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Modulation of the enantioselectivity of *Candida antarctica B* lipase via conformational engineering: kinetic resolution of (±)-α-hydroxy-phenylacetic acid derivatives

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Abstract—The modulation, via immobilization and engineering the reaction medium, of the enantioselectivity exhibited by the lipase from *Candida antarctica B* (CAL-B) in the hydrolysis of α-hydroxy-phenylacetic acid derivatives is shown. The enzyme was purified and immobilized using different protocols to obtain immobilized enzyme preparations with different orientations and micro-environments. The catalytic properties (activity, specificity, enantioselectivity) of the resulting derivatives were found to be quite different from each other. The enantioselectivity (E value) strongly depends on the type of derivative and the conditions employed. Thus, the enzyme immobilized on cyanogen bromide (CNBr) presented $E=7.4$, while the PEI derivative yielded $E=67$ in the hydrolysis of α-hydroxy-phenylacetic acid methyl ester under similar conditions. Moreover, the enantioselectivity of the PEI derivative decreased from 67 to 14 on lowering the reaction temperature from 25 to 4°C at pH 5, while the E of some other derivatives improved significantly under similar experimental changes. Similar changes in the E values were observed in the hydrolysis of (*RS*)-2-butyroyl-2-phenylacetic acid. Using this substrate, the interfacially adsorbed enzyme (octadecyl) afforded an E value of only 2 at pH 5, while the glutaraldehyde derivative presented a high enantioselectivity (E >400) under all conditions studied. The corresponding (*S*)-ester and (*R*)-acid were obtained with excellent enantiomeric excess using the glutaraldehyde derivative, while using the interfacially immobilized one there was no appreciable enantioselectivity. Thus, using differently immobilized derivatives and different experimental conditions, lipase enantioselectivity could vary from negligible to up to 400. The experimental conditions were also found to have varying effects on the different lipase derivatives. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Chiral carboxylic acids are important building blocks for the synthesis of many pharmaceutical drugs[1] and natural compounds such as pheromones[2] and pesticides,[3] in which substances of very high enantiomeric excess (>99% ee) are often needed. In particular, pure isomers of (*R*)- and (*S*)-α-hydroxy-phenylacetic acid (mandelic acid) and their esters are very useful in organic synthesis. (*R*)-Mandelic acid is used for the synthesis of very interesting cephalosporin antibiotics such as Cephamandole and Cephonicid.[4] Enantiomerically pure acids may also be used in the resolution of racemates by selective precipitation.[5]

(*RS*)-2-Butyroyl-2-phenylacetic acid is used as a displacer with the Cyclobond-II chiral stationary phase, eliminating much of the trial and error traditionally involved in the development of a displacement chromatographic separation on cyclodextrin silica stationary phases.[6] Moreover, (*S*)-mandelic acid derivatives have been used as chiral solvating agents to induce significant non-equivalence in the ^{1}H NMR spectra of several heterocyclic β-dimethylamino esters and amides.[7] Other mandelic acid derivatives are used in the synthesis of 3-methoxy-4-hydroxy benzaldehyde (Vanillin), one of the most important flavoring and fragrance agents in beverage, food and perfumery chemistry, and for synthesis of *iso*-Vanillin, a very important building block used in the synthesis of the PDE4 inhibitors.[8]

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are perhaps the most frequently used enzymes in organic chemistry because they couple a broad range of specificity with high regio- and enantioselectivity, and thus,

