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Interfacial adsorption of lipases on very hydrophobic support (octadecyl–Sepabeads): immobilization, hyperactivation and stabilization of the open form of lipases

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Abstract

Octadecyl–Sepabeads (Mitsubishi Chemical Corporation) was used to immobilize the lipases from *Candida antarctica* (fraction B), from *Mucor miehei* and from *Candida rugosa* via interfacial adsorption. The activity and stability properties of the derivatives obtained using this strategy by "stabilization of the open structure of the lipase" were compared to other more conventional immobilized derivatives (e.g. those obtained by multipoint covalent attachment) where the lipases are likely to be immobilized exhibiting its closed structure. Lipases adsorbed on hydrophobic supports exhibited a clear hyper-activation compared to the soluble enzyme or other types of derivatives. Their specific activities were greatly improved after immobilization (*M. miehei* lipase derivative was even 20 times more active than the soluble enzyme). Furthermore, lipases adsorbed on hydrophobic supports were very stable against heat and organic solvents inactivation. For example, *C. antarctica B* lipase octadecyl derivatives preserved 100% of the activity after 200 h of incubation at pH 7 and 50 °C. In addition, these derivatives remained also fully active after a very long incubation (200 h) in 50% dioxane at pH 7 and 25 °C. In spite of being immobilized by simple physical adsorption these lipase derivatives were more stable than multipoint covalently immobilized derivatives and much more stable than their respective soluble enzyme. It seems that the "open structure" of lipases, adsorbed on hydrophobic supports, is much more active and much more stable than the corresponding "closed" structure even when the closed structure is undergoing a very intense multipoint covalent attachment.

Keywords: Lipase immobilization; Interfacial immobilization; Sepabeads; Open structure of lipases

1. Introduction

Lipases (glycerol ester hydrolases E.C. 3.1.1.3) natural function is the hydrolysis of triglycerides. However, lipases may be used in vitro to catalyze many different reactions, often very different from the natural ones (regarding conditions, substrates, etc). Thus, lipases may be used in the industry not only to modify oils and fats [1–3], but also to synthesize fatty esters as cosmetic or surfactants [4–7] and to produce many different intermediates for organic synthesis (e.g. resolution of racemic mixtures) [8–12]. In fact, lipases are very likely the most used enzymes in organic chemistry because these enzymes combine a broad substrate

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specificity with a high enantio- and regio-selectivity [13–16].

Lipases have a very complex catalytic mechanism. In aqueous homogeneous solutions, lipases are in equilibrium between two forms. A main closed and inactive form, where a flap or lid secludes the active center from the medium and an open and active form, where this lid is moved to allow substrate accessibility to the active center [17]. In the presence of drops of the natural substrates (oils), lipases are adsorbed to the hydrophobic interface via the very hydrophobic area formed by the internal face of the flap and the surroundings of the active center [18,19]. This adsorption promotes a dramatic change of the enzyme structure, leading to an apparently more ordered enzyme structure [17].

Some problems arise from this mechanism of action when industrial immobilized lipase bio-catalysts are prepared. When inside a porous structure, lipase molecules become inaccessible to external interfaces; therefore, there is no possibility of enzyme interfacial adsorption in aqueous solutions. In fact, conventionally immobilized lipase preparations are usually utilized in almost anhydrous media, where the lipase may become activated by the direct interaction with the organic solvent phase [20,21].

However, many interesting reactions catalyzed by lipases may be advantageously carried out in aqueous systems, for example hydrolytic resolutions of racemic mixtures [14,15]. The interfacial adsorption of lipases on hydrophobic supports has been proposed as a simple method to prepare immobilized lipase derivatives useful to be used in any media [22]. The hypothesis behind this immobilization strategy is to take advantage of the complex mechanism of lipases (an apparent problem) as a tool that permits the immobilization of lipases via an "affinity-like" strategy [22]. Using a hydrophobic support that somehow resembles the surface of the drops of the natural substrates, and very low ionic strength, lipases become selectively immobilized on these supports. The adsorption involves the hydrophobic areas surrounding the active center and the internal face of the flap (other water soluble proteins are not adsorbed on the support under these mild conditions) [19–22]. These adsorbed lipases present an open form, with the active center accessible for the small substrates, in fact, immobilized enzymes usually exhibit a significantly enhanced enzyme activity (by the "interfacial activation mechanism"). That is, we have an immobilized lipase where the open form of the lipase has been "fixed" and does not depend on the presence of external hydrophobic interfaces.

