33.3 mM), EC-CALB (90 mg of biocatalyst/ml of reaction mixture) and toluene. The reaction was allowed to proceed for 70 h in each cycle. The immobilized derivative EC-CalB was recovered by centrifugation and was reused for the next batch reaction under the same conditions without further washings.

3.3. Enzyme stability

EC-CalB immobilized preparations were re-used in four consecutive reaction batches under optimal conditions in order to evaluate enzyme operational stability. Fig. 6 shows that each reaction cycle resulted in around a 10% decrease in enzyme activity. Stability assays of immobilized enzyme preparations in the absence of substrates showed that enzyme activity remained unaltered after 100 h of enzyme incubation. Therefore, the decrease in enzyme activity during enzyme operation might be associated with the deleterious effect of vinyl acetate, which can chemically modify the enzyme. However, the low decrease of enzyme activity after each reaction cycle suggests that the enzyme activity was not seriously affected in a reaction cycle and that differences between different preparations in one cycle should be not attributed to enzyme inactivation during the reaction.

3.4. Immobilization of CalB on non-conventional heterofunctional Eupergit C

Fig. 7 shows the optimization of the degree of modification of epoxy groups from Eupergit C supports with different functional groups (EDA, IDA or IDA-Cu^{2+}) to immobilize CalB via different orientations [61]. Higher enzyme adsorption rates were observed at higher degrees of chemical modification of the epoxy groups. However, covalent immobilization rates decreased when the modification of the support was increased because fewer epoxy groups were available for covalent reaction with the protein [49,53]. In all cases, maximum covalent immobilization rates were obtained with modification degrees of the support of around 20% of the epoxy groups. These results agree with the previously reported by Mateo et al. [49], who showed that a compromise between adsorption rate and covalent reaction is necessary for efficient immobilization of the protein when using these kinds of heterofunctional supports.

CalB was also adsorbed in both EC-IDA and EC-EDA at pH 7.0. This result is remarkable because isoelectric point (Ip) of CalB is