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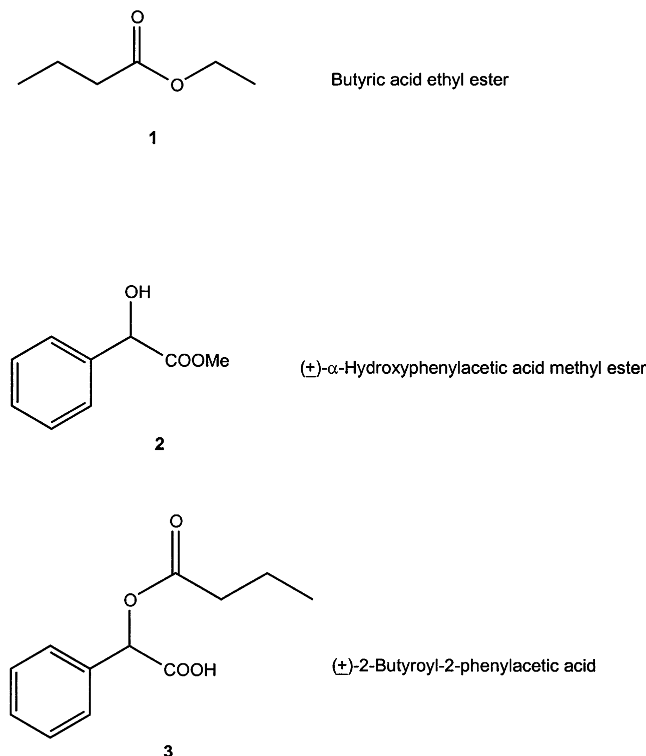
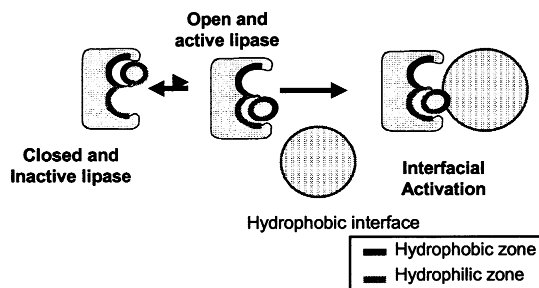
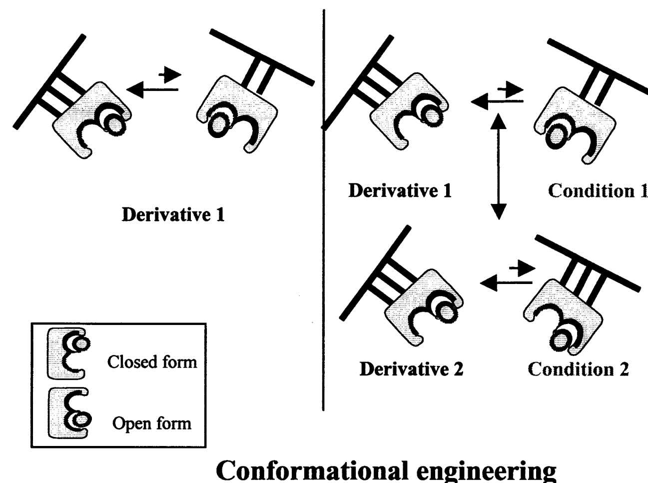


they have been employed as catalysts for kinetic resolutions of racemates (e.g. racemic secondary alcohols, racemic carboxylic acids, etc).[9–13] However, when using lipases in organic chemistry, we must bear in mind that their mechanism of catalysis involves dramatic conformational changes of the enzyme molecule.

Lipases may exist in two different structural forms. One of them, in which the active site of the lipase is secluded from the reaction medium by a helical oligopeptide chain called the 'lid', is considered an inactive (closed) form. The other, in which the lid is displaced and the active site is exposed to the reaction medium, is considered to be an active (open) form of the lipase. In homogeneous aqueous media, the lipase molecule is in equilibrium between these two structures, with the closed form predominating. However, upon exposure to a hydrophobic substrate such as a lipid droplet, only the open form of the lipase can interact with it and thus, the equilibrium shifts towards the open form (interfacial activation)[14,15] (Scheme 1). This equilibrium between two very dissimilar molecular forms also exists in most immobilized lipases.



Scheme 1. Catalytic mechanism of lipases in aqueous media.



Scheme 2. Intended conformational engineering of lipases.

The alteration of this equilibrium or the exact shape of the open form could change the catalytic properties of the enzyme. We propose that this could be achieved via immobilization techniques involving different areas of the enzyme, conferring different rigidity to the enzyme structure or even generating a certain special microenvironment surrounding the enzyme. This could reduce the freedom of the enzyme lid to move, altering the shape of the final open form of the lipase (Scheme 2).

Additionally, changes in the reaction conditions can cause marked effects on the properties of a lipase, perhaps because the conditions may promote a change in the global interactions of the open and closed forms of the lipase, again altering the exact shape of the open structure of the enzyme. The interaction between conditions and immobilization protocols could also be used to produce varying results.

This modulation of the enzyme properties by trying to alter the exact form of the active-site of lipase via physicochemical tools could be denominated 'conformational engineering' and it has been used successfully to modulate the behavior of different enzymes which undergo drastic conformational changes during catalysis, (e.g. penillin G acylase[16] and lipases[17]).

