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Preparation of new lipases derivatives with high activity–stability in anhydrous media: adsorption on hydrophobic supports plus hydrophilization with polyethylenimine

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Abstract

A novel method to prepare immobilized lipases derivatives is hereby proposed. Lipases are firstly adsorbed on supports having large internal surfaces covered by hydrophobic groups (e.g. polyacrylic resins covered by C18 moieties). Then, immobilized lipases are incubated in the presence of polyethyleneimine (PEI) at a pH value over the isoelectric point of the enzyme in order to cover the lipase surface with this polymer. In this way, we try to minimize all possible direct interactions between immobilized lipase and organic solvents when using these derivatives in anhydrous media.

Lipases from *Rhizomucor miehie* (RML) and *Candida rugosa* (CRL) were immobilized according to the proposed protocol. These derivatives were very active and very stable when catalyzing esterifications and transesterifications in anhydrous media. For example, RML derivatives exhibited a very high synthetic activity (more than 1000 Units/g immobilized biocatalyst) even when catalyzing the esterification of lauric acid with octanol at water activity values very close to zero. On the contrary, covalently immobilized derivatives exhibited a much lower synthetic activity under similar conditions (less than 10 Units/g of immobilized biocatalyst). Moreover, these new RML derivatives preserve 100% activity after incubation for 3 days in anhydrous butanone in the presence of molecular sieves. Under the same conditions, commercial immobilized RML lost more than 90% of activity in less than 10 min. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

1.1. Immobilized lipases in organic solvents

The impressive possibilities of lipases as catalysts of biotransformations in organic solvents have been

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extensively described in literature [1–3]. In most cases, these studies have been performed by using lipase aggregates (lyophilized lipases, enzymes "straight from bottle", enzyme precipitated on solid supports, etc.). Although such reaction systems are too complex to be analyzed at a molecular level, these lipase preparations have been found to be suitable biocatalysts, at least for preliminary tests at a laboratory scale.

However, immobilization of isolated lipase molecules on pre-existing solid porous supports (e.g. by covalent immobilization, by strong physical adsorption) may offer additional advantages both at laboratory as well as at industrial scale [4]:

- full dispersion of the molecules of immobilized lipase allows an accurate study of the behavior of lipases in organic solvents at a "molecular" level;
- pre-existing supports may provide better mechanical and dynamical properties to the final biocatalyst;
- the performance of "directed and controlled" immobilization protocols may provide "new tools to improve" activity, selectivity and stability of lipases [5,6].

1.2. Sepabeads as very rigid supports suitable for immobilization of lipases

These polyacrylic resins are prepared via a very intense polymerization in the presence of porogenic agents. In this way, they exhibit a macroporous structure (e.g. 400 A of pore diameter) but being very rigid. Opposite to most of organic supports, Sepabeads exhibit almost identical morphology in dry form, in aqueous media or in organic solvents. That is, these dry supports are not swollen when incubated in water and they do not shrink after a further drying or a further incubation in organic media. In this way, lipases can be fairly immobilized on these supports in aqueous. These immobilized derivatives can be firstly assayed in water media and then dried and assayed again in anhydrous organic solvents with no changes in the support morphology. In addition to that, these supports are very resistant to mechanical stirring and they can be easily filtered and reused again for many reaction cycles.

1.3. Conventionally immobilized lipases (e.g. covalent immobilization, ionic adsorption) in organic media

The use of conventionally immobilized lipases in anhydrous organic solvents may promote interesting physic-chemical phenomena.

On one hand, lipases are enzymes that undergo some dramatic conformational changes during catalysis [7,8]. In aqueous medium, the enzyme is in an equilibrium between a closed inactive structure and an open and active one. However, in the presence of hydrophobic interfaces, this equilibrium completely shifts towards the open form(s). In the presence of anhydrous organic solvents, the enzyme may undergo interfacial activation promoted by the interface of the solvent that is able to penetrate inside the porous structure of the biocatalyst. On the other hand, the enzyme surface may be also completely surrounded by an hydrophobic solvent and this phenomenon may promote dramatic inactivations of the immobilized enzyme. In this way, activity and stability properties of these lipase derivatives may dramatically depend on the used organic solvent [9–11] (Fig. 1).

