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Immobilization of lipases for non-aqueous synthesis

Manish Petkar [a], Arvind Lali [a,*], Paolo Caimi [b], Moreno Daminati [b]

[a] *Chemical Engineering Division, Institute of Chemical Technology, University of Mumbai, Matunga, Mumbai 400019, Maharashtra, India*
[b] *Resindion SRL, Binasco, Milan, Italy*

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Abstract

Immobilization of lipases involves many levels of complications relating to the structure of the active site and its interactions with the immobilization support. Interaction of the so called hydrophobic 'lid' with the support has been reported to affect synthetic activity of an immobilized lipase. In this work we evaluate and compare the synthetic activity of lipases from different sources immobilized on different kinds of supports with varying hydrophobicity. *Humicola lanuginosa* lipase, *Candida antarctica* lipase B and *Rhizomucor miehei* lipase were physically adsorbed onto two types of hydrophobic carriers, namely hydrophilic carriers with conjugated hydrophobic ligands, and supports with base matrix hydrophobicity. The prepared immobilized enzymes were used for acylation of *n*-butanol with oleic acid as acyl donor in iso-octane with variable water content (0–2.8%, v/v) as reaction medium. Enzyme activity and effect of water on the activity of the immobilized derivatives were compared with those of respective soluble lipases and a commercial immobilized lipase Novozyme 435. Both *R. miehei* and *H. lanuginosa* immobilized lipases showed maximum activity at 1.39% (v/v) added water concentration. Sepabeads, a methacrylate based hydrophilic support with conjugated octadecyl chain showed highest immobilized esterification (synthetic) activity for all three enzymes, and of the three *R. miehei* lipase displayed maximum esterification activity comparable to the commercial enzyme.

Keywords: Enzyme; Esterification; Hydrophobic; Immobilization; Lipase; Sepabeads

1. Introduction

Lipases (glycerol ester hydrolases E.C.3.1.1.3) are widespread in nature and have been shown to catalyze hydrolysis/synthesis of wide range of soluble and insoluble carboxylic acid esters and amides. Lipases find useful applications in hydrolysis of fats and oils [1,2], for synthesis of fatty esters [3,4] and many different organic intermediates for organic synthesis, e.g. resolution of racemic mixtures [5–7]. One of the major advantages for popularity of lipases is the high regio and stereo-specificity they can display in organic synthesis.

Catalytic action of lipases is rather complex and the shape and structure of the enzyme around the active site varies significantly from one lipase to another. In most lipases, a part of the enzyme molecule covers the active site with a short α-helix called 'lid' or 'flap'. The side of the α-helical lid facing the catalytic site, as well as the protein chains surrounding the catalytic site is composed mainly of hydrophobic side chains [8–10]. With the lid covering the catalytic site a lipase is inactive in absence of a hydrophobic interface and is said to in 'closed' form. In the presence of a hydrophobic substrate, lipases are 'adsorbed' on to the hydrophobic interface that promotes dramatic changes in the enzyme structure leading to 'open' form of the lipase resulting in hydrolytic reactions [11]. The required hydrophobic interface is easily available in biphasic hydrolysis of fats but has to be made available suitably when intention is to conduct esterification in an organic solvent. This requirement along with the constraints of cost has necessitated use of lipases in immobilized forms. However, it has been difficult to prepare an immobilized lipase preparation that exhibits broad substrate specificity for organic synthesis and numerous studies on the topic have appeared over last three decades. Type of support used for immobilization, conjugation chemistry, conjugation conditions, and selection of reaction medium all have complex effects on the enzyme activity for a particular substrate. Consequently, the more successful immobilized lipases are very expensive which has limited their usage especially for large-scale synthesis applications.

Lipases immobilized on porous adsorbents show low hydrolytic activity due to diffusional limitations of the oil-water biphasic substrate system. On the other hand, these immobilized

* Corresponding author. Tel.: +91 22 24145616x292; fax: +91 22 24145614.
E-mail address: aml@udct.org (A. Lali).

