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Modulation of *Mucor miehei* lipase properties via directed immobilization on different hetero-functional epoxy resins Hydrolytic resolution of (*R*,*S*)-2-butyroyl-2-phenylacetic acid

Jose M. Palomo, Gloria Muñoz, Gloria Fernández-Lorente, Cesar Mateo, Manuel Fuentes, Jose M. Guisan[1], Roberto Fernández-Lafuente*

Department of Biocatalysis, Institute of Catalysis, CSIC, Campus UAM, Cantoblanco, 28049 Madrid, Spain

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Abstract

Purified lipase from *Mucor miehei* (MML) has been covalently immobilized on different epoxy resins (standard hydrophobic epoxy resins, epoxy-ethylenediamine, epoxy-iminodiacetic acid, epoxy-copper chelates) and adsorbed via interfacial activation on octadecyl-Sepabeads support (fully coated with very hydrophobic octadecyl groups). These immobilized enzyme preparations were used under slightly different conditions (temperature ranging from 4 to 25 °C and pH values from 5 to 7) in the hydrolytic resolution of (*R*,*S*)-2-butyroyl-2-phenylacetic acid.

Different catalytic properties (activity, specificity, enantioselectivity) were found depending on the particular support used. For example, the epoxy-iminodiacetic acid-Sepabeads gave the most active preparation at pH 7 while, at pH 5, the ethylenediamine-Sepabeads was superior.

More interestingly, the enantiomeric ratio (*E*) also depends strongly on the immobilized preparation and the conditions employed. Thus, the octadecyl-MML preparation was the only immobilized enzyme derivative which exhibited enantioselectivity towards *R* isomer (with *E* values ranging from 5 at 4 °C and pH 7 to 1.2 at pH 5 and 25 °C).

The other immobilized preparations, in contrast, were *S* selective. Immobilization on iminodiacetic acid-Sepabeads afforded the catalyst with the highest enantioselectivity ($E = 59$ under optimum conditions).

Keywords: Conformational engineering; Covalent immobilization; Interfacial activation; Lipase enantioselectivity; (*R*,*S*)-2-Butyroyl-2-phenylacetic acid

1. Introduction

Lipases (triacyglycerol ester hydrolases, EC 3.1.1.3) are perhaps the most popular enzymes in biocatalysis because they couple a wide specificity to a high regio- and enantioselectivity; therefore, they may be used in many different reactions [1–7].

However, lipase catalysis involves dramatic conformational changes of the enzyme molecule. Lipases may be in two different structural forms. One of them, where 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

* Corresponding authors. Tel.: +34-91-585-4809; fax: +34-91-585-4760.
E-mail addresses: jmguisan@icp.csic.es (J.M. Guisan), rfl@icp.csic.es (R. Fernández-Lafuente).
[1] Co-corresponding author.