In this paper, we have used a new commercial hydrophobic support (Sepabeads from Mitsubishi Chemical Corporation) which has some advantages compared to other available hydrophobic supports. The preparation of the Sepabeads (a very intense crosslinking in the presence of porogenic agents) accounts for the internal geometry of the support, which presents pores having large surfaces where the proteins may interact intensively. Also, it is very rigid (therefore, adequate to be used in packed or stirred reactor) and it does not swell when it changes from aqueous buffer to anhydrous media (therefore, could be used in any reaction media). The support used in this work is an epoxy acrylic matrix with the surface covered by octadecyl groups, yielding a very hydrophobic support surface. We have studied some properties (activity, stability) of different lipases immobilized on this support, compared that to other preparations (covalently attached derivatives).

2. Materials and methods

2.1. Materials

The lipases from *Candida antarctica* (fraction B) (CAL-B; Novozym 525L), and *Mucor miehei* (MML; Novozym 388) were obtained from Novo Nordisk (Denmark). Lipase from *Candida rugosa* (Type VII) (CRL; specific activity, 875 U/mg solid) was from Sigma. Octadecyl–Sepabeads was generously donated by Resindion Srl (Mitsubishi Chemical Corporation, Milan, Italy) while agarose 10BCL was kindly donated by Hispanagar SA (Burgos, Spain). Octyl–Sepharose 4BCL was purchased from Pharmacia Biotech (Uppsala, Sweden). Glutaraldehyde–agarose [23] and Glyoxyl–agarose [24] were prepared as previously described. Tritón X-100, *p*-nitrophenyl propionate (*p*NPP) and ethyl butyrate were from Sigma. Other reagents and solvents used were of analytical grade.

2.2. Methods

2.2.1. Hydrolysis of pNPP

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 *p*NPP 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 2.5 ml of substrate solution. One international unit of *p*NPP activity was defined as the amount of enzyme that is necessary to hydrolyze 1 μmol of *p*NPP per minute (IU) under the conditions described previously.

2.2.2. Hydrolysis of ethyl butyrate

This assay was performed by using a pHstat, measuring the release of butyric acid promoted by the enzymatic hydrolysis of ethyl butyrate 50 mM at pH 7 and 25 °C.

2.2.3. Purification of lipases

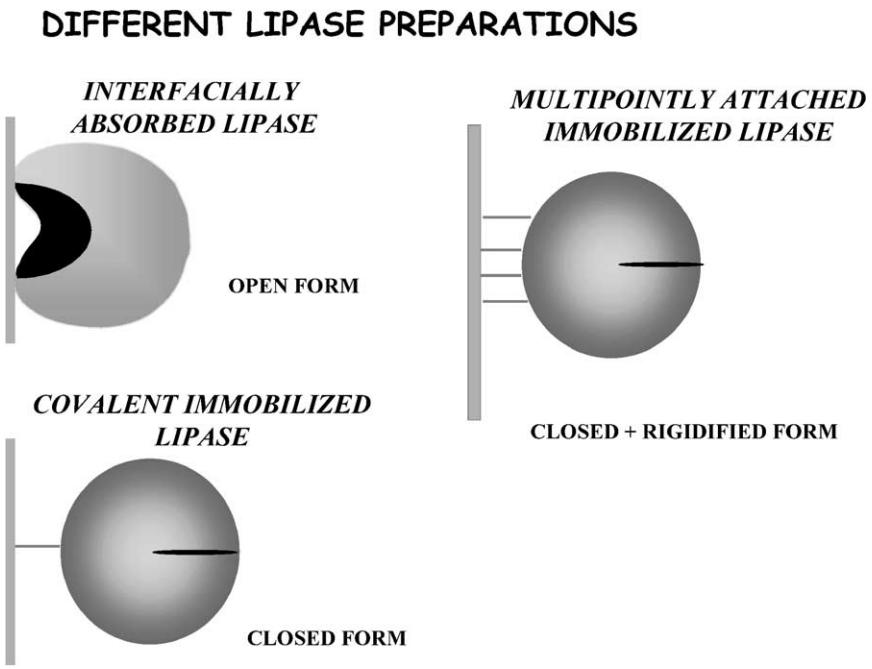
To purify the lipases from any other contaminant protein (e.g. esterases) the different lipase preparations were adsorbed on octyl–agarose following the procedure previously described [22]. In order to desorb the enzymes, the adsorbed lipases were washed with Tritón X-100 in 5 mM sodium phosphate buffer at pH 7 and 4 °C (different Tritón X-100 concentrations were necessary for the different lipases: 1% for *C. antarctica B* lipase, 0.5% for *C. rugosa* lipase, 0.07% from *M. miehei* lipase).