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lipase preparations when used in near anhydrous media, become partially activated by direct interaction with organic solvent of an appropriate log *P* value and can be used for ester synthesis. However, to obtain an immobilized lipase that has broad specificity and esterification activity in a variety of organic solvents, it is necessary to immobilize a lipase in 'open' form. This may be achieved by using solid supports with surface activating groups and/or using proper conjugation chemistry to prepare immobilized lipase derivatives [12]. On account of the relatively high surface hydrophobicity of lipases, simple adsorption of lipases on suitably hydrophobic supports has been the more popular strategy over covalent conjugation methods. Supports reported for lipase immobilization can be divided under two categories: hydrophobic matrices and hydrophilic matrices with conjugated hydrophobic ligands. Most of the work reported on lipase immobilizations has used expensive and fragile gel based hydrophilic matrices [13]. Use of lipase immobilized on such gel matrices restricts their large-scale industrial applications. Further, lipase immobilized on gel matrices cannot be used for non-aqueous synthetic applications. Industrially successful immobilized lipases are based on rigid and stable supports (e.g. Novozyme 435, immobilized *Candida antarctica* lipase B and RM-IM, immobilized *Rhizomucor miehei* lipase). Many others have also reported immobilization of different lipases on different solid supports like Celite, Octyl-silica, Aminopropyl silica, Eupergit C250L, porous Polypropylene, and Accurel EP-100 [14,15].

Despite a large amount of work reported on use of lipases for synthesis, there have been far fewer reports on how best to immobilize a given lipase. Further, the effect of support surface hydrophobicity on the resultant immobilized activity of a lipase in relation to its active site structure has not been reported. In the present paper we report the effect of immobilization of lipases from different sources on different porous, rigid hydrophobic supports with varying surface hydrophobicity, as well as on hydrophilic carriers with conjugated hydrophobic ligands, for non-aqueous synthesis. Thus surface hydrophobicity of support matrix has been shown to have effect on the esterification activities of the different immobilized lipases through the possible conformational changes caused by interfacial activation of a lipase upon binding on the solid interface.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Tributyrin (Glyceryl tributyrate) as lipase substrate was obtained from HiMedia Laboratories Ltd., India. Oleic acid (*cis*-9-Octadecanoic acid), *n*-butanol and iso-octane (2,2,4-trimethylpentane) were purchased from S.D. Fine Chemicals, India. All other chemicals were standard laboratory grade.

2.1.2. Enzymes

R. miehei lipase as Palatase (referred hereafter as RM-lipase), *Humicola lanuginosa* lipase as Lipolase 100T (HL-lipase) and *C. antarctica* lipase B (CAL B) as Novozyme 525L were obtained as gifts from Novo Nordisk, Denmark. Novozyme 435 as immobilized *C. antarctica* lipase B was also obtained from Novo Nordisk, Denmark.

2.1.3. Polymeric solid supports

Hydrophilic acrylate based porous Sepabeads EC-Butyl, Sepabeads EC-Octyl, Sepabeads EC-Decyl and Sepabeads EC-Octadecyl enzyme carrier resins used in the work are products of Resindion srl, Italy. These resins provide the respective hydrophobic ligands covalently conjugated on to the otherwise hydrophilic surface. Fully hydrophobic cross-linked polystyrene-DVB matrix based porous adsorbents Diaion HP20 and Diaion SP207 used in the work are products of Mitsubishi Chemical Corp. Japan also provided by Resindion srl.

2.2. Methods

2.2.1. Tributyrin hydrolytic activity assay

The hydrolytic activity of lipase solutions was determined using the hydrolysis of tributyrin (glyceryl tributyrate) using a modification of the method suggested by Wang et al. [16]. Two hundred and fifty microlitres of appropriately diluted solution of enzyme was added to 1062 μl of 0.1 M phosphate buffer (pH 7.0) followed by 188 μl of tributyrin. The solution was vortexed on a tabletop cyclo-mixer for 15 min at room temperature, and the reaction was stopped by addition of 10 ml of methanol and the mixture immediately titrated against 0.05 M alcoholic NaOH solution using phenolphthalein as indicator. The activity was calculated as micromoles of acid liberated per minute and one hydrolytic lipase unit was taken as the amount of lipase, which released 1 μmole of free acid per minute under assay conditions.