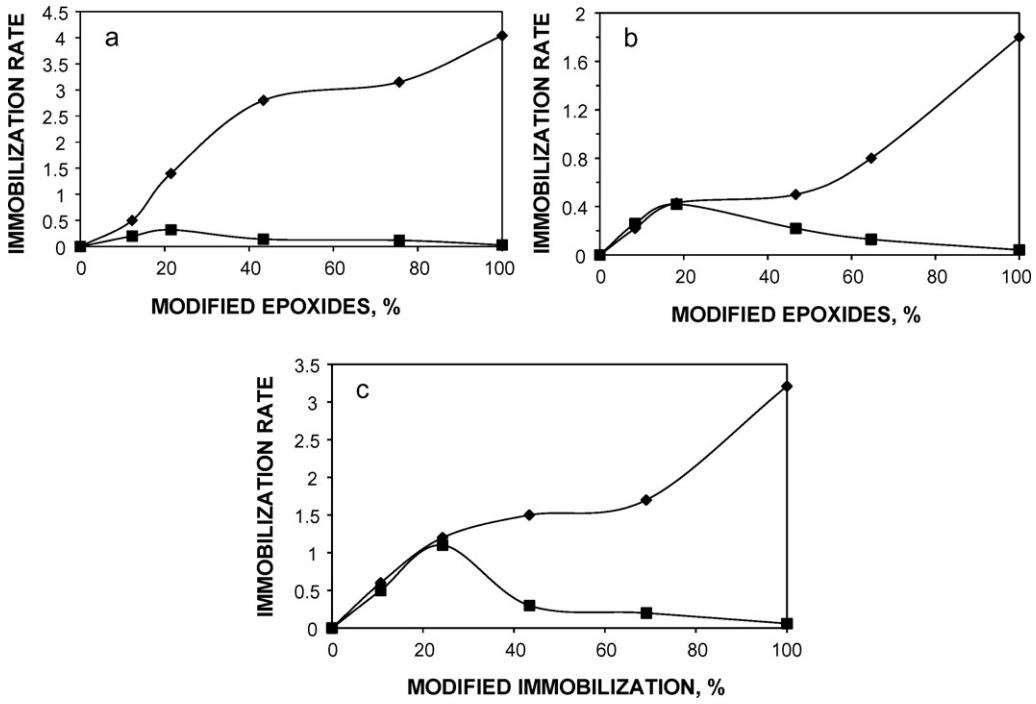


Fig. 7. Optimization of the modification of the epoxy groups of Eupergit C for the covalent immobilization of proteins. (a) EC-EDA, (b) EC-IDA, (c) EC-IDA-Cu^{2+}. The figure represents the immobilization of CalB at pH 7 and 5 mM sodium phosphate. Experiments were carried out as described in Section 2.2. The dotted line curve represents the rate of total immobilization (adsorption + covalent attachment) of the enzyme to the support. The solid line shows the rate of covalent immobilization of the support. That was determined by adding 0.5 M of NaCl to a sample of the immobilization suspension of (a), (b) and 100 mM imidazole for (c). This treatment released no covalently bound enzyme molecule from the support. Immobilization rate was defined as % of enzyme immobilized in the first hour.

6.0 [41]. At first glance, CalB should be not immobilized on EC-IDA supports because this support has properties of an anion exchanger and at pH 7.0, CalB is charged negatively as is the support. However, adsorption rate was only twofold higher using EC-EDA than EC-IDA (using fully modified supports) (Fig. 7). This effect could be due to EC-IDA supports acting as a sort of mixed ionic exchanger at pH 7, having positive charge near to the support surface (due to the tertiary amine bond) and two negative charges (the two carboxylic groups) from IDA. Mixed ionic supports have been reported to be able to immobilize enzymes that cannot be adsorbed on any of their equivalent standard ionic exchangers [62]. This mixed ionic exchange could be the reason for significant immobilization of CalB on EC-IDA at pH 7.

Thus, EC-IDA could immobilize CalB at zones containing both negative and positive charges at pH 7, while EC-EDA will immobilize CalB via the area having more negatively charged groups. On the other hand, EC-IDA-Cu^{2+} supports will immobilize CalB in zones rich in histidine moieties (although other groups may be also participating in the protein adsorption) [63,64].

Fig. 8 shows the immobilization courses of CalB onto the three optimized supports. EC-EDA and EC-IDA-Cu^{2+} supports immobilized around 40% of the offered enzyme after 48 h, while the IDA-support immobilized almost 20%. During immobilization, the activity of the immobilization suspension decreased by around 10%, indicating that enzyme activity of the immobilized enzyme molecules decreased by around 20–30%. Incubation of the immobilized preparations at high ionic strength (EC-EDA and EC-IDA) or in the presence of imidazol (EC-IDA-Cu^{2+}) solutions released non-covalently immobilized enzymes; with desorption percentages of 20, 12 and 10%, respectively. Final recovered enzyme activities of 70% for EC-EDA, 65% for EC-IDA and 75% for EC-IDA-Cu^{2+} were observed.

3.5. Effect of the different immobilization protocols on p-NPP/atenolol CalB activities and synthetic yields

It has been previously described that enzyme immobilization following different protocols may greatly affect their substrate specificity, mainly in aqueous medium [25,61]. Tables 1–2 compare hydrolytic activities of different CalB biocatalysts in aqueous media using *p*-NPP as substrate and *trans*-esterification activity using vinyl acetate and atenolol as substrates in organic medium. It should be noted that decreases of enzyme activity after each reaction cycle were quite similar for all immobilized enzyme preparations (8–12%, results not shown).

EC-CalB showed an activity of 15.4 U/g on *p*-NPP hydrolysis, and 0.11 U/g in the transesterification reaction in hexane. This activity ratio of approximately 140 was very similar for

Table 2
Activity and enantiospecificity ratios (*E*) of different immobilized derivatives of CalB during acylation of racemic atenolol in toluene.

