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Modulation of Immobilized Lipase Enantioselectivity *via* Chemical Amination

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Abstract: The aspartic and glutamic carboxylic groups of the surface of three different immobilized lipases (those from *Candida antarctica* (form B) (CAL-B), *Thermomyces lanuginose* (TLL) and *Pseudomonas fluorescens* (PFL) have been modified with ethylenediamine (after activation of the carboxylic function with carbodiimide). The exchange of groups with a negative charge for positively charged groups permitted us to greatly alter the activity, specificity and stereoselectivity of the lipase. Thus, in some cases, the activity of the lipases increased after the chemical modification while in other cases the activity was strongly reduced. Moreover, the effect of the experimental conditions on the activity was also strongly altered. Remarkably, the enantioselectivity of the enzymes in the hydrolysis of different mandelic acid derivatives was strongly modulated. For example, amination of the CNBr-CAL-B preparation greatly increased the enantioselectivity of the enzyme in the hydrolysis of (±)-2-hydroxyphenylacetic acid methyl ester, from an E value of 2 without modification up to $E > 100$, affording (*R*)-mandelic acid in high purity ($ee > 99\%$ at 50% conversion) at pH 7 and 4 °C. Thus, the chemical modification of lipases seems to be a very powerful tool to improve their performance as enantioselective biocatalysts.

Keywords: amination of protein surface; chemical modification of immobilized lipases; improved activity; improved enantioselectivity; lipase modulation; mandelic acid

Introduction

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are the most used enzymes in organic chemistry because they can recognize a wide diversity of substrates with a high regio- and enantioselectivity. In this way, they have been successfully employed as catalysts for kinetic resolutions of racemates (e.g., racemic secondary alcohols, racemic carboxylic acids, etc) and in asymmetric reactions.[1]

Lipases may exist in two different structural forms.[2] One of them, in which the active site of the lipase is secluded from the reaction medium by a helical oligopeptide chain called "lid", is considered an inactive (closed) form. The other one, which presents the lid displaced and the active site exposed to the reaction medium, is considered to be the lipase in an active (open) form. In homogeneous aqueous media, the lipase molecule exists as an equilibrium between these two structures. This equilibrium is, however, shifted towards the closed form. Upon interaction with a hydrophobic surface, such as a lipid droplet or other hydrophobic surface, the open form of the lipase becomes adsorbed to it and this equilibrium shifts towards the open form (interfacial activation).[3]

The alteration of this equilibrium or the shape of the open form of the lipase could change the catalytic properties of the lipase, such as its selectivity. For example, this has been already accomplished by using different immobilization protocols.[4] Here, taking into account that the movement of the lid involves different interactions among residues placed on the protein surface, (e.g., electrostatic interactions),[2] the alteration of this movement by chemical modification of the protein surface has been proposed. The modification of carboxylic groups of the superficial Asp and Glut residues by chemical amination[5] may produce an alteration in the interaction between the lid and the rest of the protein (e.g., forming new ionic bridges or promoting repulsion between groups formerly involved in ionic bridges).

Furthermore, it has been shown that small changes in the reaction conditions could exert an especially intense effect on the properties of the lipases.[4,6] This should be a consequence of the change in the global interactions of the open-closed forms of lipases, alter-

ing the exact shape of the open structure of the lipase. This effect of the experimental conditions could also be altered on the chemically modified lipases.

In this work, we have tried to study the prospects of the chemical modification of lipases, previously covalently immobilized in a very mild fashion on a CNBr-agarose support,[7] through amination by ethylenediamine for the modulation of the properties of the lipase, especially its enantioselectivity.

The use of lipases that have been immobilized under dissociation conditions (e.g., in the presence of detergents) permitted us to avoid undesired lipase-lipase interactions in the study of properties of the lipase (e.g., specific lipase-lipase aggregation).[8] The immobilization of the lipases on CNBr-agarose at 4 °C and pH 7 in the presence of 0.1 % detergent permitted only a much reduced enzyme-support multi-interaction, keeping the lipase's properties almost unaltered and assuring the immobilization of lipase's individual molecules. Moreover, the use of immobilized proteins prevents any inactivation of the lipases due to aggregation.[5]