2.2.2. Esterification activity assay

It is difficult to calculate the hydrolytic activity of solid immobilized catalyst since oil–water emulsions cannot effectively penetrate the pores of the immobilized catalyst. Thus it is not appropriate to conduct the tributyrin hydrolysis assay for an immobilized lipase. Esterification activity of immobilized lipases was estimated through an esterification reaction. Two hundred milligrams of the vacuum dried immobilized lipase material was added to a screw capped vial containing a mixture of 0.32 ml oleic acid and 0.27 ml dry *n*-butanol in 3 ml dry iso-octane and 0.05 ml distilled water. The vials were placed in a controlled temperature shaker at 30 °C and shaken at 250 rpm. The reaction was stopped after 1 h by addition of 10 ml of methanol, and the reaction mixture was immediately titrated for unreacted fatty acid against 0.05 M alcoholic NaOH using phenolphthalein as indicator. Immobilized esterification activity was expressed as μmoles of ester formed per min under reaction conditions.

2.2.3. Optimization of added water for esterification reaction

Optimal water content required for an immobilized lipase to perform esterification reaction was optimized for all the immobilized lipase preparations using a procedure similar to

the assay procedure mentioned above. Thus 200 mg of vacuum dried immobilized lipase was placed in a screw-capped vial containing a mixture of oleic acid (0.32 ml), *n*-butanol (0.27 ml) in iso-octane (3.0 ml). The solvents and reagents were dried using molecular sieves prior to use. Variable amount of distilled water was added and checked for the effect of added water on esterification activity of immobilized lipase.

2.2.4. Protein determination

Protein content in a lipase preparation was determined by the Bradford protein assay method using Bradford reagent from bio-rad, USA at a wavelength of 595 nm using a standard calibration curve of BSA [17].

2.2.5. Physical adsorption of lipase on hydrophobic surface

All the resins, namely Sepabeads EC-Butyl, Sepabeads EC-Octyl, Sepabeads EC-Decyl, Sepabeads EC-Octadecyl, and Diaion HP20 and Diaion SP207 were degassed and washed with distilled water and then equilibrated with 0.1 M phosphate buffer of pH 7.0. To a 5 ml of washed resin, undiluted lipase preparation equivalent of 5000 hydrolytic units was added and shaken on a rocker at room temperature. Adsorption of the lipase activity with time was estimated from initial activity and activity in the periodically withdrawn samples of the supernatant enzyme solution. The time required for equilibrated adsorption for each lipase and support was determined and used for further work on respective lipases and supports.

3. Elucidation of lipase catalytic sites

Lipases are a special class of esterases that hydrolyze fatty acid esters like triglycerides at lipid/water interface. All the lipases have a α/β-hydrolase fold structure with a catalytic triad similar to the one found in serine proteases [18]. The active site is generally buried under a lid or flap containing an amphiphilic α-helix, making the active site inaccessible to the substrate in the so-called closed conformation. Opening of the lid twists and exposes a large hydrophobic surface, whereas the previously exposed hydrophilic domain becomes buried inside the protein [19,20]. Lipases under this study were *H. lanuginosa* lipase, *R. miehei* lipase and *C. antarctica* lipase B. It is noteworthy that the three dimensional structure of HL-lipase is highly homologous to that of RM-lipase [20]. Crystal structures of both the lipases in the closed and open conformations have revealed that