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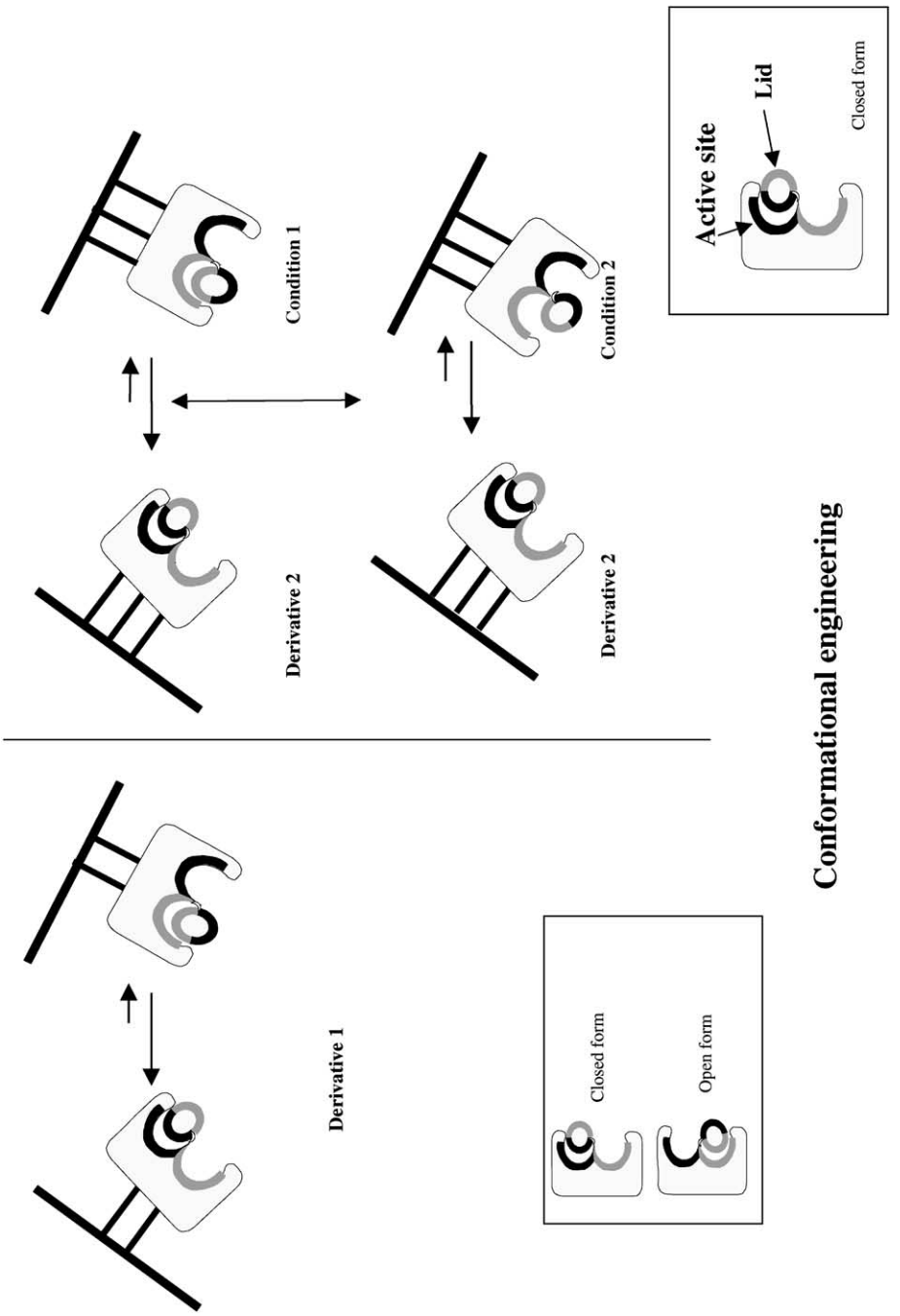
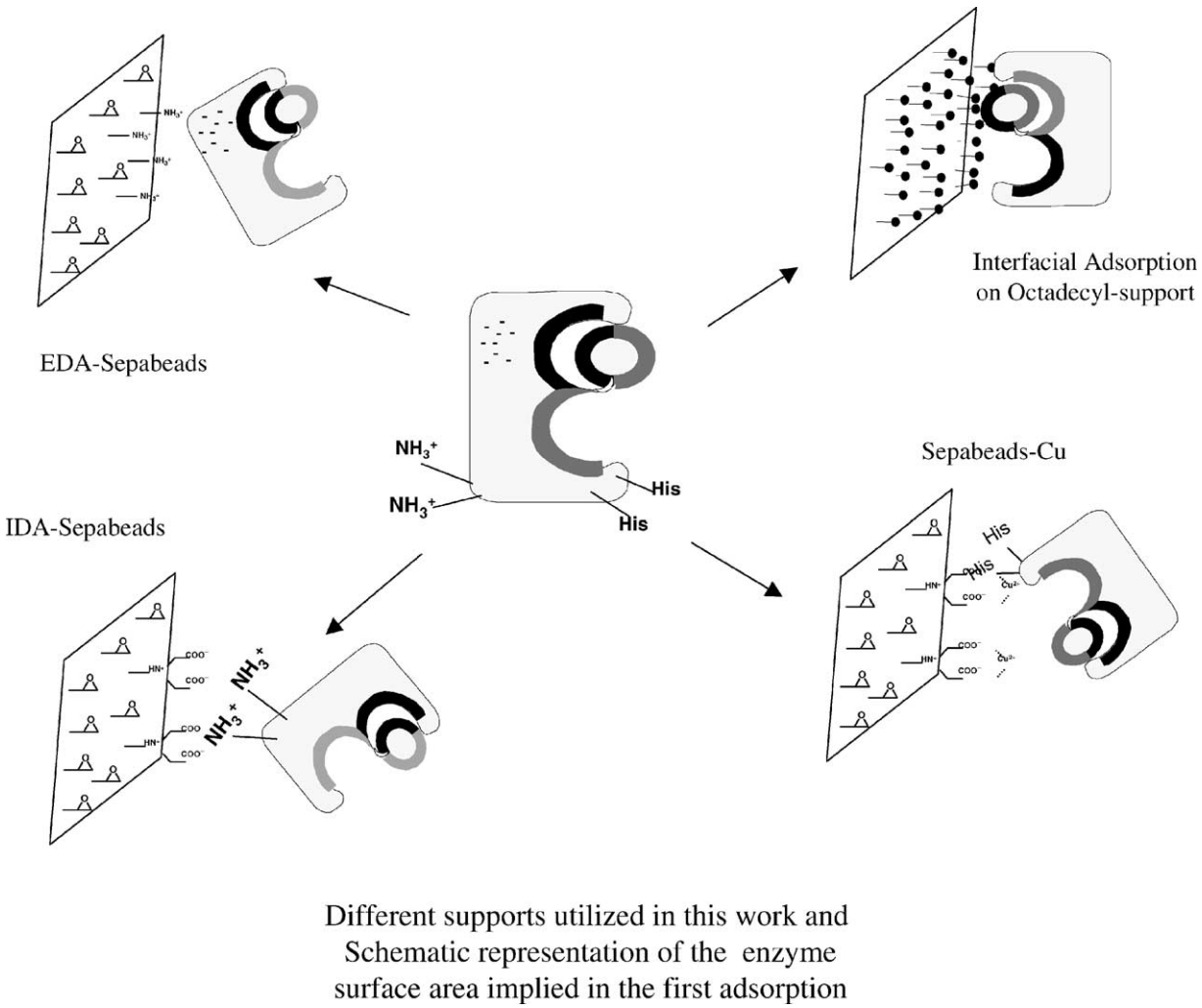