To check the feasibility of this technique, we have used the kinetic resolution of two derivatives of α-hydroxyphenylacetic acid as a model reaction, (±)-α-hydroxyphenylacetic acid methyl ester (**1**) (asymmetric centre in the acyl donor site) and (±)-2-*O*-butyryl-2-phenylacetic acid (**2**) (asymmetric centre in the acyl acceptor site). (*R*)-α-Hydroxyphenylacetic acid (mandelic acid) (**3**) is an important precursor of cephalosporin antibiotics such as Cefamandole and Cefonicid.[9] Moreover, it is a key intermediate in the synthesis of (+)-goniodiol, a potent and selective cytotoxic agent against human lung carcinoma[10] (Scheme 1) and other interesting products.[11]

Three widely used lipases from different sources were selected as model enzymes, those from *Candida antarctica* (form B) (CAL-B),[12] *Thermomyces lanuginose* (TLL),[13] and *Pseudomonas fluorescens* (PFL).[14]

Results and Discussion

Analysis of the Lipase Surface

The surface of the three lipases was studied to determine the presence of Asp or Glu residues on areas near the active centre, and the suitability of the proposed strategy to alter the lipase's performance (Figures 1–3).

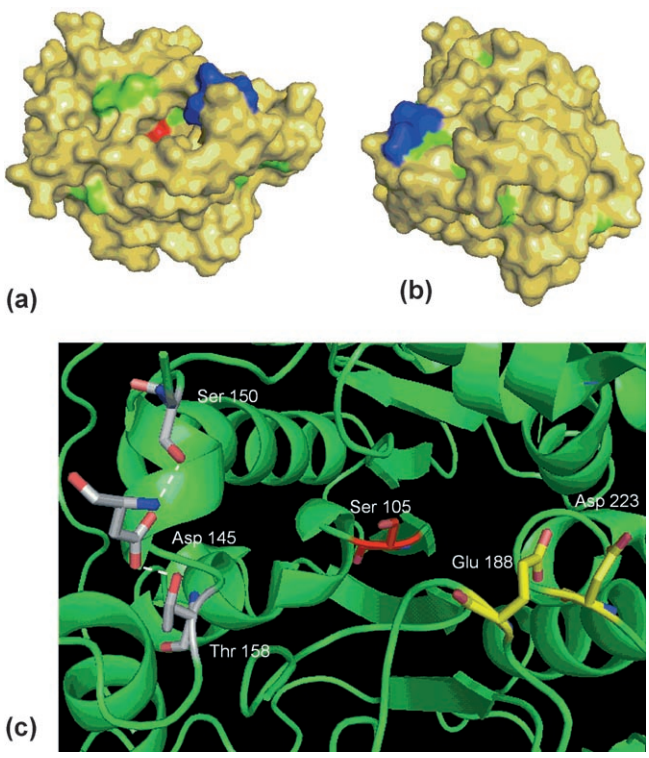
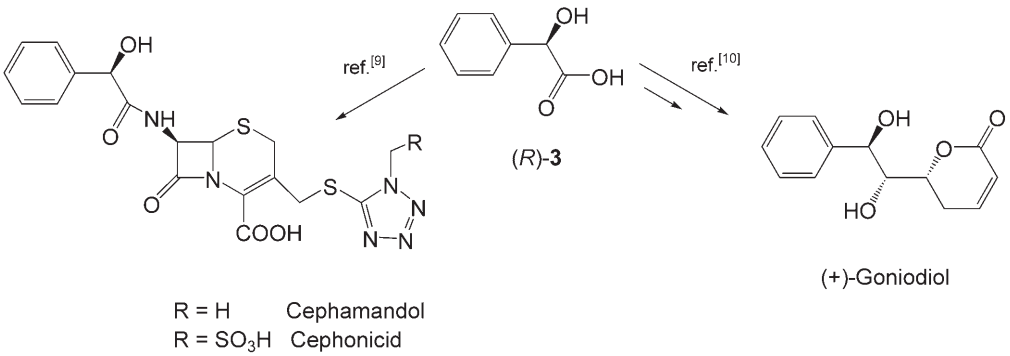


Figure 1. 3D surface model structure of CAL-B. **a**) lid face, **b**) lateral face. Asp or Glu residues (green), lid oligopeptide (blue), catalytic serine (red); **c**) Representation of the active site surrounded of the CAL-B marking the key amino acid. The 3D structure was obtained from the Protein Data Bank (PDB) using Pymol *vs.* 0.99. The pdb code for CAL-B is TCA.



Scheme 1. Synthesis of biologically active compounds from (*R*)-mandelic acid.