Enzyme derivative	Activity[a]	Enantiopreference	Conversion (%)	ee (%)	E[b]	Maximum yield (%)
CalB Eupergit C	0.11 ± 0.03	R	22.5	96	65	76
LIS-CalB Eupergit C	0.0037 ± 0.0005	R	21.2	60	6	27
EC-EDA-CALB	0.072 ± 0.005	R	26.1	85	29	55
EC-IDA-CALB	0.042 ± 0.003	R	29.6	74	19	41
EC-IDA-Cu-CALB	0.034 ± 0.003	R	29.6	66	13	27

[a] Enzymatic activity was defined as (μmol/(min g biocatalyst)).
[b] Enantiomeric ratio was calculated as described by Chen et al. [58]. Reaction conditions were 16.6 mM atenolol, 33.3 mM vinyl acetate and immobilized derivatives (90 mg of biocatalyst/ml of reaction mixture) and toluene for a total volume of 3 ml.

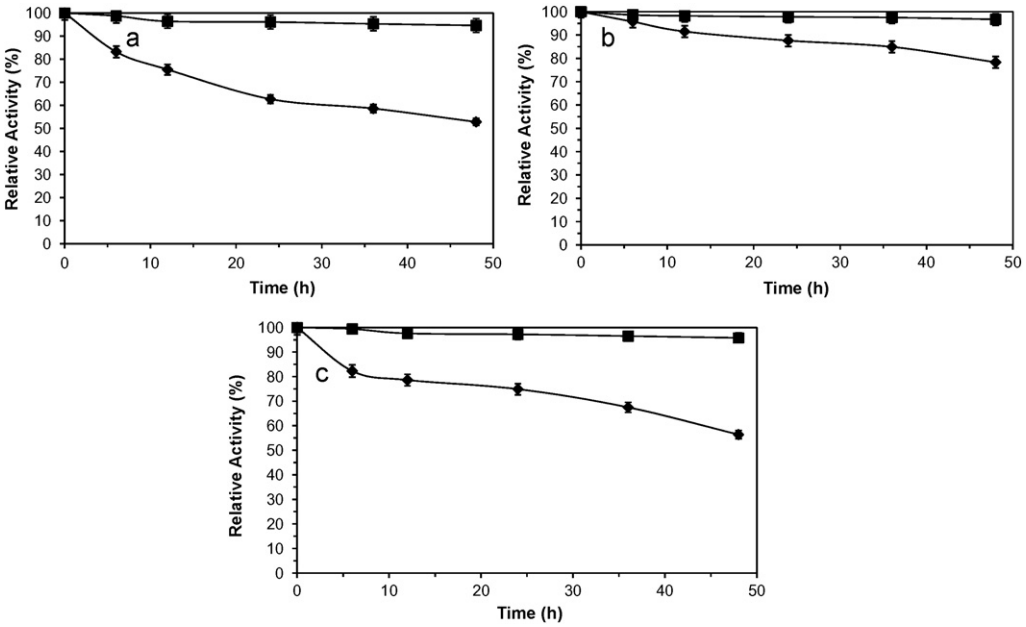


Fig. 8. Immobilization courses of CalB on different EC supports. (a) Immobilization of CalB on EC-EDA. (b) Immobilization courses of CalB on EC-IDA. (c) Immobilization courses of CALB on EC-IDA-Cu^{2+}. Experiments were carried out in sodium phosphate pH 7 as described in Section 2. (■) Suspension and (♦) supernatant.

Table 3
Activity and enantiospecificity ratios (*E*) of different immobilized derivatives of CalB during acylation of racemic atenolol in hexane.

Enzyme derivative	Activity[a]	Enantiopreference	Conversion (%)	ee (%)	*E*
EC-CALB	0.090 ± 0.002	R	26	89	43
LIS-EC-CALB[b]	0.0025 ± 0.0002	R	21.2	56	4
EC-EDA-CALB	0.048 ± 0.005	R	25	85	25
EC-IDA-CALB	0.011 ± 0.002	R	29.6	61	17
EC-IDA-Cu-CALB	0.030 ± 0.004	R	27	51	12

[a] Enzymatic activity was defined as (μmol/(min g biocatalyst)).
[b] Enantiomeric ratio was calculated as described by Chen et al. [58]. Reaction conditions were 16.6 mM atenolol, 33.3 mM vinyl acetate and immobilized derivatives (90 mg of biocatalyst/ml of reaction mixture) and hexane at a total volume of 3 ml.