Scheme 1. Conformational engineering of lipases.

and the active site exposed to the reaction medium, is considered the lipase in an active (open) form. In the absence of hydrophobic interfaces, the lipase molecule is in equilibrium between the open-active and the closed-inactive structures of the immobilized lipases. This equilibrium shifts upon interaction with a hydrophobic interface, such as a lipid droplet, towards the open form exposing the active site providing free access for the substrate (interfacial activation) [8–12].

Bearing in mind these dramatic conformational changes, it is very likely that lipase catalytic properties may be dramatically modified if this equilibrium or the exact shape of the open form of the lipase is altered in any way.

This could be achieved via immobilization techniques involving different areas of the enzyme surface and, therefore, giving different orientation of the enzyme regarding the support surface, giving different rigidity to the enzyme structure or even generating a certain special microenvironment surrounding the enzyme. This could reduce the mobility of the lid, altering the exact shape of the final open form of the lipase (Scheme 1). In fact, enzyme derivatives from lipases exhibiting different catalytic properties may



Scheme 2. Enantioselective hydrolysis of (*R*,*S*)-2-butyroyl-2-phenylacetic acid catalyzed by different MML derivatives.

be found in the literature [13–17]. Also, the change of reaction conditions could have a dramatic effect on the properties of the lipases, perhaps because the change in the global interactions of the open/closed forms of lipase could alter the exact shape of the lipase open structure. Interaction between conditions and immobilization protocols could also offer a variety of results (Scheme 1).

This modulation of lipase properties by altering the exact form of their active site via physicochemical tools could be denominated "conformational engineering" and it has been used successfully to modulate the behavior of different enzymes which suffer drastic conformational changes during catalysis, such as *Penicillin G. acylase* [18,19] and some lipases [15–17].

In this paper, different immobilization techniques have been used to prepare immobilized preparations of the lipase from *Mucor miehei* (MML) involving reaction of the protein with surfaces containing different groups, promoting different degrees of enzyme rigidity or altering the micro-environment, together with a study on the effect of the reaction conditions on the different enzyme preparations.

Octadecyl-Sepabeads have been used to achieve the immobilization plus interfacial activation of lipases interfacial activation [20,21].