The open structure of the CAL-B (Figure 1) shows several Asp and Glu groups that are placed in important areas of the protein. Going into detail, it may be remarked that Asp 145 – the amino acid placed in the lid – can establish hydrogen bonds with Ser 150 and Thr 158 in both lipase conformations (Figure 1c). Moreover, Asp 223 and Glut 188 are two negatively charged groups very near to the active centre, so in this case the chemical amination will change this negatively charged group into a larger and positively charged group, perhaps influencing the movements of this area of the protein during catalysis.

Figure 2 shows the different sides of the 3D-structure of TLL. We can observe that this enzyme presents a very high number of Asp/Glut residues on its surface, even presenting some of them in the "lid" or in areas near to the active centre.

Finally, PFL presents Asp and Glu residues close to the lid but not so many in the surroundings of the active site (Figure 3).

Thus, it may be expected that the three different lipases have enough Asp and Glu groups in critical positions to be good models for the potential application of this strategy to modulate lipases *via* chemical modification of these groups.

Effect of the Chemical Amination of Immobilized Lipases on Catalytic Activity

Table 1 shows the initial activity displayed by different lipase preparations in the hydrolysis of *p*-NPB (an ester with a good leaving group), at pH 7 and 25 °C. In the case of CAL-B and TLL immobilized on agarose activated with CNBr, an increase in the specific activity was observed after chemical treatment. A direct correlation between the modification degree and the activity increase was found, multiplying the activity by three in the case of TLL.

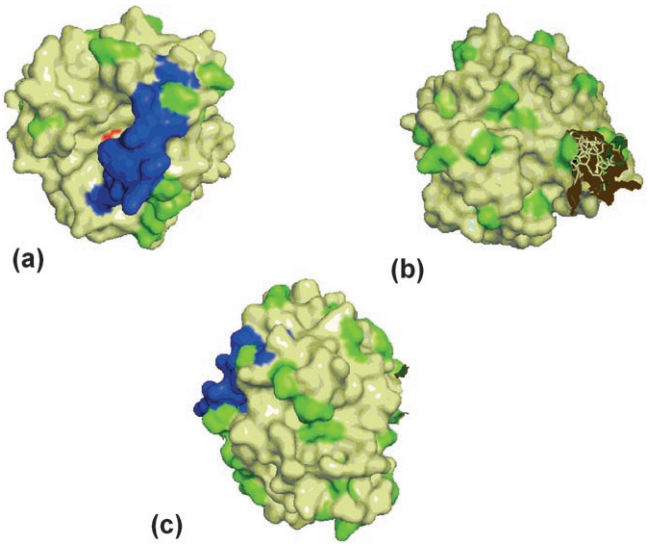


Figure 2. 3D surface model structure of TTL. **a**) lid face, **b**) lid opposite face **c**) lateral face. Asp or Glu residues (green), lid oligopeptide (blue), catalytic serine (red).The 3D structure was obtained from the Protein Data Bank (PDB) using Pymol *vs.* 0.99. The pdb code for TLL is 1DTB.

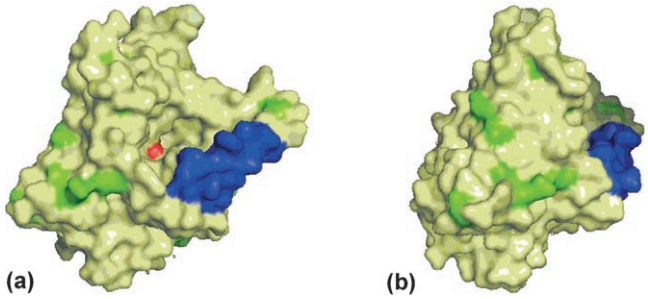


Figure 3. 3D surface model structure of PFL. **a**) lid face, **b**) lateral face. Asp or Glu residues (green), lid oligopeptide (blue), catalytic serine (red).The 3D structure was obtained from the Protein Data Bank (PDB) using Pymol *vs.* 0.99. The pdb code for PFL is 2LIP.

Table 1. Effect of the amination in the enzymatic activity of different lipases immobilized on CNBr in the hydrolysis of pNPB at pH 7. Other specifications as described in methods.

Lipase	Modification	Relative activity[a] [%]
CALB	NO	100
CALB	PARTIAL	100
CALB	TOTAL	200
TLL	NO	100
TLL	PARTIAL	190
TLL	TOTAL	350
PFL	NO	100
PFL	PARTIAL	60
PFL	TOTAL	30

[a] Ratio between the activity of the unmodified enzyme and the modified enzyme in %.

However the CNBr-PFL preparation underwent a significant decrease in enzyme activity depending on the modification degree. Thus the immobilized enzyme maintained only 30 % of the initial activity after full amination.