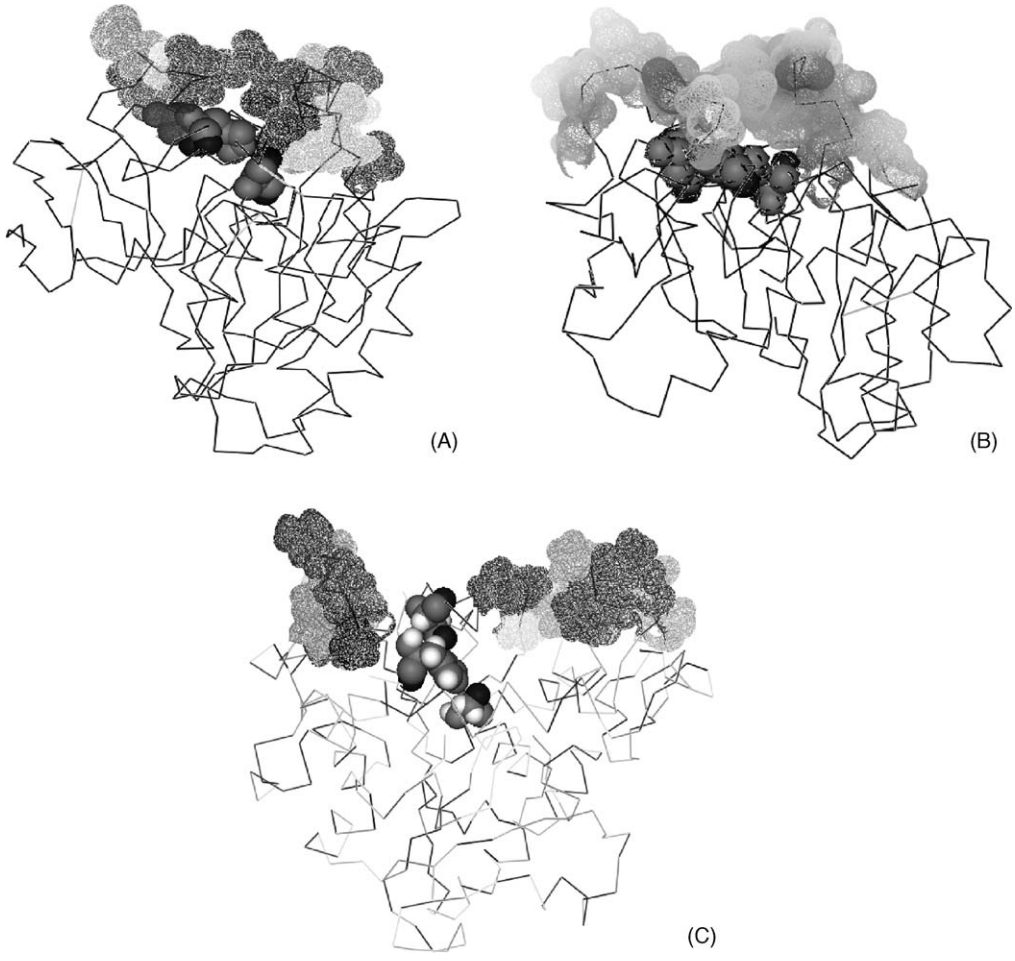

Fig. 1. Schematic representation of the solvent accessible van der Waals' surface of the lid and adjacent residues in the wire frame representation of (A) RM lipase (3TGL); (B) HL lipase (1TIB); (C) CAL B lipase (1LBS). Active site residues are displayed as CPK models; and lid or neighboring residues are displayed as Van der Waals surface.

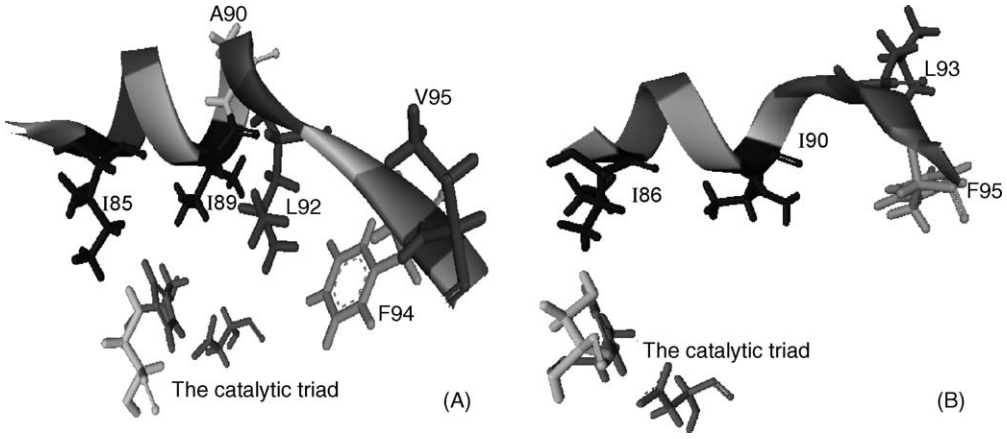

Fig. 2. Representation of active site conformation of un-opened (A) RM lipase (3TGL) and (B) HL lipase (1TIB) presented as CPK model with the helical lid shown with the hydrophobic residues on it marked out in solid C^{α} ribbon presentation.