Following these protocols, a quantitative immobilization of lipase activity was observed and the SDS–PAGE analysis of the protein adsorbed to the octyl–Sepharose [22] only showed a single band with a molecular weight corresponding to that of the different native lipases.

2.2.4. Lipases immobilization on different supports

Three different derivatives were prepared (Scheme 1). Enzyme loading never was higher than 1 mg/ml of support (that is around 1–2% of the maximum loading) in order to prevent diffusion problems.

2.2.4.1. Preparation of octadecyl–Sepabeads lipase derivatives. The purified lipase was diluted to a ten-fold factor using distilled water (to dilute the Tritón X-100) and octadecyl–Sepabeads was added.



Scheme 1. Different lipase preparations.

Samples of suspension and supernatant were periodically withdrawn in order to check the immobilization protocol. In some cases, no purified 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.

2.2.4.2. Lipases immobilization on glyoxyl–agarose. This immobilization protocol was selected to prepare multipoint covalently immobilized lipases preparations, where a certain rigidity of the enzyme structure could be expected [25,26]. Glyoxyl–agarose was added to the different enzyme solutions, and the pH was increased to 10.5. After 17 h, the enzyme–support multi-interaction was ended by adding 1 mg of sodium borohydride per ml of suspension.

2.2.4.3. Lipases immobilization on glutaraldehyde–agarose. Glutaraldehyde–agarose was added to the purified enzyme. After 5 h of immobilization, 1 volume of 100 mM sodium bicarbonate containing 2 mg/ml of sodium borohydride at pH 8.5 was added as an end point to the enzyme support reaction.

2.2.5. Stability of the different lipase derivatives preparations

The different derivatives were incubated at the conditions described (pH, *T*, presence of organic co-solvents) and samples of these inactivating reactions were periodically withdrawn and their activity assayed as described previously.

2.2.6. Temperature–enzyme activity profile of different lipases preparations

The temperature effect on the enzyme activity of the lipases preparations was checked in the hydrolysis of ethyl butyrate 50 mM in 50 mM sodium phosphate at pH 7. The buffer was pre-incubated to reach the desired temperature before adding the ethyl butyrate and the enzyme.

3. Results and discussion

3.1. Effect of immobilization on enzyme activity

Fig. 1 shows the immobilization courses for the lipase from *M. miehei* on different supports. Here, no purified preparations were used to avoid any possible effect of Tritón X-100 on enzyme behavior. While the covalent immobilization on glutaraldehyde– and glyoxyl–agarose presented a negligible effect on the enzyme activity (even promoted a slight decrease) the interfacial immobilization promoted a significant increment in enzyme activity. In fact, there was a progressive increment in activity (by five-fold factor).