Therefore, the effect of this modification on the activity of the lipases in the hydrolysis of simple substrates was quite dissimilar when comparing different enzymes, from a noticeable increase to a significant decrease.

Effect of the Amination on the Enantioselectivity of the CNBr-CALB Immobilized Preparation Catalyzed Hydrolysis of (±)-1 and (±)-2

The specific activities and enantiomeric ratio values (E) of the CNBr-CALB immobilized preparation with

Table 2. Effect on the the amination grade in the enantioselectivity of the CNBr-CALB immobilized preparation.

Aminated Biocatalyst	Experimental Conditions	Substrate	Initial Rate[a]	c[b]	E Ratio
Not modified	pH 7, 25 °C	**1**	180	16	4.4
Partially modified		**1**	139	20	8.5
Fully modified		**1**	90	16	10
Not modified	pH 5, 25 °C	**1**	93	15	8
Fully modified		**1**	83	14	12
Not modified	pH 7, 4 °C	**1**	54	14	2
Fully modified		**1**	28	15	>100
Not modified	pH 5, 4 °C	**1**	18	18	45
Fully modified		**1**	15	15	>100
Not modified	pH 7, 25 °C	**2**	0.83	15	1
Fully modified		**2**	0.26	11	2.6
Not modified	pH 7, 4 °C	**2**	0.11	10	1
Fully modified		**2**	0.05	12	2.5

[a] the initial rate in UI/h ($\times 10^{-3}$).
[b] c = conversion.

different amination degrees in the kinetic hydrolysis of (±)-**1** and (±)-**2** at different conditions are shown in Table 2.

In the hydrolytic resolution of (±)-**1**, the effect of the modification on the enzyme activity was quite different to that detected using pNPB. The partial amination of the enzyme surface produced a moderate decrease of the enzyme activity (around 25%), and the full modification produced a further decrease in the enzyme activity (50%). The decrease in temperature from 25 to 4 °C decreased the enzyme activity by around 65% for non- and full aminated enzymes. Nevertheless, the effect of changing the pH value – from pH 7 to pH 5 – was very different. The non-modified enzyme suffered a decrease in activity up to 50% and 33% at 25 °C and 4 °C, respectively, while the fully aminated enzyme almost maintained its activity unaltered at 25 °C and retained more than 50% of its initial activity if the change in the pH was performed at 4 °C.

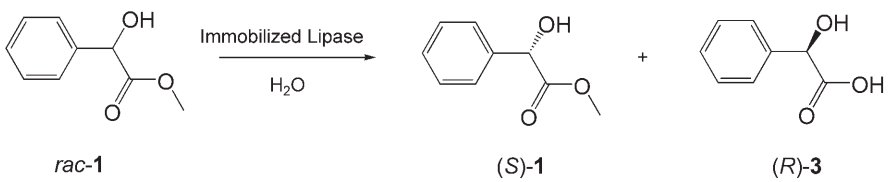
Using substrate (±)-**2**, which presents a negative charge at pH 7 by the carboxyl group (Scheme 3), the situation is slightly different. Full chemical modification of the immobilized lipase produced a reduction in the activity in 69% at 25 °C and around 50% at 4 °C.

Interestingly, the substrate specificity of the enzyme changed when comparing the activities of the aminated and non-aminated enzymes. Thus, at pH 7 and 25 °C, the modified enzyme showed higher activity towards pNPB than the non-modified enzyme while the expressed activity was lower with substrates (±)-**1** and (±)-**2**.

If we divided the enzyme activity corresponding to substrates (±)-**1** or (±)-**2** by the enzyme activity against pNPB, we can calculate the specificity of the enzyme by the different substrates. To simplify the comparison among the different preparations, we have given a relative ratio of 1 to the value obtained using the non-modified enzyme. Thus, fully aminated enzymes give a value of 0.5 and 0.3, respectively, that is, the enzyme activity is negatively relatively affected by the amination when using less suitable substrates for the enzyme.