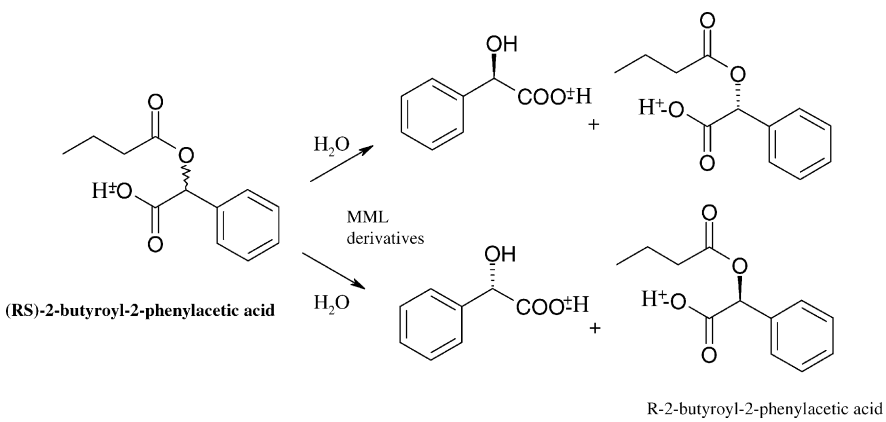
Immobilization on different epoxy supports [22], partially functionalized with different groups has been used to obtain different covalent enzyme derivatives. The first step in the immobilization on epoxy supports is a rapid physical adsorption (promoted by the groups added to the epoxy support), followed by chemical reaction [22,23] between the already adsorbed enzyme and the epoxy groups remaining in the support. This immobilization mechanism permits, using the same chemistry reaction with epoxy groups, to immobilize enzymes on supports through the part of the protein surface with the highest positive charge (using iminodiacetic-epoxy supports), the highest negative charge (using ethylenediamine-epoxy supports), or with more density of histidine residues (using metal ion-chelate-epoxy supports), . . . [22]. In this way, it is possible to obtain immobilized enzyme preparations that present different local environments on the support.

We have used as a model compound 2-butyroyl-2-phenylacetic acid (Scheme 2). This compound was used as chiral displacer with the cyclobond-II chiral stationary phase [24]. Optically pure isomers of *R* and *S* mandelic acid and their esters are very useful in organic synthesis.

2. Materials and methods

2.1. Materials

The lipase from *M. Miehei* (Novozym 388) (MML) was purchased by Novo Nordisk (Denmark). Octadecyl-Sepabeads and Sepabeads EP (epoxide) were kindly donated by Resindion srl (Milan, Italy). This support did not swell when changed from dry to wet condition. Iminodiacetic-epoxy-Sepabeads



Scheme 3. Different immobilized derivatives of *Mucor miehei* lipase.

(IDA), ethylenediamine-epoxy-Sepabeads (EDA) and copper-chelates-epoxy-Sepabeads (IDA-Cu^{2+}) were prepared as previously described [22] (Scheme 3). Octyl-agarose 4BCL was purchased from Pharmacia Biotech (Uppsala, Sweden). Triton X-100 and *p*-nitrophenyl butyrate (pNPB) were obtained from Sigma. (*R*,*S*)-2-butyroyl-2-phenylacetic acid was kindly donated by Dr. Marco Terreni (University of Pavia, Italy). Other reagents and solvents used were of analytical grade.

Protein concentration was measured using Bradford's method [25]. The calibration curve was obtained with bovine serum albumin (BSA) for determining protein concentrations in the range of 0.1–1.5 mg/ml.

2.2. Methods

2.2.1. MML purification

The lipase preparation was adsorbed on octyl-agarose [20] to purify the lipase from any other contaminant protein (e.g. esterases). In a standard experiment, 1 ml of octyl-agarose was added to 15 ml of standard lipase preparation in 5 mM sodium phosphate buffer at pH 7.4 °C. A blank suspension was prepared by adding 1 ml of Sepharose 4B-CL. Activity of suspensions and supernatants was assayed periodically by using the pNPB assay described below. After immobilization, the adsorbed lipase was abundantly washed with distilled water. To desorb the enzyme, the adsorbed lipase on octyl-agarose was washed with Triton X-100 0.6% in 5 mM sodium phosphate buffer at pH 7 and 25 °C. Triton was diluted 500-fold with sodium bicarbonate 5 mM.