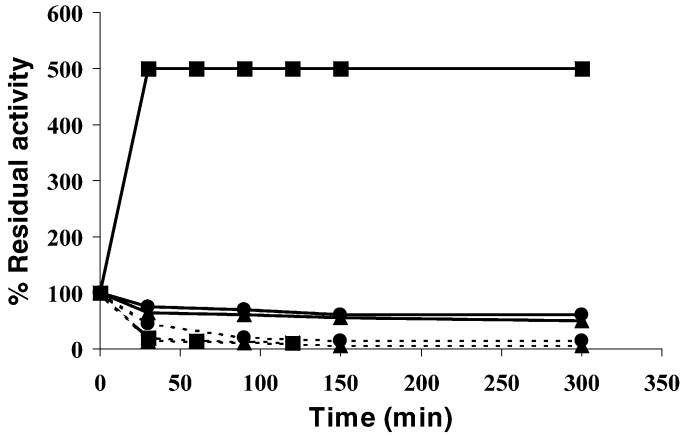


Fig. 1. Immobilization courses of *Mucor miehei* lipase on different supports. Experiments were carried out using crude enzyme preparations. Other specifications as described in Section 2.2. Soluble enzyme kept unaltered its activity under all the immobilization conditions. Octadecyl–Sepabeads (squares), glyoxyl–agarose 10BCL (circles), glutaraldehyde–agarose 10BCL (triangles).

Table 1
Relative activity of enzymatic derivatives with *p*NPP and ethyl butyrate

Lipases	Enzymatic derivatives	Relative activity	
		*p*NPP (0.4 mM)	Ethyl butyrate (50 mM)
CAL-B	Soluble	1	1
	Glyoxyl	0.9	0.8
	Octadecyl	0.95	2
	Glutaraldehyde	0.85	0.85
CRL	Soluble	1	1
	Octadecyl	1	4
	Glutaraldehyde	0.3	0.75
MML	Soluble	1	1
	Glyoxyl	0.7	0.73
	Octadecyl	5	20
	Glutaraldehyde	0.5	0.6

All enzymatic derivatives had the same enzyme loading (1 mg/ml). The experiments were performed as described in Section 2.2.

Similar results were found using the enzyme from *C. rugosa* and *C. antarctica B*. Table 1 shows the obtained results using purified enzyme, using both *p*NPP and ethyl butyrate substrates. The lipase from *C. rugosa* could not be immobilized on glyoxyl–agarose because of the lack of stability of the enzyme at pH 10.

Therefore, the hyperactivation of the different enzymes, when they were immobilized by interfacial adsorption, was clearly evidenced as well as the fact that this effect could not be directly related to the dispersion of the enzymes on the support (that should occur in all the immobilized derivatives). Hyperactivation was always more significant using ethyl butyrate as substrate, perhaps because it is a small and hydrophobic compound which can easily accede to the active site of the enzyme.

3.2. Effect of immobilization on enzyme stability

Fig. 2 shows the thermal inactivation of the different CAL-B derivatives. The glutaraldehyde–agarose derivative presented a higher stability than that of the soluble enzyme. Glyoxyl–agarose derivative was more stable than the glutaraldehyde preparation, suggesting that some multipoint covalent attachment between the enzyme and the support has been introduced increasing the enzyme rigidity and, therefore, its stability. However, the thermal stability of the interfacially immobilized derivative was much higher than the stability of the glyoxyl one. In fact, the interfacially adsorbed derivative remained fully active under conditions where the soluble enzyme was fully inactivated and even the glyoxyl derivative presented <70% of the initial activity.

Interfacially immobilized CAL-B derivative was also more stable in the presence of organic co-solvents (under conditions in which the enzyme remained