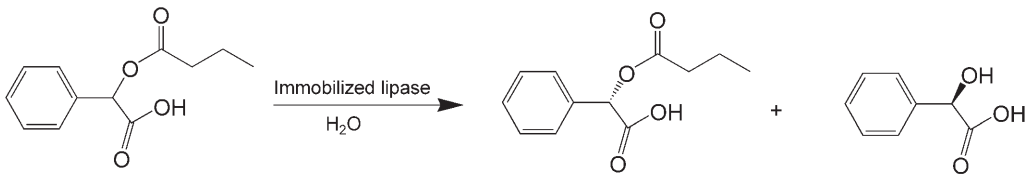
These results in enzyme activity and specificity suggested that the amination of the CNBr-CALB preparation had altered its catalytic properties, but the most interesting property that we intend to alter (and if possible to improve) is the enantioselectivity. Thus, the enantioselectivity of the different preparations was evaluated in the hydrolysis of (±)-**1** (Table 2). All enzyme preparations favoured the hydrolysis of the *R* isomer, but the E values depended strongly on the preparation employed (Scheme 2).

The unmodified CNBr-CALB preparation presented a quite low E value at pH 7 and 25 °C ($E = 4.4$). The decrease in the pH value to pH 5 improved the enantiomeric ratio up to 8, although when the reac-



rac-**1** (*S*)-**1** (*R*)-**3**

Scheme 2. Enzymatic kinetic resolution of (±)-**1**.



rac-**2** (*S*)-**2** (*R*)-**3**

Scheme 3. Enzymatic kinetic resolution of (±)-**2**.

tion was performed at 4 °C, the E value of the biocatalyst reached 45. At pH 7, the decrease in temperature produced a decrease in the E value from 4.4 to 2. Partial amination permitted us to increase the E value to 8.5.

The fully aminated CAL-B immobilized preparation gave better values of enantiomeric ratio than the unmodified enzyme in all cases, permitting us to obtain an $E > 100$ ($ee > 99\%$) at 4 °C at both pH values (Table 2).

Interestingly, this fully aminated enzyme, in opposition to the behaviour of the unmodified CNBr-CALB, improved the E value at pH 7 when decreasing the T, from $E = 10$ to > 100.

In the hydrolysis of (±)-**2** (Table 2), the unmodified CNBr-CALB preparation was not selective at all, whereas full chemical amination permitted us to obtain a slight selectivity towards the *R* isomer (Scheme 3).

Effect of the Amination on the Enantioselectivity of the CNBr-TLL Immobilized Preparation Catalyzed Hydrolysis of (±)-1 and (±)-2

Results are summarized in Table 3. Full modification of the immobilized enzyme promoted a slight decrease in the enzyme activity compared to substrate (±)-**1** (by 10 %) at the different conditions evaluated, while the partial amination produced an important drop in the enzyme activity (by a 7-fold factor). This result was unexpected since the lowest modification presented the highest effect on lipase activity, suggesting that the decrease in activity is not due to the direct effect on relevant groups in the protein's active centre (that obviously will be also modified in the more aminated enzyme) but due to an alteration of the enzyme structure, perhaps by altering the exact shape of the open form of the enzyme. In fact, these lipases having only 50 % of the carboxylic groups modified may be the ones with more "new" interactions.

Using substrate (±)-**2**, full modification of external carboxylic acids in the immobilized lipase produced a slight increase in the activity at pH 7 and 25 °C (but only by 10 %). However, at pH 5 and 25 °C, the activities of the different immobilized preparations showed an increase, higher in the fully aminated one, by more than a two-fold factor.

In this case the substrate specificity of the enzyme has been changed. The full modification of the enzyme, which improves the activity against pNPB by a three-fold factor, presented an almost null effect on the activity against the other substrates at pH 7, with a slight decrease using (±)-**1** and slight increase using (±)-**2**.

When the enantioselectivity factor of the different TLL-immobilized preparations was studied in the hydrolysis of (±)-**1** (Scheme 2), the unmodified CNBr-TLL preparation presented a low E value at pH 7

Table 3. Effect on the amination grade in the enantioselectivity of the CNBr-TLL immobilized preparation.

Aminated Biocatalyst	Experimental Conditions	Substrate	Initial Rate[a]	c[b]	E Ratio
Not modified	pH 7, 25 °C	**1**	0.9	15	3.5
Partially modified		**1**	0.13	12	1.2
Fully modified		**1**	0.8	13	6.5
Not modified	pH 5, 25 °C	**1**	0.34	10	1.9
Fully modified		**1**	0.31	17	3.6
Not modified	pH 7, 25 °C	**2**	0.40	18	3.0
Fully modified		**2**	0.44	17	6.0
Not modified	pH 5, 25 °C	**2**	0.70	10	1.1
Fully modified		**2**	1.75	13.5	1.3

[a] The initial rate in UI/h ($\times 10^{-3}$).
[b] c = conversion.