the lid consists of a α-helix (residues 85–91) and two hinge regions (residues 83–84 and 91–95) [11]. The three-dimensional structures of the lipases were taken from the ProteinDataBank as 3TGL, 1TIB and 1LBS for *R. miehei* lipase, *H. lanuginosa* lipase, and *C. antarctica* lipase B, respectively. The active sites in RM-lipase, HL-lipase and CAL B lipase as visualized on DS Modeling Visualizer (Accelrys, USA) are shown as CPK representations in Fig. 1. The enzyme structures are presented in the Figure along with the van der Waals surface around the active site. It can be seen that in both RM-lipase and HL-lipase there is a presence of a 'lid' structure that covers the active sites, while such a lid is non-existent in CAL B lipase. Fig. 2 presents the three-dimensional selective schematic diagrams of the catalytic sites of RM-lipase and HL-lipase in 'un-opened' conformations along with the covering helical lid and associated hydrophobic residues shown in solid C^{α} ribbon presentation. These two pictures indicate the positions of the hydrophobic residues that are part of the respective lids in the closed forms of the two enzymes. Thus one can hypothesize that while HL-lipase and RM-lipase will need a 'lid opening' mechanism for their activation, CAL B lipase does not need any such facilitation. The open conformation of RM-lipase upon activation by diethyl phosphate was downloaded from PDB as 4TGL and is given as Fig. 3. Fig. 3A presents picture of the active site of RM-lipase along with the 'opened' lid position, while Fig. 3B clearly shows the 'opened' catalytic site. Comparison of these pictures with Figs. 1A and 2A brings out how the hydrophobic residues have been 'pulled' away for the RM-lipase to become active. It has been reported that catalytic sites on *C. antarctica* lipase B (CAL B) and *Pseudomonas glumae* lipase have near non-existent lids covering the catalytic sites while on the other hand, RM-lipase and HL-lipase do possess these lids [21,22]. For this reason, catalytic activity of CAL B resembles more of an esterase than that of a lipase.

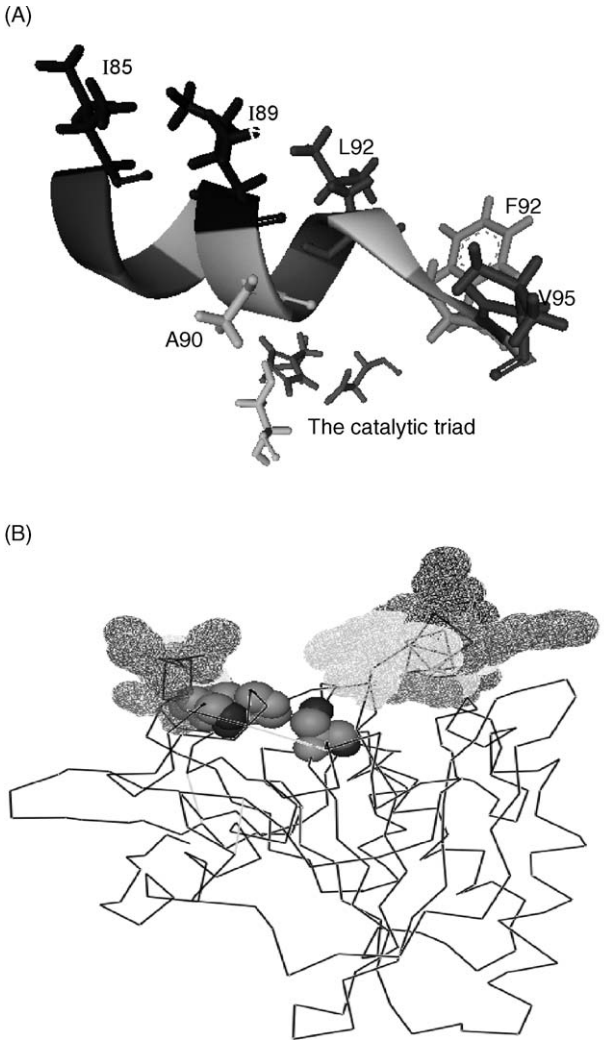

Fig. 3. Hydrophobic interfacial activation of RM-lipase. (A) Representation of active site conformation of open RM lipase (4TGL) presented as CPK model with the twisted and opened α-helical lid shown with hydrophobic residues in C^{α} ribbon representation. (B) The exposed catalytic site on RM-lipase with twisted and opened 'lid' van der Waals surface.