The lipase from *Candida antarctica* fraction B (CAL-B) is one of the most widely used lipases, because of its high activity and stability. It is commercialized both in free and immobilized form by diverse commercial suppliers. The enzyme has a molecular weight of 33 kDa, with an isoelectric point of 6.0; the 3D structure and amino acid sequence has been resolved by Uppenberg.[18] In addition, due to the fact that CAL-B presents a limited available space in the pocket of the active-site, this enzyme exhibits a high degree of selectivity. Thus, the enantioselectivity predicted by Uppenberg showed that the (*R*)-enantiomer should be preferred, behavior reliably predicted by Kazlauskas' rule.[19]

Herein, we have tried to study 'conformational engineering' in the modulation of the properties of this very

interesting lipase, using the kinetic resolution of (*RS*)-α-hydroxy-phenylacetic acid methyl ester **2** as a model reaction to study the possibility of altering the acyl donor site of the enzyme while (*RS*)-2-butyroyl-2-phenylacetic acid **3** has been used to study the nucleophilic site of the enzyme (Scheme 3).

2. Results and discussion

2.1. Specificity of different derivatives from CAL-B for different substrates

The initial activity displayed by different derivatives from CAL-B in the hydrolysis of different substrates, a simple aliphatic ester (substrate **1**) and two chiral compounds, one of them having the stereogenic center in the acyl-donor (substrate **2**) and the other in the nucleophile site (substrate **3**), are shown in Table 1.

For all of the CAL-B derivatives, the highest activity was found using substrate **1** and the lowest activity was found using substrate **3**. More interestingly, the specificity of the different immobilized derivatives were found to be quite different.

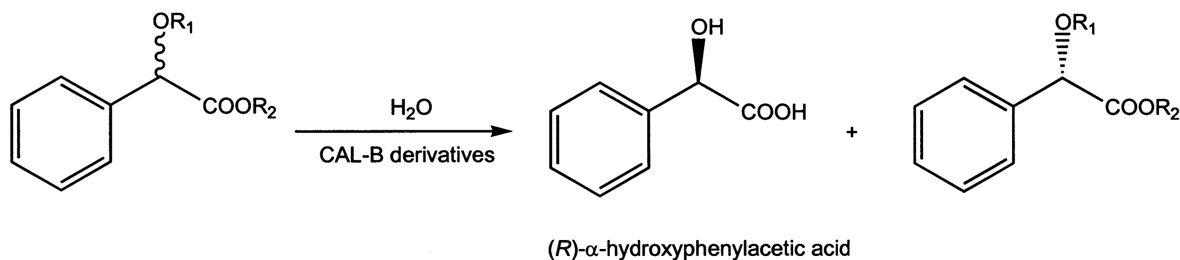
First, the results obtained at pH 7 will be discussed.

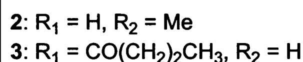
Using substrate **1**, the highest initial rate was obtained using the interfacially adsorbed derivative, which was almost a 4-fold factor more active than the PEI derivative, 5 or more fold higher than glutaraldehyde, glyoxyl or CNBr derivatives and approximately a 100-fold more active than the Eupergit-Cu derivative.

Using the (*R*)-isomer of substrate **2**, the situation was quite different. In this case the PEI derivative exhibited the highest initial rate, being over eight times more active than the interfacially activated derivative (octadecyl), more than 4-fold more active than the glutaraldehyde derivative and even more than 60 times more active than the Eupergit-Cu derivative. Intermediate differences were found with the other derivatives.

The initial rates achieved with derivatives catalyzing the hydrolysis of substrate **3** were much lower than the rates obtained with the other substrates. The highest initial rate was found when using the glutaraldehyde derivative, being twice as active as the glyoxyl one and over 50 times more active than the other derivatives. Moreover, it is remarkable that Eupergit-Cu, the less active derivative with other substrates, becomes almost twice as active as the interfacially immobilized, PEI or CNBr derivatives.

Table 1 also shows the effect of changing the pH from 7 to 5 when using substrates **2**–**3**. Using the (*R*)-isomer of substrate **2**, the effect of this decrease in pH on the initial rate was negligible for all derivatives. While some enzyme derivatives suffer certain decreases in the enzyme activity (octadecyl, PEI derivatives), the activity of other enzymes is slightly increased (glutaraldehyde, glyoxyl derivatives).





Scheme 3. CAL-B-catalyzed hydrolysis of substrates **2**–**3**.