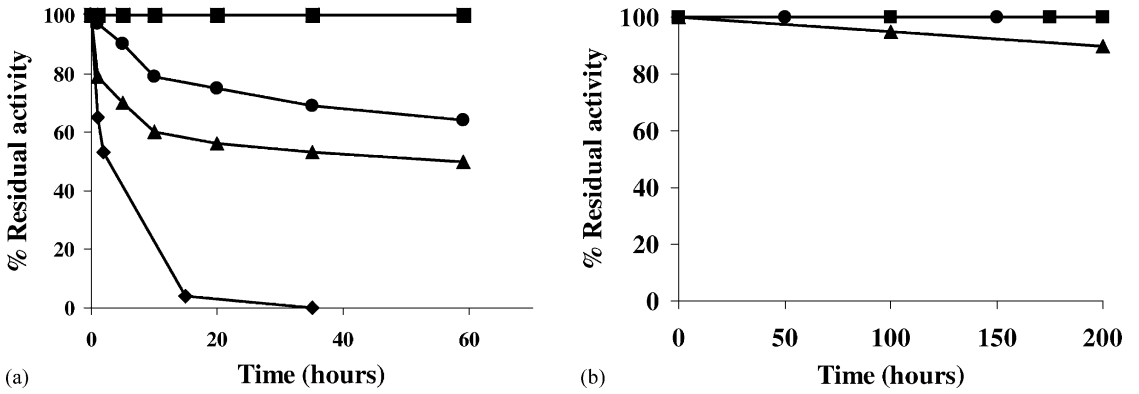


Fig. 2. Stability of different derivatives from *C. antarctica B* lipase. (a) Thermostability of derivatives from *C. antarctica B* lipase. Inactivation was performed at pH 7 and 50 °C. Experiments were carried out as described in Section 2.2. Octadecyl–Sepabeads (squares), glyoxyl–agarose 10BCL (circles), glutaraldehyde–agarose 10BCL (triangles), soluble enzyme (rhombus). (b) Stability against co-solvents of derivatives from *C. antarctica B* lipase inactivation was performed at 25 °C and pH 7 in the presence of 50% dioxane. Experiments were performed as described in Section 2.2. Octadecyl–Sepabeads (squares), glyoxyl–agarose 10BCL (circles), glutaraldehyde–agarose 10BCL (triangles).

Table 2
Stability of *C. rugosa* lipase and *M. miehei* lipase on different conditions

Enzyme	Derivative	Thermal inactivation (%)	Co-solvent inactivation (%)
CRL	Soluble	0 (after 2 h)	nd
CRL	Glutaraldehyde	70	30
CRL	Octadecyl	100	80
MML	Soluble	10	nd
MML	Glutaraldehyde	20	30
MML	Glyoxyl	40	60
MML	Octadecyl	60	100

The table shows the percentage of activity exhibited after 100 h of incubation of the enzyme at pH 7 and 45 °C (thermal inactivation) or at pH 7 and 25 °C in the presence of 40% dioxane (co-solvent inactivation). Experiments were carried out as described in Section 2.2.

adsorbed on the support) than their covalent counterparts (glyoxyl derivative being once again more stable that glutaraldehyde), even though the presence of the organic solvents could somehow weaken the adsorption strength of the enzyme on the support, which is based in hydrophobic interactions.

In the case of the interfacially adsorbed derivative of CRL, its activity remained unaltered after 100 h at 45 °C (Table 2), while the soluble enzyme was fully inactivated (in fact, all the activity disappeared after only 2 h) and the glutaraldehyde derivative presented just over 60% of the initial activity (Table 2).

The stability in the presence of dioxane was lower for this enzyme compared to CAL-B since the hydrophobically adsorbed enzyme presented 80% of activity after 100 h of incubation in these conditions, while the glutaraldehyde derivative maintained only 30% of the initial activity (Table 2).

MML derivatives seemed to be thermally more unstable than the other enzymes ones (Table 2). Thus, at 100 h of incubation, the interfacially adsorbed enzyme activity was reduced to 40% while glutaraldehyde derivative and soluble enzyme decreased the activity around 80%. However, the enzyme was quite resistant to the action of dioxane. Interfacially adsorbed derivative preserved its activity unaltered after 100 h in 40% dioxane, while glutaraldehyde derivative decreased its activity to 70%. Glyoxyl derivative was more stable than glutaraldehyde derivative, but less stable than the interfacially adsorbed one.

These results show that the interfacially adsorbed enzyme was always the most stable preparation, even when compared to multipointly immobilized preparations, in both thermal and organic co-solvent inactivation.