4. Results and discussion

It is well known that lipases are activated at natural hydrophobic oil and water interface [9]. During enzymatic hydrolysis of triglycerides aliphatic hydrophobic tails of the oil imparts a 'pulling effect' on the α-helical 'lid' of the lipase away from

Table 1
Details of solid adsorbents used for immobilization

Type of support	Polymer base	Functional group concentration	Pore diameter/volume	Particle size (mm)
Sepabeads EC type	Polymethacrylate	Minimum 110 μmol/g (wet)	>1000 Å	0.15–0.3
Diaion HP20	Styrene DVB copolymer	–	1.3 ml/g approximately	0.3–0.8
Diaion SP207	Styrene DVB copolymer	–	1.1 ml/g approximately	0.3–0.8

Table 2
Esterification (butyl oleate) activity of enzymatic derivatives of RM-lipase at 1.39% water concentration

Lipases	Immobilized derivative	Hydrolytic activity (μmole/min/ml of catalyst)		Esterification activity (μmole/min/g of catalyst)
		Load	Immobilized[a]	
RM-lipase	EC-Octadecyl	1050	1037	67.8
	EC-Decyl	1050	923	40.4
	EC-Octyl	1050	908	37.1
	EC-Butyl	1050	845	22.6
	HP20	1050	793	13.5
	SP207	1050	788	13.5
[b]CAL B	Novozyme 435	NA	NA	71.3

Experiments performed as described in Section 2.2.
[a] Estimated by material balance.
[b] Commercial immobilized lipase.

the active site resulting in an 'open' conformation of the lipase. Absence of such an interface in 'dry' esterification reactions results in poor esterification activity of most lipases. Lipase preparations intended for esterification reactions therefore have been devised through 'mimicking' of the interface through their immobilization on to solid supports that provide a suitably hydrophobic surface.

However, the concept of immobilization of lipases in active 'open' form can be conceptually possible only if the surface hydrophobicity of the solid matrix surface in just about right to render an irreversibly adsorbed or conjugated lipase 'open' while not denaturing the entire protein structure. In this work, it was decided to study the effect of surface hydrophobicity on esterification activity of immobilized lipase preparations with two types of hydrophobic supports; hydrophilic supports with conjugated straight chain alkyl ligands, giving surface hydrophobicity with free hydrophobic groups to the matrix, and secondly supports having base matrix hydrophobicity due to the cross-linked hydrophobic monomers. Sepabeads EC-Butyl, Sepabeads EC-Octyl, Sepabeads EC-Decyl, and Sepabeads EC-Octadecyl were the supports of the former type, while the latter type supports used were Diaion HP20 and Diaion SP207. Table 1 gives the details of the resin adsorbents used. Three lipases from *R. miehei*, *H. lanuginosa*, and *C. antarctica* fraction B were immobilized on the different supports. The loaded hydrolytic activities and the esterification activities of the immobilized preparations are summarized in Tables 2–4 at the optimum water concentration of 1.39% (v/v) (see later below). The Tables also indicate the corresponding esterification activity of Novozyme 435 under similar conditions. It can be seen that, in case of the both immobilized RM-lipase and HL-lipase, increasing the *n*-alkyl chain length from butyl to octadecyl increased the esterification activity. Synthesis activity of the CAL B is almost independent of the surface hydrophobicity and showed good activity on both types of matrices. RM-lipase showed that hydrophobic ligand conjugated Sepabeads are more useful than plain polystyrene-DVB

Table 3
Esterification (butyl oleate) activity of enzymatic derivatives of HL-lipase at 1.39% added water concentration

Lipases	Immobilized derivative	Hydrolytic activity (μmole/min/ml of catalyst)		Esterification activity (μmole/min/g of catalyst)
		Load	Immobilized[a]	
HL-lipase	EC-Octadecyl	1314	844	51.1
	EC-Decyl	1314	429	20.2
	EC-Octyl	1314	459	24.2
	EC-Butyl	1314	439	10.8
	HP20	1314	763	47.1
	SP207	1314	438	36.3
[b]CAL B	Novozyme 435	NA	NA	71.3

Experiments were performed as described in Section 2.2.
[a] Estimated by material balance.
[b] Commercial immobilized lipase.

Table 4
Esterification (butyl oleate) activity of enzymatic derivatives of CAL B at 1.39% added water concentration

Lipases	Immobilized derivative	Hydrolytic activity (μmole/min/ml of catalyst)		Esterification activity (μmole/min/g of catalyst)
		Load	Immobilized[a]	
CAL B	EC-Octadecyl	1118	1016	59.8
	HP20	1118	1098	55.3
	SP207	1118	1028	52.3
[b]CAL B	Novozyme 435	NA	NA	71.3

Experiments performed as described in Section 2.2.
[a] Estimated by material balance.
[b] Commercial immobilized lipase.

based Diaion supports. HL-lipase on the other hand, preferred Sepabeads EC-Octadecyl the most but gave lower activities on other ligands than on the two Diaion supports.