Table 1. Specific activity of different CAL-B derivatives-catalyzed hydrolysis of substrates **1**–**3** at 25°C

Enzyme derivatives	Activity[a] **1**	Activity[a] (*R*)-**2**		Activity[a] (*RS*)-**3**	
	pH 7	pH 7	pH 5	pH 7	pH 5
Octadecyl	1141	7	5	6.1×10^{-4}	2×10^{-3}
PEI	323	60	58	6.6×10^{-4}	8.33×10^{-4}
Glutaraldehyde	250	13	15	0.0186	0.0318
Glyoxyl	184	7.72	9.91	8.3×10^{-3}	4.5×10^{-4}
CNBr	182	1.47	nd	4.5×10^{-4}	5.8×10^{-4}
Eupergit-Cu	14	0.75	nd	1.06×10^{-3}	1.66×10^{-3}

[a] Specific hydrolytic activity (μmol mg_{prot}^{-1} min^{-1}). The relative error was estimated to be ±3%.

However, the results were very different with substrate **3**. The most significant change was an almost 4-fold increase in initial rate for the octadecyl derivative at pH 5 (although with substrate **2** a decrease in the enzyme activity was observed) while the activities of the other derivatives (Eupergit-Cu and glutaraldehyde) increased by approximately a 2-fold factor with this pH change. However, the PEI and CNBr derivatives had the same initial rate under both conditions and the activity of the glyoxyl derivative was reduced almost by half at pH 5.

In the case of the octadecyl derivative, the increase in activity could be related to the extremely hydrophobic microenvironment surrounding the active site of the interfacially adsorbed lipase, formed by the hydrophobic pockets of the lipase and the hydrophobic surfaces of the support, which make the enzyme more selective towards the most hydrophobic substrate (at pH 5 ionization of the substrate **3** is lower).

2.2. Enantioselectivity of different derivatives from CAL-B-catalyzed hydrolysis of substrate 2

The *E* values of different immobilized derivatives from *C. antarctica B* lipase in the hydrolysis of substrate **2** at pH 7 and 25°C are shown in Table 2.

The preferred isomer for all derivatives was the (*R*)-isomer. The PEI derivative afforded the highest *E* value (67), while the CNBr derivative presented the lowest one (7.4). The enantioselectivities for the other derivatives were very similar with slight differences (between 10 and 20).

Moreover, the enantiomeric ratio for the CAL-B derivatives presented changes when the reaction conditions were altered. Thus, by increasing the salt concentration of the reaction media, the *E* value doubled for the Eupergit-Cu derivative, while the enantioselectivity of the PEI derivative decreased slightly. Other derivatives presented only marginal alterations of the *E* value with increasing ionic strength.

Fig. 1a shows the enantioselectivity for different CAL-B derivatives at pH 7 and different temperatures. Most of them showed an improvement in *E* values when the temperature was decreased. However, it is remarkable that the PEI derivative presented the opposite behavior. Thus, while at 4°C, PEI derivative had an *E* value lower than that of the glyoxyl derivative; at 25°C the situation was inverted. The PEI derivative is not very stable, thus it was not possible to further increase the reaction temperature. At pH 5 (Fig. 1b) the tendency of

Table 2. Enantioselectivity of CAL-B derivatives in the hydrolysis of a substrate **2** at pH 7, 25°C with different ionic strength

Enzyme derivatives		25 mM sodium phosphate buffer		100 mM sodium phosphate buffer	
	Preferred enantiomer	Ee	*E*	Ee	*E*
CNBr	*R*	60	7.4	–	nd
Octadecyl	*R*	70	12	70	12
Glutaraldehyde	*R*	75	16	80	21
Glyoxyl	*R*	78	19	79	20
PEI	*R*	91	67	88	53
Eupergit-Cu	*R*	79	20	68	10

Ee = enantiomeric excess; *E* = enantioselectivity.