2.2.2. SDS-PAGE analysis

SDS-PAGE electrophoresis was performed as described by Laemmli [26] in a SE 250-Mighty Small II electrophoretic unit (Hoefer Co.) using gels of 12% polyacrylamide in a separation zone of 9 cm × 6 cm and a concentration zone of 5% polyacrylamide. Gels were stained with Coomassie brilliant blue method. Low molecular weight markers were used (14,400–94,000 D) from Pharmacia (Fig. 1).

2.2.3. Hydrolysis of pNPB

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 initiate the reaction, 0.1 ml of lipase solution or suspension was added to 2.5 ml of substrate solution. One international unit of pNPB activity was defined as the amount of enzyme that is necessary to hydrolyze 1 μmol of pNPB per minute (IU) under the conditions described above.

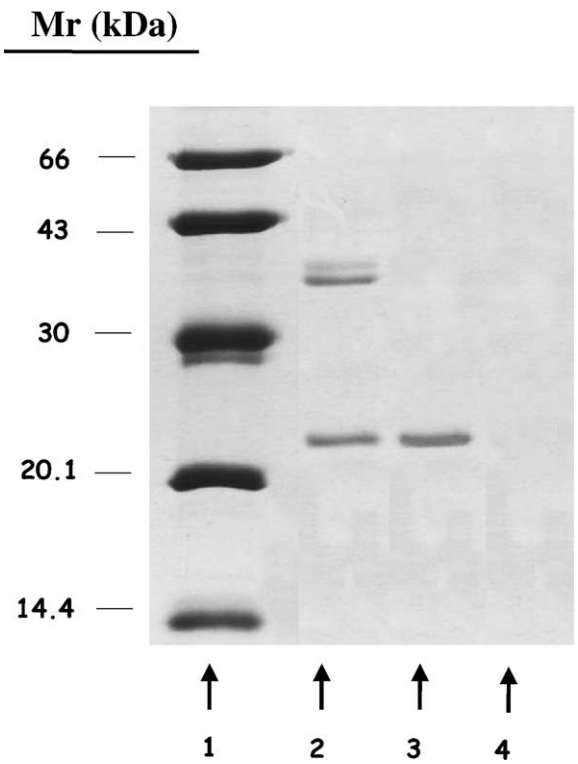


Fig. 1. SDS-PAGE gel of different MML *preparations*. Lane 1—molecular weight markers; lane 2—commercial MML preparation; lane 3—proteins adsorbed on octyl derivative; lane 4—octyl-agarose derivative after desorption. Experiments were performed as described in Section 2.2.

2.2.4. Immobilization of MML on different supports

Enzyme loading was about 1.7 mg protein/ml of wet support (that is around 2–3% of the maximum loading) in order to prevent diffusion problems and more than 95% of the free protein was immobilized in all cases. Protein concentration was determined according to Bradford [25]. The schematic representation of the different supports is given in Scheme 3.

2.2.4.1. Preparation of octadecyl-Sepabeads lipase derivative. The purified lipase was diluted 10-fold using distilled water (to dilute the Triton X-100) and octadecyl-Sepabeads were added. Samples of suspension and supernatant were periodically withdrawn in order to check the course of immobilization. In some cases, unpurified enzyme preparations were used (using identical enzyme loading), the results being almost identical, evidencing that this support could also be used for the selective immobilization of lipases, at least in the three cases studied. After immobilization, the supports were washed with an excess of distilled water.

2.2.4.2. Immobilization on modified-epoxy-Sepabeads. Seven grams of support (EDA-Sepabeads, IDA-Sepabeads or IDA-Cu^{2+}-Sepabeads) was suspended in 25 ml of solutions of purified enzyme (protein concentration was 0.52 mg/ml) in 25 mM sodium phosphate buffer at pH 7 and 25 °C. Periodically, samples of supernatant and suspension were withdrawn and the enzyme activity was determined. To check that the immobilization was actually covalent, the immobilized enzyme preparations were incubated under conditions where physically adsorbed protein molecules were released. After immobilization, immobilized enzyme preparations were incubated in the presence of 3 M glycine at pH 8.5 during 24 h to block the remaining epoxy groups and to promote the hydrophilization of the support surface. Finally, preparations were washed with an excess of distilled water.

2.2.5. Enzymatic hydrolysis of (R,S)-2-butyroyl-2-phenylacetic acid

The activities of different immobilized derivatives of lipase from *M. miehei*were assayed by adding 0.5 g of wet immobilized preparations to 6 ml of 2.5 mM substrate in 10 mM sodium phosphate buffer at different conditions (pH, *T*) under mechanical stirring. During the reaction, the pH value was maintained constant by automatic titration using a pH-stat. Blank experiments were performed using suspensions of the different matrices without enzyme.