Table 4. Effect on the amination grade in the Enantioselectivity of the CNBr-PFL immobilized preparation.

Aminated Biocatalyst	Experimental Conditions	Substrate	Initial Rate[a]	c[b]	E Ratio
Not modified	pH 7, 25 °C	**1**	0.22	11	1
Partially modified		**1**	0.036	12	1.1
Fully modified		**1**	0.33	17	1.7
Not modified	pH 7, 25 °C	**2**	0.41	19	2.0
Fully modified		**2**	0.32	18	3.4
Not modified	pH 5, 25 °C	**2**	0.15	12	29
Fully modified		**2**	0.11	13	12

[a] The initial rate in UI/h ($\times 10^{-3}$).
[b] c = conversion.

($E = 3.5$), which was reduced when the reaction was performed at pH 5 ($E = 1.9$). The amination on the TLL immobilized preparation improved the E values by around a two-fold factor (Table 3). Curiously, a partial amination gave a decrease in the E value (from 3.5 to 1.2).

Using substrate (±)-**2** (Scheme 3), the amination produced an improvement in the E value for the TLL preparation, especially at pH 7 (from 3 to 6) (Table 3).

Effect of the Amination on the Enantioselectivity of CNBr-PFL Catalyzed Hydrolysis of (±)-1 and (±)-2

The specific activities and enantiomeric ratio values of the CNBr-PFL immobilized preparation with different amination degrees in the kinetic hydrolysis of (±)-**1** and (±)-**2** at different conditions are shown in Table 4.

Full modification of the enzymes promoted a significant increase in the enzyme activity (by a 1.5-factor) in the hydrolysis of (±)-**1**, while the partial amination of the enzyme provoked a very significant reduction in the enzyme activity (by 85 %). Again, this inverse relation between activity variation and modification degree suggested that the chemical modification did not have a direct effect on chemical groups related to the enzyme activity, but may be altering the percentage or the shape of the open form of the lipase. In contrast, the effect of full modification on the enzyme activity was negative when using substrate **2**, reducing the activity by around 25 % at both pH values.

Following this procedure, the enzyme specificity was altered again, in this case favouring substrate **1** (relative specificity was 4.5 for the full modified preparation comparing substrate (±)-**1** and pNPB, and 1 for the non-modified enzyme).

With respect to the enantioselectivity towards (±)-**1** (Table 4), the unmodified CNBr-PFL preparation did not present a significant E at pH 7, that is improved after full amination of the preparation, detecting a certain selectivity towards the *R* isomer (Scheme 2). At pH 5, all preparations presented a very low activity against substrate **1** and could not be used in this reaction.

Using substrate (±)-**2** (Scheme 3), the aminated immobilized preparation presented better E value at pH 7 compared to the unmodified one ($E = 3.4$ *versus* 2). When the reaction was performed at pH 5, the enantioselectivity of both preparations increased, although the highest E value obtained was found when using the non-aminated one ($E = 29$) (Table 4).

Production of Enantiomerically Pure (*R*)-Mandelic Acid

The chemical amination of the CNBr-CAL-B immobilized preparation has permitted us to obtain an enantioselective biocatalyst (E > 100 %) for the prepara-

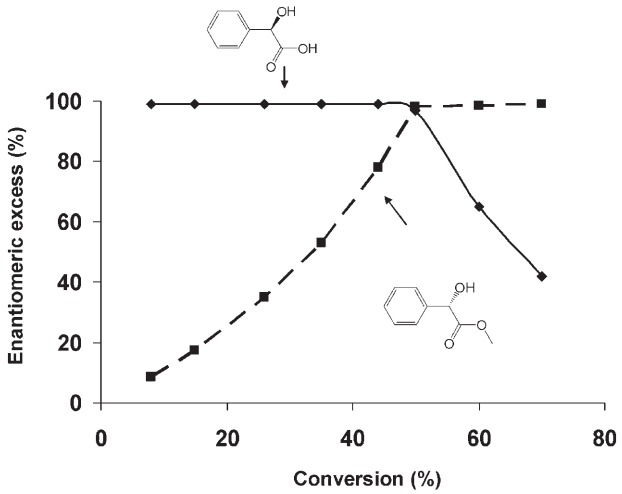


Figure 4. Evolution of enantiomeric excess (*ee*) of **1** and **3** *versus* hydrolysis conversion of (±)-**1** catalyzed by aminated BrCN-CAL-B immobilized preparation. Experiments were performed using 10 mM of substrate at pH 7 and 4 °C. *ee* of [(*R*)-**1**] (square), *ee* of released acid [(*R*)-**3**] (rhombus).

tion of enantiomerically pure (*R*)-mandelic acid [(*R*)-**3**], a precursor of different biologically active compounds (Scheme 1). Figure 4 shows the catalyst's performance; the *ee* value of both the acid and the ester is around 99% at 50% conversion.