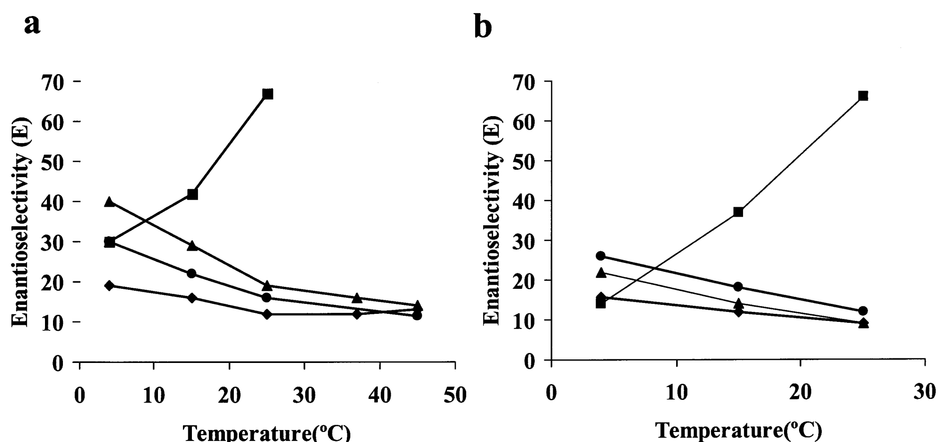


Figure 1. Effect of temperature on the enantioselectivity of different CAL-B-immobilized derivatives in the hydrolysis of substrate **2**; (a) enantioselectivity (*E*) at pH 7; (b) enantioselectivity (*E*) at pH 5. Glutaraldehyde (circles), glyoxyl (triangles), octadecyl (rhombus), PEI (squares). Experiments were performed in 25 mM sodium phosphate buffer with 10 mM of substrate at pH 7 as described in Section 4.

the *E* values of the derivatives was similar to that exhibited at pH 7, and the PEI derivative presented even more drastic changes in the *E* value on increasing the reaction temperature (from 12 at 4°C to 67 at 25°C).

Fig. 2 shows the enantioselectivities of some CAL-B derivatives at different concentrations of dioxane (pH 7 and 25°C). Again, the results were quite different when comparing the different derivatives. The hydrophobic derivative (octadecyl) maintained the same *E* value at different concentrations of dioxane. PEI derivative presented a dramatic decrease in the *E* value at 20% dioxane (from 67 to 25). Eupergit-Cu presented a slight decrease with increasing percentages of dioxane.

The most dramatic effect was found in the *E* value of the glutaraldehyde derivative increasing by more than a 3-fold factor at 15% dioxane. The same effect was found in glyoxyl-agarose derivative though its *E* value only doubled. Thus, the glutaraldehyde derivative was five times less enantioselective than PEI in aqueous buffer (in the absence of dioxane), while the *E* values achieved with both derivatives were very similar at 15% dioxane.

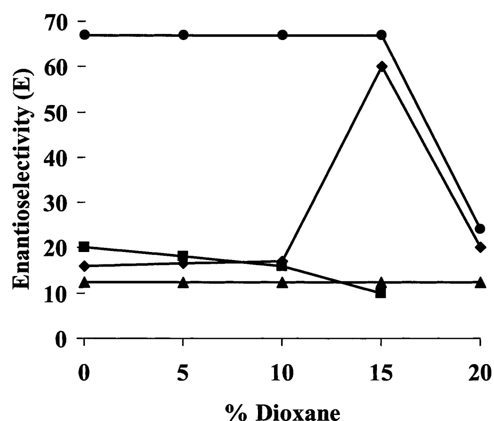


Figure 2. Enantioselectivity of different CAL-B derivatives at different concentrations of dioxane in the hydrolysis of substrate **2**. Glutaraldehyde (rhombus), octadecyl (triangles), PEI (circles), Eupergit-Cu (squares). Experiments were performed in 25 mM sodium phosphate buffer and 10 mM of substrate at pH 7 and 25°C as described in Section 4.

2.3. Enantioselective hydrolysis of substrate 3 catalyzed by different derivatives of *C. antarctica B* lipase

The enantioselectivity of different CAL-B derivatives in the hydrolysis of substrate **3** is shown in Table 3. This Table shows a high heterogeneity of results, comparing the different derivatives. However, all of them presented an enantiopreference for hydrolysis of the (*R*)-isomer.

Glutaraldehyde derivative presented the highest *E* value (*E* >400), no (*S*)-isomer of the product could be detected after full hydrolysis of the (*R*)-isomer of the substrate, at both pH 5 and 7. The lowest enantioselectivity was found using the CNBr derivative, which seemed to be unable to discriminate between both enantiomers.

The *E* value achieved with the other derivatives studied, was significantly higher when the pH was 7 instead of 5, the Eupergit-Cu derivative being the best one (*E*=36).

Using this substrate, the effect of the ionic strength on the enantioselectivity of the different derivatives was not evident in any case.

