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Dedicated to Professor Florin Dan Irimie on the Occasion of His 65th Anniversary

COVALENT IMMOBILIZATION OF LIPASES ON ACTIVATED HOLLOW SILICA MICROSPHERES

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ABSTRACT. This study explores the covalent immobilization of three lipases (Lipase AK, from *Pseudomonas fluorescens*; Lipase PS, from *Burkholderia cepacia*; and CrL, from *Candida rugosa*) on four supports prepared by functionalization of mesoporous hollow silica microspheres (M540) with various bisepoxides as activating agents for production of novel lipase biocatalysts for enantiomer selective biotransformations of secondary alcohols. The influence of length, rigidity and hydrophobicity of the bisepoxide activating agents was investigated on the efficiency of immobilization and catalytic properties of the resulted twelve lipase biocatalysts. The hollow silica particles modified with the most beneficial bisepoxide activating agents resulted in novel biocatalysts capable for kinetic resolution of racemic 1-phenylethanol *rac*-**1a** and racemic octan-2-ol *rac*-**1b** with high activity and enantioselectivity.

Keywords: *hollow silica microspheres, bisepoxide surface activation, kinetic resolution, lipase, immobilization*

INTRODUCTION

Due to the effective catalytic properties and green nature of the enzymes and to the progress of modern biotechnology, enzyme industry witnessed a rapid development over the past decades [1,2]. Nowadays, several different enzymes are available for the practicing organic chemists and biotechnologists for a variety of different transformations [1–5].

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Hydrolytic enzymes such as lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) play a prominent role among biocatalysts [6,7]. Lipases are one of the most used enzymes, applied as biocatalysts in synthetic organic chemistry using their hydrolytic capacity in aqueous medium as well as their catalytic ability of the reverse reaction in organic solvents for various synthetic biotransformations related to carboxylic acid esters [8–11].

However, similarly to other enzymes, native lipases can easily become deactivated and their recovery for reuse may be challenging [12–14]. Therefore, especially in large-scale processes, lipases are often applied in immobilized form in order to facilitate their recovery and enhance their activity, thermal and operational stability [15–17]. Enzyme immobilization can be carried out by different methods and the selection of the mode of immobilization is crucial for preventing the loss of enzyme activity by stabilizing of its active conformation [18–20].

Surface-modified silica gel-based particles proved to be efficient supports in our practice for the immobilization of several lipases [21–26]. Therefore, in this study our aim was to extend the immobilization of further lipases [Lipase AK from *Pseudomonas fluorescens* (Lipase AK), Lipase PS from *Burkholderia cepacia* (Lipase PS) and Lipase from *Candida rugosa* (CrL)] using bisepoxide-activated mesoporous hollow silica microspheres to produce efficient biocatalysts for kinetic resolution of secondary alcohols.

RESULTS AND DISCUSSION

Previous results of our research group indicated that surface grafted mesoporous silica gel supports can be advantageously applied for easy-to-perform adsorptive immobilization of lipases leading to biocatalysts with useful activity and selectivity in kinetic resolution of racemic secondary alcohols [27]. Lipases could be immobilized by sol-gel-entrapment or entrapment within nanostructured polymeric systems including electrospun fibers [17,19]. Such entrapment-based immobilization methods proved to be advantageous in enzyme stabilization, as the enzyme molecules could be rigidified by the embedding matrix. Covalent immobilization carried out on bisepoxide-activated aminoalkyl polymer resins resulted in hydrophobic stabilization of the immobilized enzyme by a relatively rigid and hydrophobic spacer arm and contributed significantly to preservation of enzyme activity and selectivity at higher temperatures [28]. To expand the usefulness of enzyme immobilization methods for industrial applications [29], the application of mechanically resistant, bisepoxide-activated mesoporous hollow silica microspheres for enzyme immobilization were extended to immobilization of three further lipases [Lipase AK from *Pseudomonas fluorescens* (Lipase AK), Lipase PS from *Burkholderia cepacia* (Lipase PS) and Lipase from *Candida rugosa* (CrL)].

Immobilization of the lipases on bisepoxide-activated M540 supports

After activation of the MatSpheres 540 hollow silica microspheres (M540) with four different bisepoxides, the three selected lipases (Lipase AK, Lipase PS and CrL) were immobilized covalently resulting in twelve different lipase biocatalysts (Figure 1).