The degree of hydrolysis was followed by reverse-phase HPLC (Spectra Physics SP 100 coupled with an UV detector Spectra Physics SP 8450) on a Kromasil C18 (25 cm × 0.4 cm) column supplied by Analisis Vinicos (Spain). At least triplicates of each assay were made. Experimental error was under 3%. The elution was performed using a mobile phase composed of acetonitrile (35%) and 10 mM ammonium phosphate buffer (65%) at pH 2.95 at a flow rate of 1.5 ml/min. The elution was monitored by recording the absorbance at 225 nm. The retention time of the acid was 3.7 min and the retention time of the ester was 23 min.

Under the studied conditions, chemical hydrolysis was found to be under the limit of detection (under 0.1%). Moreover, adsorption of the substrate/product on the immobilization matrices was also found to be under the limit of detection (under 3%).

2.2.6. Determination of the enantiomeric excess (ee)

At different conversion degrees, the enantiomeric excess (ee_p) 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 by recording the absorbance at 225 nm. The retention time of the *S* isomer was 39 min, while the retention time of the *R* isomer was 42 min. Special interest presented the ee at 50% of hydrolysis (corresponding to the maximum yield), the value with more practical relevance.

2.2.7. Determination of the eantioselectivity value (E)

From the data obtained above, it was possible to calculate the initial rate of hydrolysis of both isomers (using hydrolysis degrees between 10 and 20%). Bearing in mind that at this substrate concentration, the enzyme kinetics is in the first order region (results not shown), the *E*-value was calculated directly from the ratio between the reaction rates of both isomers.

3. Results

3.1. Immobilization of Mucor miehei lipase

The immobilization courses of *M. miehei* lipase on the different Sepabeads supports are shown in Fig. 2. Using octadecyl-Sepabeads immobilization was extremely rapid (Fig. 2a), with no soluble lipase activity detectable after 1 h of incubation and promoted a two-fold hyperactivation of the immobilized enzyme.