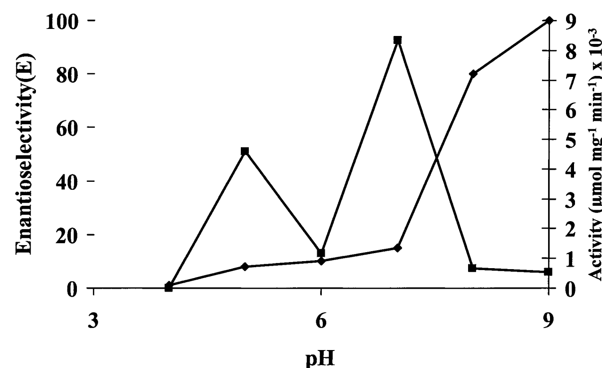


Figure 3. Effect of pH on the activity and enantioselectivity of CAL-B immobilized on Glyoxyl-agarose-catalyzed hydrolysis of substrate **3**. Experiments were performed in 25 mM sodium phosphate (pH 6–8), sodium acetate (pH 4–6), and sodium carbonate buffer (pH 8–9) using 0.5 mM of substrate as described in Section 4.

Table 3. Enantioselectivity of CAL-B derivatives-catalyzed hydrolysis of substrate **3** at 25°C

Enzyme derivatives		pH 5		pH 7 buffer	
	Preferred enantiomer	Ee	*E*	Ee	*E*
CNBr	*R*	6	1	nd	nd
Octadecyl	*R*	25	2	56	6
Glyoxyl	*R*	62	8	74	15
PEI	*R*	76	17	nd	nd
Glutaraldehyde	*R*	>99.5	>400	>99.5	>400
Eupergit-Cu	*R*	73	14	86	36

Ee = enantiomeric excess; *E* = enantioselectivity.

2.4. Effect of pH on the behavior of glyoxyl-CAL-B derivative when using compound 3 as substrate

Fig. 3 shows the activity and enantioselectivity exhibited by the glyoxyl derivative in the hydrolysis of substrate **3** at different pH.

The highest initial rate for this derivative was found at pH 7, but another maximum was observed with half the activity at pH 5. This effect might be explained considering the pK_a of the substrate (at first glance, the ionized substrate should enter the hydrophobic active site of the enzyme with more difficulty) and the actual capacity of the enzyme to hydrolyze the substrate.

The *E* value increased with increasing pH, having the maximum value at the highest pH studied (pH 9) ($E=100$) while at acidic pH (pH 4) this enzyme derivative hardly showed any enantiopreference. When the pH was increased the *E* value presented a slight increase up to pH 7, an inflexion point, wherein the *E* value suffered a dramatic increase.

3. Conclusions

We have been able to resolve compound **3** (ee higher than 99.5% after 50% hydrolysis of the racemic mixture), thus the corresponding (*S*)-ester and (*R*)-acid were obtained using the glutaraldehyde derivative. In the case of compound **2**, the highest ee achieved was 91%, after 50% hydrolysis using PEI immobilized lipase under optimal conditions.

The obtained results suggest that the properties of a lipase may be strongly modulated by conformational engineering: directed immobilization (altering the enzyme's rigidity and environment) and manipulation of the experimental conditions.

Thus, under fixed conditions, the same lipase immobilized on different supports (e.g. having different orientation or microenvironment) exhibited very different catalytic properties: different activity (even by a 100-fold factor) and different *E* value (*E* could be varied from 1 with a given derivative to more than 400 for another one; e.g. resolution of substrate **3** at pH 5 with lipase immobilized in CNBr or glutaraldehyde respectively). It is difficult to obtain such changes so simply with any other technique.

Also, slight changes of the reaction conditions can dramatically alter the properties of the lipase; for example, the *E* value for CAL-B immobilized on PEI support improved from 14 to 67 simply by increasing the temperature from 4 to 25°C, whilst the *E* value for lipase immobilized on a glutaraldehyde support improved from 19 to 64 by adding 15% of dioxane.

Finally, the effect of experimental conditions may vary for different derivatives: many examples show how the same change can improve, decrease or have no effect on the *E* value achieved with different enzyme preparations.

These results seem to support the initial hypothesis showed in Scheme 2. The fact that we are using a purified lipase may imply that the differences between different derivatives can only be explained by the varying behavior of enzyme/s immobilized on different supports.

Moreover, this process of altering the properties of enzymes that undergo dramatic changes in conformation during catalysis seems to be something that could be generally employed to manipulate their catalytic performance.