1.4. Design of new lipase derivatives

In this paper, we discuss the preparation of new immobilized lipase derivatives with a very low sensitivity (positive or negative) towards the organic solvents even when working in anhydrous media. In order to avoid those two possible direct interactions between immobilized lipases and organic solvents, a new protocol for preparation of immobilized lipase derivatives is hereby proposed.

First, lipases are selectively adsorbed, at low ionic strength, on solid porous supports completely covered by a dense layer of hydrophobic moieties (e.g. polyacrylic resins (Sepabeads) covered by octadecyl moieties) Lipases become adsorbed and activated "against" these well-defined and controlled support surfaces [12,13]. In this way, it can be expected that

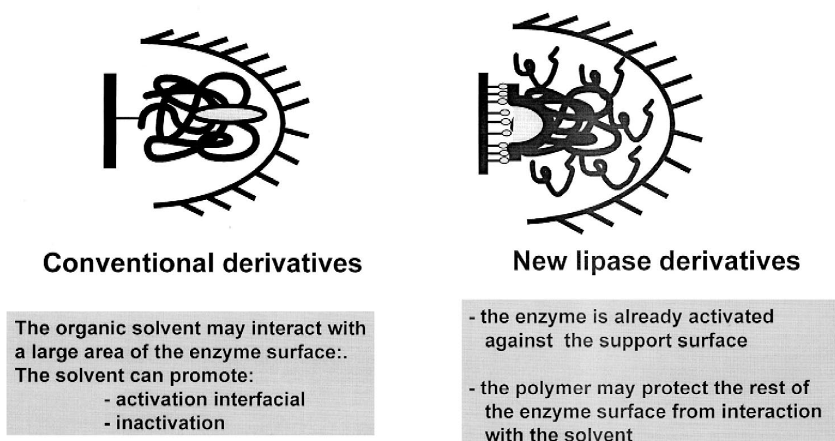

Fig. 1. Different lipase derivatives in anhydrous organic media.

the active center of the enzyme, very near to the support surface, may not undergo interfacial activation with the organic solvent.

Immobilized lipases are further incubated in the presence of a random coil polycationic polymer, polyethyleneimine (PEI) at low ionic strength and at a pH value higher than the isoelectric point of the enzyme. In this way, the whole surface of immobilized lipase molecules (except the hydrophobic active center activated against the support) may become fairly covered by a layer of a very hydrophilic polymer (Fig. 1). Hence, it may become protected from direct interaction with the organic solvents [14] (e.g. protected from deleterious effects on solvent interfaces).

2. Experimental

2.1. Materials

Purified lipase from *Rhizomocur miehie* (RML) was from Fluka, lipase from *Candida rugosa* (CRL), *p*-nitrophenyl propionate and olive oil emulsion were from Sigma. Sepabeads supports (amino and decaoctyl) were a kind gift from Resindion (Mitsubishi Chemical Corporation). Glutaraldehyde-Sepabeads supports were prepared using the amino-Sepabeads (Resindion — Mitsubishi Chemical Corporation). Activation was carried out as previously described for amino-agarose gel [15]. Lipozyme was obtained from Novo Nordisk — Spain. Other reagents were analytical grade.

2.2. Methods

2.2.1. Immobilization of lipases on C18-Sepabeads

Commercial samples of soluble lipase were dissolved in 5 mM phosphate pH 7.0 at 4°C. Solutions were centrifuged to eliminate insoluble material. Then, 1000 ml of lipase solution (containing 10 g of CRL commercial preparation or 200 mg of pure RML) and 10 g of C18-Sepabeads were mixed. The solution was gently stirred for 3 h at 4°C and all lipase (and no other proteins contained in the sample) becomes interfacially adsorbed on the support [12,16]. Then, immobilized lipase derivative was washed with 5 mM phosphate pH 7.0 to eliminate all

the no-lipase proteins. Derivatives were filtered under vacuum in order to remove all buffer except that filling the pores of the derivatives. All lipase activity resulted immobilized under these conditions.