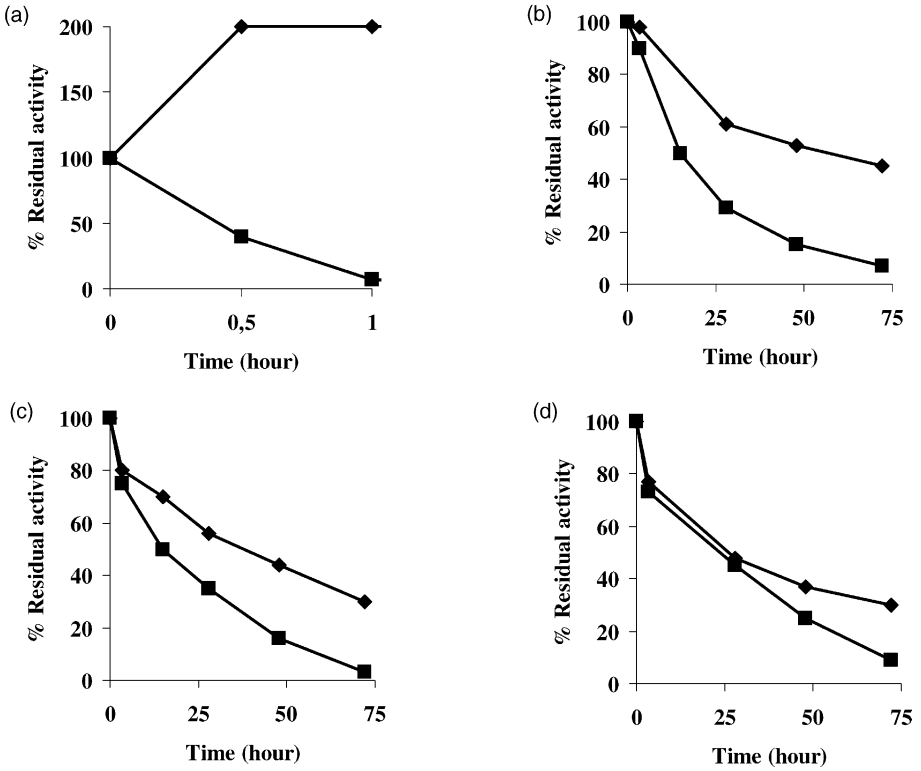


Fig. 2. Immobilization course of MML on different supports: (a) immobilization on octadecyl-Sepabeads support. Octadecyl-Sepabeads (rhombus), supernatant (squares). (b) Immobilization on IDA-Cu^{2+}-Sepabeads support; IDA-Cu^{2+}-Sepabeads (rhombus), supernatant (squares). (c) Immobilization on IDA-Sepabeads; IDA-Sepabeads (rhombus), supernatant (squares). (d) Immobilization on EDA-Sepabeads; EDA-Sepabeads (rhombus),supernatant (squares). Experiments were carried out as described in Section 2.2.

The immobilization rates on the epoxy-functionalized Sepabeads were much slower (50% of the enzyme immobilized in 15–20 h in all cases) and a decrease in enzyme activity was observed (by 55% in the IDA-Cu^{2+}-epoxy support, 70% in the IDA and EDA-epoxy).

3.2. Activity of the different immobilized enzyme preparations in the hydrolysis of (R,S)-2-butyroyl-2-phenylacetic acid: effect of the experimental conditions

At 25 °C and pH 7 (Table 1), the highest activity was found using the IDA-Sepabeads preparation. This activity was about 25-fold higher than in IDA-Cu^{2+} Sepabeads preparation and more than a 10-fold factor than that of the interfacially activated preparation. However, using pNPB as substrate, octadecyl derivative exhibited six- to seven-fold more activity than the IDA-epoxy one. This shows a dramatic dependence of the activity of the enzyme on the immobilization protocol.

Moreover, the EDA-epoxy enzyme preparation was found to be twice as active as the interfacially adsorbed one towards this substrate. This could be

Table 1
Enzymatic activity[a] of different MML immobilized derivatives in hydrolysis of (*R*,*S*)-2-butyroyl-2-phenylacetic acid

Enzyme derivative	25 °C, pH 7	4 °C, pH 7	25 °C, pH 5
Octadecyl	0.04	0.027	0.07
IDA-Cu^{2+}	0.02	0.0015	0.0057
IDA	0.55	0.067	0.0326
EDA	0.096	0.02	0.076

Experiments were performed as described in Section 2.2.

[a] Enzymatic activity was defined as μmol/(min $mg_{protein}$). The experimental error was estimated to be ±3%.

related to the presence of a charged group in the substrate which could hinder its entry in the very hydrophobic environment formed by the support surface and the hydrophobic pockets of the enzyme.

At 4 °C, the octadecyl-derivative activity decreased (compared to that at 25 °C) less than two-fold, while the activity of the covalent immobilized derivatives decreased much more significantly (around a five-fold factor for the EDA-epoxy preparation and more than 13-fold for the IDA-Cu^{2+}-epoxy preparation). The lower temperature might affect mainly the possibility of opening the lid (at low temperature the hydrophobic interactions may be intensified), and this should not affect the interfacially activated preparation. Differences in the conformational rigidity of the different immobilized preparations could also explain this different effect on enzyme activity.

On decreasing the pH value from 7 to 5, a decrease in the activity of the immobilized enzyme preparations was observed, except in the case of the octadecyl one in which activity increased almost two-fold. This could be related to the higher "hydrophobicity" of the substrate at this more acidic pH value, being able to compensate the negative effect on the enzyme activity.

Among the covalently immobilized preparations there are also some very significant differences. Activity moved from a slight decrease (e.g. by 22% in the case of EDA derivative) to a 17-fold factor in the case of the IDA derivative.

Thus, the results suggested that the properties of the different preparations were very different and that the influence of the experimental conditions also depended on the type of support used.

3.3. Enantioselectivity of the different preparations: effect of the experimental conditions

The differences in *E* values observed with the different immobilized preparations were even more interesting (Table 2).

All the covalently immobilized preparations hydrolyzed the *S* isomer more rapidly than the *R* isomer. At pH 7 and 25 °C, the IDA-Cu^{2+}-epoxy preparation gave an *E*-value of 30 (enabling to achieve an ee of the product of 84% after 50% of hydrolysis of the racemic substrate) while the IDA-epoxy preparation gave a low value ($E = 5$), that permitted to achieve an ee of only 51% after 50% hydrolysis.