Increasing activity of immobilized RM-lipase and HL-lipase with length of alkyl chain conjugated on to acrylate based Sepabeads can be attributed to the increase in the 'pulling effect' of alkyl chain on the lid opening of the lipase at catalytic site. The fact that HL-lipase immobilized on Diaion HP20 and Diaion SP207 also showed reasonable activities may be due to the interaction of the hydrophobic residues other than the 'lid' away from the active site causing partial blocking of the 'lid'. In case of immobilized CAL B all the immobilized derivatives showed similar activities irrespective of the hydrophobic surface.

The three-dimensional structures of open and closed forms of RM-lipase (3TGL and 4TGL) in Figs.3 and 2A, respectively, show that the residues on the hydrophobic lid face the active site in the closed conformation, and are positioned away from the active site in the open conformation obtained in the presence of an hydrophobic environment. The density and orientation of the hydrophobic residues in the lid of HL-lipase (1TIB) differs from that on RM-lipase (Fig. 2A and B). It is noteworthy that HL-lipase has hydrophobic lid residues oriented away from the active site and on the outside surface of the lid in the closed state. This is perhaps the reason that HL-lipase is seen to require a 'larger' hydrophobic 'pull' to open the lid than RM-lipase, and as a result Diaion matrices perform better than low alkyl chain length ligands for HL-lipase.

CAL B however, gave good catalytic activity irrespective of the density and chemical nature of the hydrophobic supports (Table 4). This is an expected result since the lid-like structure that covers the active site of other lipases is absent in CAL B.

Esterification activities of the immobilized preparations on Sepabeads EC-Octadecyl at the optimum initial water concentrations for the three enzymes are summarized in Table 5. As measure of efficiency of enzyme immobilization, the ratio of esterification activity of the preparation and the immobilized hydrolytic activity is also presented in the Table. It may be seen that this ratio for both RM-lipase and HL-lipase preparation was comparable with that of immobilized CAL B. It may also be noted that esterification activities of all three-enzyme preparations is of the same order as that of the commercial Novozyme 435.

Effect of water concentration on ester synthesis activity of lipases has been a subject of much work [23,24]. Hydration of the enzyme in water is essential for its activity and hence distribution of water between the solvent and the enzyme plays an important role in determining the enzyme activity in a particular solvent. In the case of immobilization of a lipase on a support matrix, the affinity of the matrix for water will also play an important role in deciding the extent and effect of water in the enzyme microenvironment. Experiments were carried out to compare the optimum water requirement for esterification activities of the immobilized lipase preparations of RM-lipase and HL-lipase on Sepabeads EC-Octadecyl. The results are presented in Figs. 4 and 5. It was observed that for both the enzymes the optimum initial water concentration was 1.39% (v/v) on all matrices. This water concentration was used for determinations of esterification activities throughout this work.

Since Sepabeads EC-Octadecyl turned out as the most promising immobilization support for all the three lipases, it was decided to optimize the adsorption time for maximum possible adsorption for the three lipases on Sepabeads EC-Octadecyl. Fig. 6 shows the time course of lipase adsorption for the three enzymes on Sepabeads EC-Octadecyl. It can be seen that after 180 min of incubation at room temperature almost 99% of the CAL B activity was adsorbed, while RM-lipase required 240 min for 99% adsorption. HL-lipase showed the slowest adsorption reaching only 64% adsorption after 270 min.

Table 5
Efficiency of immobilized enzymatic derivatives of CAL B, RM-lipase and HL-lipase on Sepabeads EC-Octadecyl resins

Lipases	Immobilized derivative	Hydrolytic activity (μmole/min/ml of catalyst) (A)	Esterification activity (μmole/min/g of catalyst) (B)	Efficiency (B/A)
CAL B	EC-Octadecyl	1016	59.8	0.059
RM-lipase	EC-Octadecyl	1037	67.8	0.065
HL-lipase	EC-Octadecyl	844	51.3	0.060