3.3. Effect of immobilization on enzyme optimal temperature

In the case of lipase from *C. rugosa*, we have assayed the optimal *T* for the free enzyme and the interfacially immobilized derivative, under conditions where the substrate was completely soluble (to prevent the interfacial adsorption of the lipase to the substrate drop). When the free enzyme was assayed the maximum activity was obtained at 45 °C while the optimal *T* for the immobilized derivative was 55 °C (Fig. 3).

Again, results suggested that the interfacial adsorption of lipases promoted a high increment in the stability of the enzyme and an improvement of the possibilities of using these enzymes in aqueous medium.

3.4. Reversibility of the lipase adsorption

A very high adsorption strength of the lipase to the support is convenient to prevent desorption of the lipase under industrially relevant conditions. However, it is convenient that the enzyme can be desorbed from the support after enzyme inactivation,

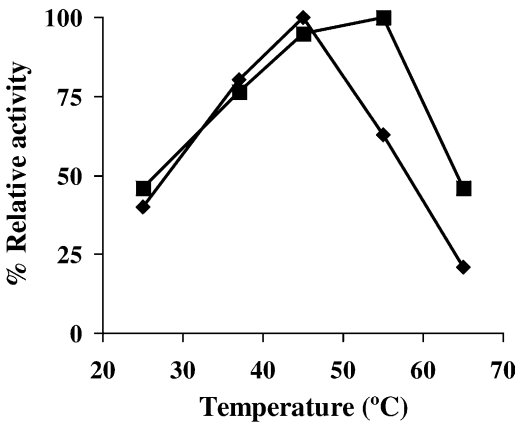


Fig. 3. Reaction temperature effect on the hydrolytic activity of *C. rugosa* lipase. Experiments were carried out using ethyl butyrate as described in Section 2.2, it was performed at pH 7; octadecyl–Sepabeads (squares), soluble enzyme (rhombus).

Table 3
Desorption of the lipases from octyl–agarose and octadecyl–Sepabeads

Lipase	Support	Tritón X-100 (%)
CAL-B	Octyl	1
	Octadecyl	4
CRL	Octyl	0.5
	Octadecyl	3
MML	Octyl	0.07
	Octadecyl	0.2

The numbers express the amount of detergent necessary to desorb 100% of lipase from the support. Experiments were performed as described in Section 2.2.

in order to reuse the support (reloading it with fresh enzyme) and take advantage of the reversibility of the immobilization technique. Table 3 shows that in all the cases the adsorption of lipase to octadecyl–Sepabeads is very strong, in fact significantly stronger than to octyl–agarose [22]. This allows to use octadecyl–Sepabeads under more drastic conditions (for instance, with higher concentrations of organic co-solvent, that may be necessary to solubilize hydrophobic substrates). Despite of the high strength of the adsorption, in all the cases, the lipase could be fully desorbed from octadecyl–Sepabeads by using moderate concentrations of detergent. Also, incubation with urea, guanidine, etc. resulted in a full desorption of the lipase.

The regenerated support could be used to immobilize new batches of lipase without significant differences in terms of loading capacity compared to the first immobilization.

4. Conclusion

Octadecyl–Sepabeads seems to be a very promising support for lipase immobilization. This support permits in one-step immobilization, purification, hyper-activation and stabilization of lipases, following a very simple protocol: the mere addition of the support to the lipase solution (even very crude preparations) at very low ionic strength.

Because of the very strong adsorption of the lipases on this very hydrophobic support, these derivatives may be used even in the presence of medium–high concentrations of organic co-solvents, under conditions where the enzyme would be released to the medium when using other commercial supports (e.g. octyl–agarose). This point may be critical in the design of many reactions catalyzed by lipases.

We would also like to remark the fact that the multipoint covalent attachment of lipases on glyoxyl–agarose promoted a significant improvement on the enzyme stability. However, even these stabilized derivatives have much lower stability than that observed using the octadecyl–Sepabeads, both in thermal and organic co-solvent inactivation assays.

These results suggest that the open form of the lipase (fixed in the interfacially adsorbed derivative) adsorbed on a hydrophobic interface may not only be more active than the closed form, but it also looks appears to be much more stable. This could be associated to the more stable form of this open structure [27,28].

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