2.2.2. Adsorption of poly(ethylenimine) on the immobilized derivatives

Immobilized derivatives were incubated in 1% of poly(ethylenimine) (20,000 Da MW) in 5 mM phosphate buffer pH 7.0. The suspension was gently stirred for 1 h and then washed with 5 mM phosphate buffer. Derivatives were filtered under vacuum in order to remove all buffer except that filling the pores of the derivatives.

2.2.3. Covalent immobilization of lipases on glutaraldehyde-Sepabeads

Immobilization of lipases on glutaraldehyde-Sepabeads was performed as described above for hydrophobic adsorption but now using 50 mM sodium phosphate as buffer. Other parameters as lipase loading, immobilization times, immobilization yields were exactly those described above.

2.2.4. Drying of lipase derivatives

2.2.4.1. Complete drying. Organic solvents were dried by overnight incubation in the presence of a high excess of dry molecular sieves (500 g of sieves per liter of solvent). Then, 5 g of lipase derivative were suspended in 50 ml of dry solvents in the presence of 20 g of dried molecular sieves for 12 h. In this way, the water filling the pores of derivatives was dissolved in the organic solvent and then adsorbed on the sieves. Sometimes drying was also performed by incubating 5 g of immobilized derivatives in a closed vessel in the presence of 20 g of anhydrous P_4O_{10} and submitted to intermittent vacuum for 100 h.

2.2.4.2. Partial drying. Five grams of immobilized lipase derivatives were incubated, in a closed vessel, in the presence of 100 ml of the adequate solution of salt during 100 h. In this way, depending of the salt used, different equilibrium positions corresponding to different water activity values were reached [17].

2.2.5. Activity of lipase derivatives in anhydrous organic solvents. Effect of water activity

Immobilized lipases and substrates were dissolved or suspended in solvent and placed in a closed vessel containing the convenient salt solution to provide equilibrium positions at different water activities. One gram of equilibrated lipase was then added to 25 ml of the substrate solution (2.5 g of lauric acid and 1.9 g of octanol) in the same closed vessel. Reaction was followed by HPLC. When working at zero level of water activity values, 20 g of dry molecular sieves were also added to the reaction mixture.

2.2.6. Stability of lipase derivatives in anhydrous organic solvents

Immobilized lipases were fully dried as described above and the activity was assayed in a transesterification in anhydrous medium (using methanol and ethyl butyrate as substrates). After a further 72-h incubation period, the activity of the immobilized lipases was assayed again under the same conditions.

3. Results

3.1. Synthetic activity of different CRL derivatives at different water activities

Conventional covalently immobilized CRL (on glutaraldehyde supports) were very poorly active (less than 1 μmole/min/g of biocatalyst) when catalyzing the synthetic reaction (esterification of octanol and lauric acid in hexane) in all range of water activity values (Fig. 2). However, our new CRL derivative (adsorbed on C18-Sepabeads plus treated with PEI) were much more active even when water activity become near to zero (35 μmole/min/g of biocatalyst). The highest activity was reached at a water activity value of 0.8 (125 μmole/min/g of biocatalyst) and it was more than two orders of magnitude higher than the one corresponding to the covalently immobilized enzyme (Fig. 2).