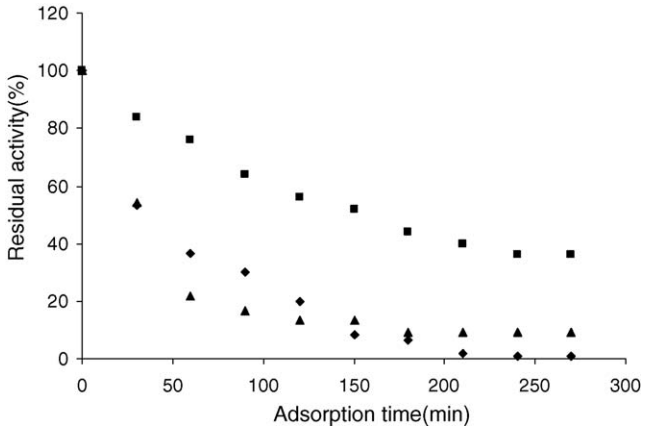

Fig. 4. Effect of immobilization time on the residual activity of free RM-lipase, HL-lipase and CAL B during immobilization on Octadecyl Sepabeads. RM-lipase (◊), HL-lipase (□), CAL B (△).

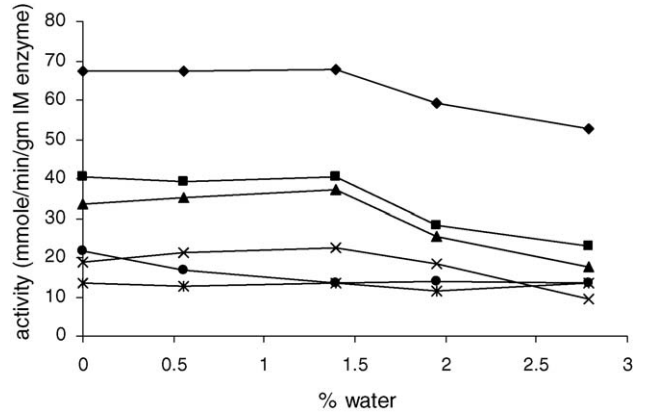

Fig. 5. Effect of added water on esterification activity of RM-lipase immobilized on different supports. Sepabeads EC-Octadecyl (◊), Sepabeads EC- Decyl (□), Sepabeads EC-Octyl (△), Sepabeads EC- Butyl (×), HP20 (+), SP207 (◯).

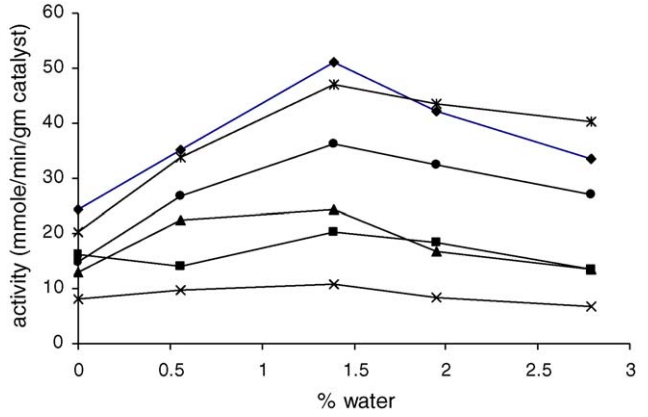

Fig. 6. Effect of added water on esterification activity of HL-lipase immobilized on different supports. Sepabeads EC-Octadecyl (◊), Sepabeads EC- Decyl (□), Sepabeads EC-Octyl (△), Sepabeads EC- Butyl (×), HP20 (+), SP207 (◯).

5. Conclusions

Lipases from *R. miehei*, *H. lanuginosa*, and *C. antarctica* lipase B species were immobilized on solid supports with varying surface hydrophobicity. Results showed that hydrophilic supports with conjugated hydrophobic ligands make better choices in general over supports prepared from cross-linked hydrophobic monomers. Further, the esterification activity of immobilized lipases like RM-lipase and HL-lipase (i.e. lipases that exist in closed form with a lid structure over their catalytic site) increased with the hydrophobicity of the conjugated ligand. Thus, esterification activities of immobilized RM-lipase and HL-lipase increased from Sepabeads EC-Butyl to Sepabeads EC-Octadecyl. Comparison of the esterification activities of the three lipases immobilized on Sepabeads EC-Octadecyl showed the support to be most effective and gave results comparable to that of the commercial Novozyme 435.

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