Conclusions

Chemical modification of lipase surfaces may be a simple and powerful tool to modify their specificity. Amination has permitted us to improve the enzyme activity against several substrates and has given a significant increment in the E values in some instances. In this way, the amination of the CNBr-CAL-B preparation permitted us to greatly modulate its enantioselectivity from an E value of 2 without modification up to $E > 100$, affording (*R*)-mandelic acid in high purity ($ee > 99\%$ at 50% conversion) at pH 7 and 4 °C.

Therefore, the results presented in this paper – on the potential of chemical modification of the lipases surface – together with the previous results on the effect of the immobilization of lipases following different strategies,[4] show that the coupled use of physicochemical tools may be a simple and rapid way of preparing a small library of biocatalysts with very different properties, significantly increasing the probabilities of finding a biocatalyst useful for the resolution of any racemate. This methodology could be considered as a very useful tool in the combinatorial biocatalysis background.[17]

Experimental Section

General Remarks

The lipases from *Candida antarctica B* (CAL-B) and *Thermomyces lanuginosa* (TTL) were from Novo Nordisk (Denmark). The lipase from *Pseudomonas fluorescens* (PFL) was from Amano. Cyanogen bromide (CNBr-activated Sepharose 4BCL) was purchased from Pharmacia Biotech (Uppsala,Sweden). *p*-Nitrophenyl butyrate (pNPB), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), ethylenediamine (EDA) and (±)-α-hydroxyphenylacetic acid methyl ester (mandelic acid methyl ester) (**1**) were purchased from Sigma. NMR data were recorded on a Bruker AC-300 (^{1}H at 250 MHz, ^{13}C at 75.5 MHz) spectrometer at room temperature. MALDI-TOF mass spectra were recorded with a Voyager-DE Pro BioSpectrometer from PerSeptive Biosystems with 2,5-dihydroxybenzoic acid (DHB) as the matrix.

Purification of the Lipases

The different lipases were purified prior to use. The used strategy was the interfacial adsorption on octyl-agarose support at low ionic strength and a second step of desorption using 1% Triton X-100 following a previously described procedure.[15]

Enzymatic Kinetic Assay

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 pNPB in 25 mM sodium phosphate buffer at pH 7 and 25 °C. To initialize the reaction, 0.05 mL of lipase solution or suspension were added to 2.5 mL of substrate solution. One international unit (IU) of pNPB activity was defined as the amount of enzyme that is necessary to hydrolyze 1 μmol of pNPB per minute under the conditions described above.

Immobilization of Lipases on CNBr-Activated Support

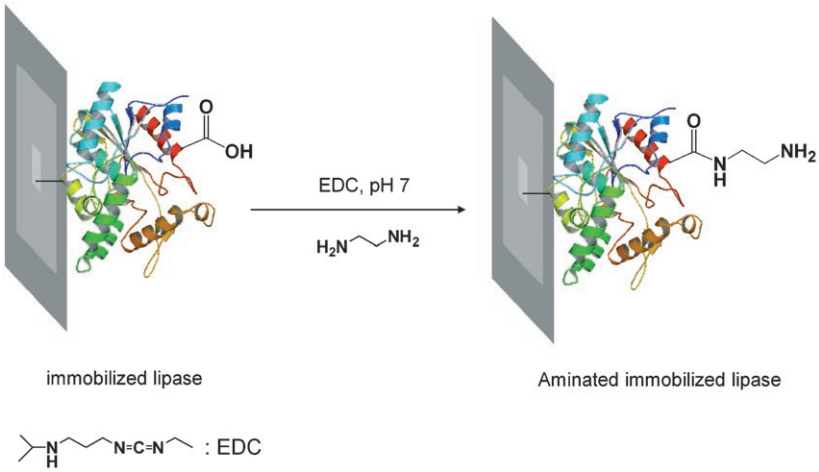
Commercial agarose support activated with CNBr was dissolved in an acidic aqueous solution (pH 2–3) for one hour. After that the solid support is filtered by vacuum. 1 mL of the purified lipase solution containing a concentration of 10 mg protein/mL was added to 20 mL of 25 mM sodium phosphate buffer solution at pH 7 containing 0.1% Triton X-100. After that, 1 g of the CNBr-agarose support was added. The mixture was then shaken at 4 °C and 250 rpm for 18 h. After that, the solution was removed by filtration and the supported lipase was washed several times with distilled water. In all cases, the immobilization percentage was more than 95%. The immobilization was followed by the assay described above.