Other derivatives, obtained by simple interfacial adsorption of the enzyme on hydrophobic supports, were also much more active than the conventional CRL derivatives (Fig. 2). However, our best deriva-

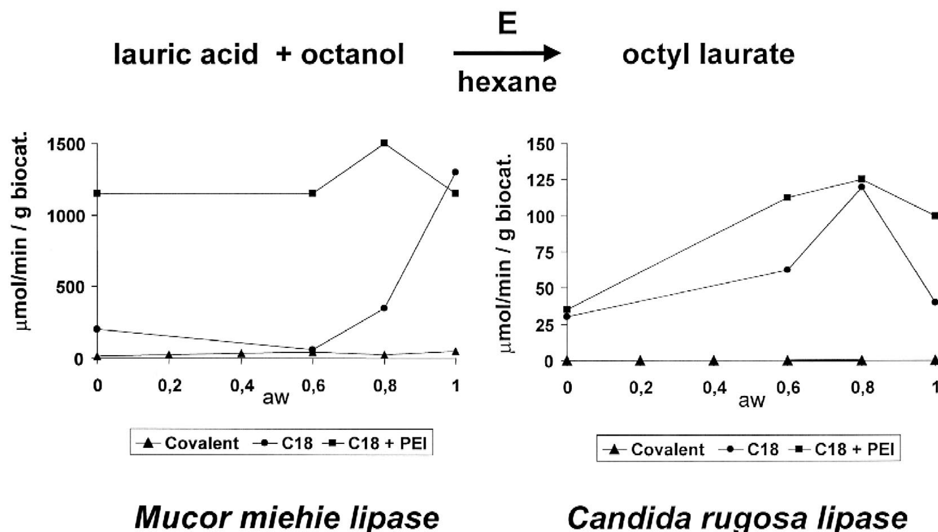

Fig. 2. Esterifications by immobilized lipases.

tives (with additional treatment with PEI) were more active and much less sensitive to different water activity values.

3.2. Synthetic activity of different RML derivatives at different water activities

Conventional covalently immobilized RML derivatives exhibited a good synthetic activity (50 μmole/min/g of biocatalyst) at a water activity value of 0.6 (Fig. 2). However synthetic activity was very dependent on the water activity of reaction medium and it decreased to less than 10 μmole/min/g of biocatalyst when water activity approached to zero.

Interestingly, our best RML derivative was much more active and much more insensitive to water activity. Synthetic activity was ranging between 1500 and 1200 μmole/min/g of biocatalyst when varying water activity from 1.0 to zero.

Again, the whole immobilization–stabilization protocol seems to be strictly necessary. Those derivatives that were not incubated with PEI were less active and more sensitive to water activity than the optimal ones (adsorbed on C18-Sepabeads and further incubated with PEI).

3.3. Stability of different CRL derivatives in anhydrous hexane

Conventional covalently immobilized CRL derivatives were very rapidly inactivated when incubated in anhydrous hexane. In less than 5 min, synthetic activity (now assayed for transesterification of ethyl butyrate with methanol) drops to zero (Fig. 3).

CRL simply interfacially adsorbed on C18-resin exhibits a significant synthetic activity (around 40 μmole/min/g of biocatalyst) after incubation in anhydrous hexane, but this activity became zero after a long-term incubation in anhydrous hexane for 72 h. However, our best derivative, with the surface protected with PEI, not only exhibits the highest activity (100 μmole/min/g of biocatalyst) at the beginning of the incubation in anhydrous hexane but it also preserved 80% of the enzyme activity after the long-term incubation for 3 days (Fig. 3).

STABILITY OF CRL DERIVATIVES IN ANHYDROUS HEXANE

ACTIVITY (IU/ mL)

	t = 0	t = 72 h
	0	0
	40	0
	100	80

MeOH + ethyl butyrate ⟶ methyl butyrate + EtOH

Fig. 3. Stability of CRL derivatives in anhydrous hexane.

3.4. *Stability of different RML derivatives in anhydrous solvents*

The three derivatives studied (commercial Lypozyme, interfacially adsorbed derivative and interfacially adsorbed and PEI-protected derivative) showed good initial transesterification activities when the experiments were carried out in anhydrous hexane (Fig. 4). The commercial sample (Lypozyme from Novo Nordisk) had 100 μmole/min/g of biocatalyst, while the other two derivatives exhibited activities near to 1000 μmole/min/g of biocatalyst (in this case, the activities of both derivatives were quite similar). The commercial derivative lost 60% of the initial activity after only 3 days of incubation under these conditions, while the other derivatives retained almost intact their activities after similar incubation period.