EC-EDA-CalB and EC-IDA-CalB immobilized preparations, while EC-IDA-Cu^{2+}-CalB showed a ratio of almost 400. Using LIS-EC-CALB, this ratio rose to more than 1500, showing that this preparation was about 10 fold less efficient in the synthetic reaction than in the hydrolytic reaction compared to the standard preparation.

The maximum yields in this type of reaction are determined by the catalytic properties of the enzyme; they are a consequence of a balance among synthesis of the desired product, hydrolysis of the activated acyl donor and hydrolysis of the target product [59,60]. Table 2 shows large differences in maximum yields achieved using the different immobilized enzyme preparations of CalB. EC-CalB achieved the highest yields, with a 76%, while LIS-EC-CalB and EC-IDA-Cu^{2+}-CalB gave only a 27%; EC-IDA-CalB gave a 41% and EC-EDA-CalB produced a 55%.

CalB biocatalysts used in this study displayed vastly different hydrolytic and transesterification rates and were also fully different regarding their transesterification yields, suggesting that the enzyme presented altered kinetic properties. These results may be explained by different specificities of the different biocatalysts (as previously described [25]), an effect of the anhydrous media on the enzyme activity that may be different for the different immobilized preparations, or differences between synthetic and hydrolytic activities caused by the distortions promoted by the different immobilization protocols.

3.6. Effect of the immobilization protocol on the enantiospecificity of CalB in the synthesis of atenolol acetate

Enantiospecificities and activities of the different CalB biocatalysts are shown in Table 2. In all cases, CalB displayed preference for the R enantiomer of atenolol. The most active and enantioselective CalB preparation in this reaction was the standard preparation, with an *E* of 65. Enantiomeric ratios (*E*) of the other immobilized CalB preparations were in the range 6–29. Thus, these results showed that immobilization protocol can strongly alter the enantiospecificity of CalB in organic media, as was previously reported in aqueous media [25].

3.7. The effect of hexane and toluene on the enantiospecificity and activity of differently immobilized CalB

As described above, EC-CalB preparations showed 20% lower activity in hexane than in toluene. All the other CalB preparations also showed lower activity in hexane, but the effects were dissimilar (Table 3). EC-IDA-Cu^{2+}-CalB had lower dependence on the solvent, decreasing only a 10% its activity when toluene was replaced by hexane in the reaction mixture, while EC-IDA-CalB was the most sensitive (decreasing the activity by 75%). The other two CalB preparations showed 30–35% decreased activity.

Enantiospecificities of reactions catalyzed by EC-CalB decreased from 69 to 43 when toluene was replaced by hexane in the reaction

mixture, while for all the other preparations the decrement on the *E* values was much lower.

Thus, the change of the solvent presented very different effects on the activities and enantiospecificities of CalB immobilized following different immobilization protocols, perhaps by modulating the conformational changes induced by the different solvents.

4. Conclusions

This paper describes the preparation of different CalB biocatalysts based on the epoxy covalent attachment but with alterations in the first step of enzyme immobilization by hydrophobic, ionic exchange and immobilized metal chelates adsorption for producing biocatalysts with the same enzyme and covalent immobilization chemistry but with a different orientation of the enzyme on the support surface [61].

The immobilized preparations of CalB exhibited very different kinetic properties, giving very different maximum synthetic yields and enantiospecificities, although in all cases the activity lost per reaction cycle did not exceed from 12%. The best preparation, EC-CalB, reached a 76% maximum yield with an *E* value of 65, while the less suitable preparation gave a maximum yield of only 27% and an *E* value of 6. EC-CalB was used four times with a total decrease in the enzyme activity of 40% that was possibly due to acetylation of the enzyme by vinyl acetate.

The modulation of CalB by immobilization had been shown in many instances in aqueous medium (enantiospecific hydrolysis in resolution of racemic mixtures [65], regioselective hydrolysis of sugars [66], specific and regioselective hydrolysis on production of 1,2 diacetin [67], etc.). In this paper, the use of different immobilization protocols has been shows as a powerful tool to alter lipase properties, also in organic medium. Further optimization of the reaction conditions and assay of new biocatalysts with a more controlled immobilization [61] may permit to further increase the performance of CalB on this reaction. Though the study presented in this paper is specific to one drug, the outcome may be used for other important chiral intermediates and chemicals.

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