Chemical Amination of Immobilized Enzymes

One g of immobilized enzyme was added to 50 mL solution of EDA 1 M in buffer at pH 4.75 (1/5 v/v). Solid EDC was added to the suspension to different final concentrations, 10^{-2} M (fully aminated biocatalyst) or 10^{-3} M (partially aminated biocatalyst).[5] After 90 min of gentle stirring at 25 °C, the immobilized-modified preparations were filtered and incubated for 4 h with a 0.1 M hydroxylamine solution at pH 7 to recover the modified tyrosines.[16] The enzyme preparations were filtered and washed with 25 mM potassium phosphate buffer at pH 7.5 and an excess of distilled water. The aminated derivatives were stored at 4 °C. Scheme 4 shows the reaction. We have checked that the colour developed by the reaction with picrylsulfonic acid with the derivatives previously modified with glutaraldehyde (to eliminate endogenous primary amino groups) were doubled in strength when using 10 mM EDA than when using 1 mM EDA for the 3 immobilized enzymes.

Synthesis of 2-*O*-Butyryl-2-phenylacetic Acid [(±)-2]

A solution of butyryl chloride (20 mmol) in diethyl ether (100 mL) was added dropwise to a stirred solution of (±)-**3** (22 mmol) in diethyl ether (200 mL) with NEt_3 (22 mmol). The mixture was stirred at 25 °C for 4 h and the reaction was followed by HPLC. The mixture was extracted with neutral aqueous solution and, after re-extraction of the water phase with Et_2O, the combined organic solvents were dried with $MgSO_4$ and the solvent was evaporated. The crude was washed several times with cold ether (5 × 2 mL) and dried under vacuum; yield: 60%. ^{1}H-NMR (250 MHz, $CDCl_3$): $\delta = 7.4$ (m, 4H), 5.9 (s,1H, CH), 2.4 (m, 2H, αCH_2), 1.7 (septet, 2H, βCH_2), 0.95 (m, 3H, CH_3); ^{13}C NMR



Scheme 4. Chemical amination of protein carboxyl groups. EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

($CDCl_3$): δ = 173 (C=O), 170 (C=O), 131.8 (C), 127.8 (CH), 127.3 (CH), 126.1 (CH),76.1 (CH), 34.2 (CH_2), 16.8 (CH_2), 12.1 (CH_3); MS (MALDI-TOF+): m/z = 245.7, calcd. for $C_{12}H_{14}O_4$ $[M+Na]^+$: 245.1.

Enzymatic Hydrolysis of Esters

The activities of the different immobilized lipase preparations on the hydrolysis reaction of different esters were performed by adding 0.2 g of catalyst in 10 mL of (±)-**1** (5 mM) and 0.5 g of catalyst in 6 mL of (±)-**2** (0.5 mM) under different conditions (pH, *T*) under mechanic stirring.

During the reaction, the pH value was maintained constant by automatic titration and the enzymatic activity (μmol of substrate hydrolyzed per hour per mg of immobilized protein) was evaluated from NaOH consumption using a pH-stat Mettler Toledo DL50 graphic system. The degree of hydrolysis was confirmed by reverse-phase HPLC (Spectra Physic SP 100 coupled with a UV detector Spectra Physic SP 8450) on a Kromasil C18 (25 × 0.4 cm) column supplied by Analisis Vinicos (Spain). At least triplicate runs of each assay were made. The elution was isocratic with a mobile phase of acetonitrile (30%) and 10 mM amonium phosphate buffer (70%) at pH 2.95 and a flow rate of 1.5 mL min^{-1}. The elution was monitored by recording the absorbance at 254 nm [substrate (±)-**1**] or 225 nm [substrate (±)-**2**].

Determination of Enantiomeric Excess

At different conversion degrees, the enantiomeric excess (*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 2.3, and the analyses were performed at a flow of 0.5 mL min^{-1} by recording the absorbance at 225 nm.

Calculation of E Value

The enantiomeric ratio (E) was calculated as the ratio between the percentage of hydrolyzed *R* and *S* isomer (from racemic mixture) at hydrolysis degrees between 15 and 20%.

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