The situation becomes more complex when using a more polar solvent (butanone) (Fig. 4). The synthetic activity of the commercial RML sample dropped to 10 μmole/min/g of biocatalyst at the beginning of the incubation and it became negligible after a 3-day incubation. On the contrary, our best RML derivative exhibited the same synthetic activity observed in hexane (around 1000 Units/ml) and again it preserves fully active after a long-term incubation in this polar anhydrous solvent. Again, the additional incubation of adsorbed lipase with PEI seems to be highly positive. Interfacially adsorbed derivatives prepared without PEI treatment showed a good initial synthetic activity, but they become rapidly inactivated after long-term incubation in butanone.

STABILITY OF MML DERIVATIVES IN ANHYDROUS MEDIA

Activity (IU / ml)

	hexane		butanone	
	t = 0	t = 72	t = 0	t = 72
	1000	1000	1000	200
	1000	1000	1000	1000
LIPOZYME	100	40	10	0

MeOH + ethyl butyrate ⟶ methyl butyrate + EtOH

Fig. 4. Stability of MML derivatives in anhydrous media.

4. Discussion

The combination of the interfacial adsorption of lipases on hydrophobic support surfaces plus an additional hydrophilization of the rest of lipase surface with PEI promotes the preparation of immobilized derivatives (e.g. of CRL or of RML) with excellent activity and stability properties in anhydrous organic media.

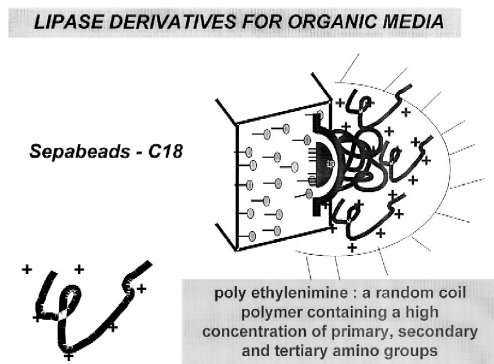

Fig. 5. Lipase derivatives for organic media.

RML derivatives exhibited an extraordinary synthetic activity (more than 1000 μmole/min/g of biocatalyst) when working in very wide range of water activity values (from 1 to 0). In addition to that, these RML derivatives preserved full activity after long-term incubation, under anhydrous conditions, in the presence of apolar (hexane) or polar (butanone) solvents. These activity–stability parameters are much better than ones corresponding to covalently immobilized derivatives in spite of having exactly the same amount of immobilized enzyme. In addition to that, these new RML derivatives were also much more active and stable than some very popular commercial derivatives.

The interfacial adsorption of lipases on hydrophobic support surfaces seems to be superior to other conventional protocols for lipase immobilization (e.g. covalent binding on glutaraldehyde supports). However, the best activity–stability values were always observed when that interfacial adsorption was combined with an additional treatment with PEI. As represented in Fig. 5, some interesting phenomena could be partially responsible of these catalytic properties:

(i) Lipase molecules undergo interfacial adsorption on the support and they can not undergone additional interfacial activation with the solvent.
(ii) The rest of lipase surface is covered by a layer of PEI and this highly hydrophilic shell protects the enzyme from additional interaction with solvent interfaces.
(iii) The highly hydrophilic environment surrounding immobilized lipases could have a positive influence in the preservation of an essential layer of water molecules surrounding each immobilized lipase.

Because of their good activity–stability properties, these new lipase derivatives could be very useful to simplify and to optimize the design of biotransformations in anhydrous systems. Now, water activity and polarity of the solvent could be selected on the basis of thermodynamical parameters and of substrate solubility but without significantly affecting the activity and stability of immobilized lipase derivatives.

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