The interfacially adsorbed enzyme presented an inverse enantioselectivity compared with the other immobilized preparations: it hydrolyzed the *R* isomer more rapidly than the *S* isomer (although with a low *E*-value of 3.4).

Also, the influence of the temperature on the obtained *E* values with the different immobilized enzymes was very different (Table 2), although all immobilized preparations maintained the same enantio-preference. While the IDA-Cu^{2+}-epoxy preparation exhibited a decrease in *E*-value from 30 to 2.2 and with the EDA-epoxy preparations the *E*-value decreased from 11 to 1.2, when the temperature decreased from 25 to 4 °C, the enzyme immobilized on the IDA-epoxy support improved its *E*-value from 5 to 59. This permitted the ee achieved at 50% hydrolysis to be around 90%, the highest one achieved with these immobilized preparations. The enantioselectivity of the preparation using octadecyl supports

Table 2
Effect of the immobilization and experimental conditions on the enantioselectivity and enentiomeric excess achieved in the hydrolysis of (*R*,*S*)-2-butyroyl-2-phenylacetic acid catalysed by MML

Enzyme derivative	Preferred enantiomer	25 °C, pH 7		4 °C, pH 7		25 °C, pH 5	
		ee[a]	*E*[b]	ee[a]	*E*[b]	ee[a]	*E*[b]
Octadecyl	*R*	40	3.4	51	5	7	1.2
IDA-Cu^{2+}	*S*	84	30	27	2.2	51	5
IDA	*S*	51	5	90	59	75	16
EDA	*S*	69	11	7	1.2	15	1.5

Experiments were performed as described in Section 2.2.

[a] Enantiomeric excess of the product at 50% of conversion.

[b] Enantioselectivity was calculated as the ratio between the initial hydrolytic activities of the different enzyme preparation. against the different enantiomers (V preferred isomer/V non-preferred isomer).

were less sensitive to the variations in the temperature (changing from 3.2 to 5).

Changing the pH from 7 to 5 caused a significant improvement of the *E* observed using the IDA-epoxy support (from 5 to 16), but caused a decrease in the *E*-value obtained for all other immobilized enzyme preparations. Octadecyl and EDA epoxy-derivatives were found to be only marginally enantioselective under these conditions, while the *E*-value of Cu-epoxy-preparation decreased from 30 to 5.

Again, the results suggested that the properties of the different immobilized preparations were very different and that the influence of the experimental conditions was also dependent on the type of preparation used.

4. Discussion

The results presented in this paper suggest that it may be possible to modulate lipase properties through directed immobilization (altering rigidity and environment) and slight changes in the experimental conditions (that is, via so called "conformational engineering" using physico-chemical tools).

Thus, under a particular set of conditions, the same lipase immobilized on different supports, having different rigidity and microenvironment, exhibited very different catalytic properties: different activity (even by a 15-fold factor) and different *E*-value (varying from 5 for one enantiomer to 59 for the other just by changing the immobilization protocol). These changes can hardly be obtained in such a simple way using any other technique. In fact, the lipase behaves as a different lipase when it is immobilized on different supports.

Moreover, slight changes in the reaction conditions can alter the lipase properties; e.g. with MML immobilized on IDA-Cu^{2+}-Sepabeads support the *E*-value increased from 2.2 to 30 just by increasing the temperature from 4 to 25 °C and with EDA-Sepabeads MML from 1.5 to 11 by changing the pH from 5 to 7.

More interestingly, an interaction between these two tools has been also found, a same variable may have different effects on different derivatives. For example, by changing the reaction Temperature, octadecyl-Sepabeads derivative hardly changed its *E*-value, while the other immobilized derivatives proved to be more sensitive.

These results seem to support the initial hypothesis showed in Scheme 1, differences between different immobilized derivatives may only be explained by the different behavior of the enzymes immobilized on the support. In fact, some modulation of the enzyme properties via directed immobilization has been previously reported for other lipases (e.g. lipase from *Ps. flourescens* [15], lipase from *Candida antarctica B* [17], lipase from *Candida rugosa* [16]) and other enzymes suffering significant conformational changes during catalysis (e.g. *Penicillin G. acylase* [18,19]).

Therefore, this mechanism of altering the properties of the enzymes seems to be a general trend in enzymes that suffer dramatic changes in the conformation during catalysis.

Acknowledgements

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