4. Experimental

4.1. General

Glyoxyl-agarose 6BCL and 10 BCL was kindly donated by the company Hispanagar SA (Burgos, Spain). Octadecyl-Sepabeads was generously donated by Resindion Srl (Mitsubishi Chem. Corp.) (Milan, Italy). Eupergit-C was kindly donated by Röhm GMBH (Degussa-Hüls Gruppe) (Darmstadt, Germany). Octyl-agarose 4BCL and cyanogen bromide (CNBr-activated Sepharose 4BCL) were purchased from Pharmacia Biotech (Uppsala, Sweden). Polyethyleneimine (PEI) (Mr 25000), glutaraldehyde, Triton X-100, *p*-nitrophenylpropionate (*p*-NPP), butyric acid ethyl ester **1**, $CuSO_4$ were purchased from Sigma. Glyoxyl-agarose,[20] glutaraldehyde-agarose,[21] Eupergit-Cu[22] and PEI-agarose[23] were prepared as previously described. (*R*)-, (*S*)- and (*RS*)-α-Hydroxyphenylacetic acid methyl ester (*R*)-**2**, (*S*)-**2** and (*RS*)-**2** were purchased from Sigma. Racemic 2-butyroyl-2-phenylacetic acid was kindly donated by Dr. Terreni (University of Pavia, Milan, Italy). Other reagents and solvents used were of analytical or HPLC grade.

4.2. Preparation of the enzyme

Lipase from *C. antarctica* (fraction B) (Novozym 525 L) was purchased from Novo Nordisk (Denmark). Protein concentration was measured using Bradford's method.[24] The calibration curve was obtained with bovine serum albumin (BSA) for determining protein concentrations in the range of 0.1–1.5 mg/mL.

To purify the lipase from any other contaminant proteins (e.g. esterases), the enzyme preparation was incubated in the presence of octyl-agarose at low ionic strength, following the previously described procedure.[25] Periodically, the activity of suspensions and supernatants was assayed using the pNPP assay as described below. After immobilization, the adsorbed lipase derivative was washed thoroughly with distilled water. To desorb the enzyme, the adsorbed lipase was washed with Triton X-100 1% in 5 mM sodium phosphate buffer at pH 7 and 25°C.

Following these protocols, a quantitative immobilization of lipase activity was observed and the SDS-PAGE analysis of the adsorbed protein derivative only showed a single band with a molecular weight corresponding to that of the native lipases. The final yield was near to 100%.

4.3. Immobilization of lipases on different supports

Different derivatives were prepared following the procedures previously described:

(i) Multipoint covalent immobilization on glyoxyl-agarose beads (through areas with the highest density of lysine (Lys) groups).[26]

(ii) Covalent immobilization on glutaraldehyde-agarose beads.[21]

(iii) Interfacial adsorption on a hydrophobic support, Sepabeads resin with the surface covered by octadecyl groups.[27] To immobilize the purified enzyme on an octadecyl support, the Triton was diluted 500-fold with sodium phosphate buffer 5 mM at pH 7.

(iv) Immobilization on CNBr-activated support using the protocol from Pharmacia (at pH 7, the immobilization should proceed at the most reactive group: the amino terminal).

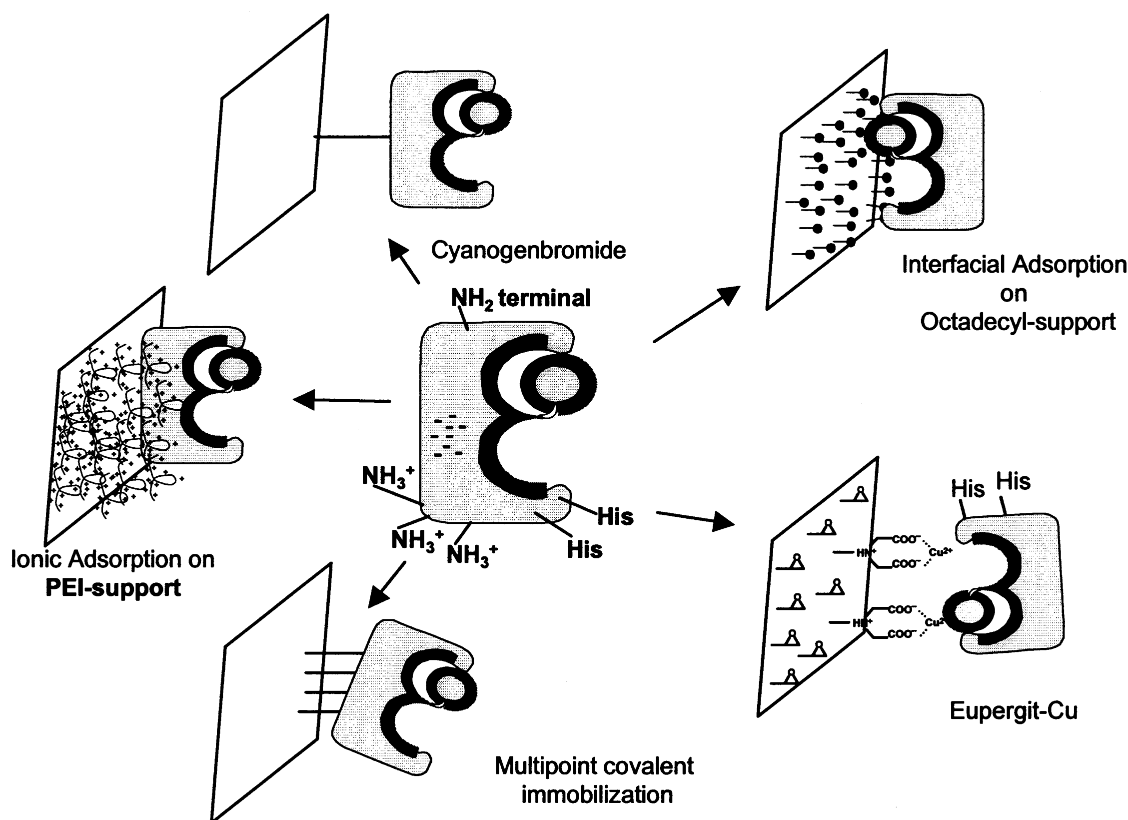
(v) Immobilization at regions of the protein rich in histidines (His) using Eupergit-C resin activated with copper chelates.[22]

(vi) Ionically adsorbed lipase on solid supports coated with PEI[23] (ionic microenvironment surrounding large areas of the protein).

A schematic representation of the different immobilization protocols is given in Scheme 4. The immobilizations were carried out at pH 7 and 25°C and periodically, activity of suspensions and supernatants was assayed by using the pNPP assay as described below. The enzyme load was 1 mg protein/mL of support (that is approx. 1–2% of the maximum load) in order to prevent diffusion problems and in all cases more than 95% of the lipase became immobilized on all different supports offered. Protein concentration was determined by the Bradford method.[24]

4.4. Hydrolysis of *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 0.4 mM pNPP in 25 mM sodium phosphate buffer at pH 7 and 25°C. To initialize the reaction, 0.05 mL of lipase solution 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 pNPP per min (IU) under the conditions described above.



Scheme 4. Different derivatives from *C. antarctica B* lipase.

4.5. Enzymatic hydrolysis of esters

The activities of different derivatives of lipase from *C. antarctica B* on the hydrolysis of different esters were investigated by adding the enzyme derivative (0.1 g) to a solution of the substrate **1** (10 mM, 150 mL); the enzyme derivative (0.2 g) to a solution of the substrate **2** (10 mM, 10 mL) ((*R*)- and (*S*)-isomers); the enzyme derivative (0.5 g) to substrate **3** (0.5 mM, 6 mL) under different conditions (pH, T, ionic strength) under mechanical stirring.

During the reaction, the pH value was maintained by automatic titration and the enzymatic activity (μmol of substrate hydrolyzed per min per mg of immobilized protein) was evaluated from NaOH consumption using a pH-stat Mettler Toledo DL50 graphic. The degree of hydrolysis was confirmed 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). Triplicates (at least) of each assay were made. The elution was isocratic with a mobile phase of acetonitrile (30%) and 10 mM ammonium phosphate buffer (70%) at pH 2.95 and a flow rate of 1.5 mL/min. The elution was monitored by recording the absorbance at 225 nm (substrate **1**) or 254 nm (substrates **2** and **3**).

4.6. Determination of enantiomeric excess (ee)

At different degrees of conversion, the ee of the released acid was analyzed by Chiral Reverse-Phase HPLC. The column was a Chiracel OD-R, the mobile phase was an isocratic mixture of 5% acetonitrile and 95% $NaClO_4/HClO_4$ 0.5 M at pH 2.1 and the analyses were performed at a flow rate of 0.5 mL/min by recording the absorbance at 225 nm.

4.7. Calculation of *E* values

For compound **2**, the *E* value was calculated as the ratio of the initial rate of hydrolysis of both pure isomers (hydrolysis of around 10% of the ester).

For compound **3**, the *E* value was calculated as the ratio between the percentage of hydrolyzed (*R*)- and (*S*)-isomer (from racemic mixture) at hydrolysis degrees of around 15%.

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