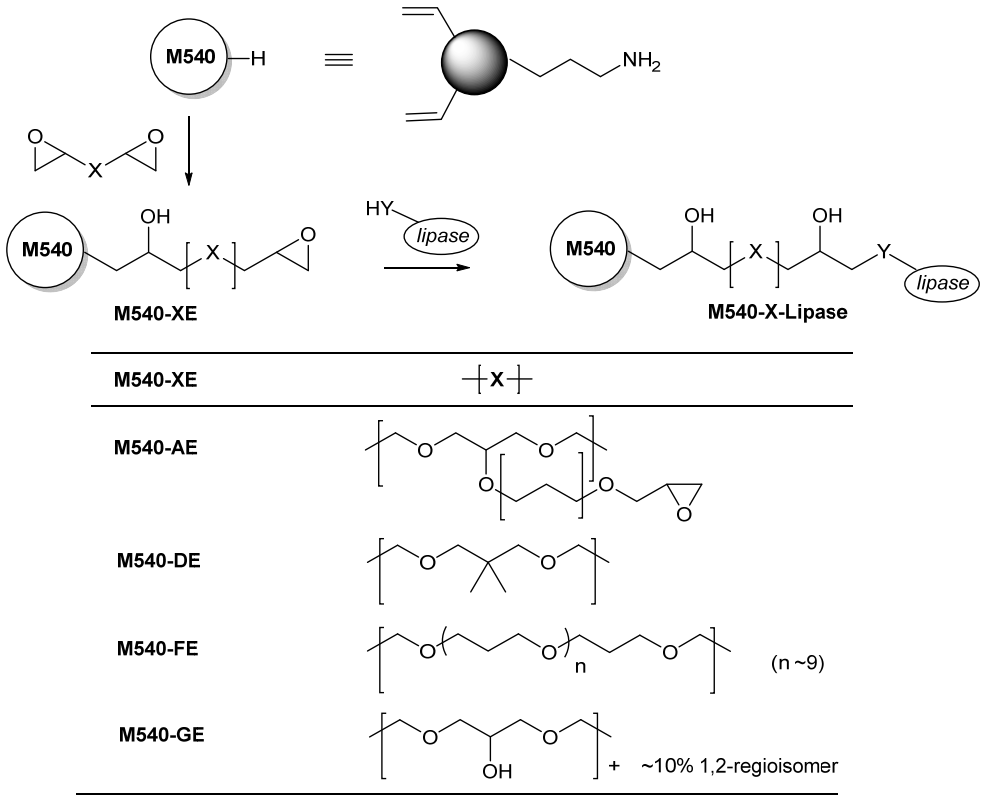

Figure 1. Lipase immobilization on bisepoxide-activated M540 supports.

The morphology of the immobilized lipase biocatalysts was investigated by scanning electron microscopy (SEM). As an example, the results with Lipase AK immobilized on M540-GE supports are shown in Figure 2. SEM investigations revealed than in all cases the biocatalyst particles were uniform in appearance with good monodispersity.

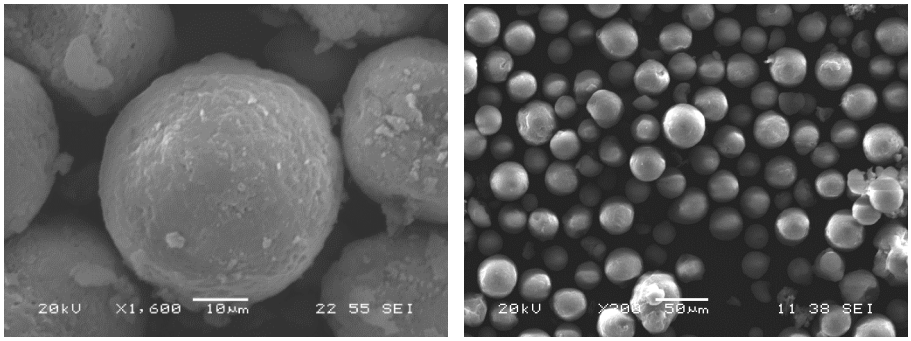

Figure 2. SEM images of Amano Lipase AK immobilized on activated M540-GE.

Kinetic resolution with lipases on bisepoxide-activated M540 supports

The catalytic performance of the immobilized biocatalysts was evaluated by investigation of their activity and selectivity in enantiomer selective acylation of racemic secondary alcohols 1-phenylethanol *rac*-**1a** and octan-2-ol *rac*-**1b** with vinyl acetate (Figure 3).

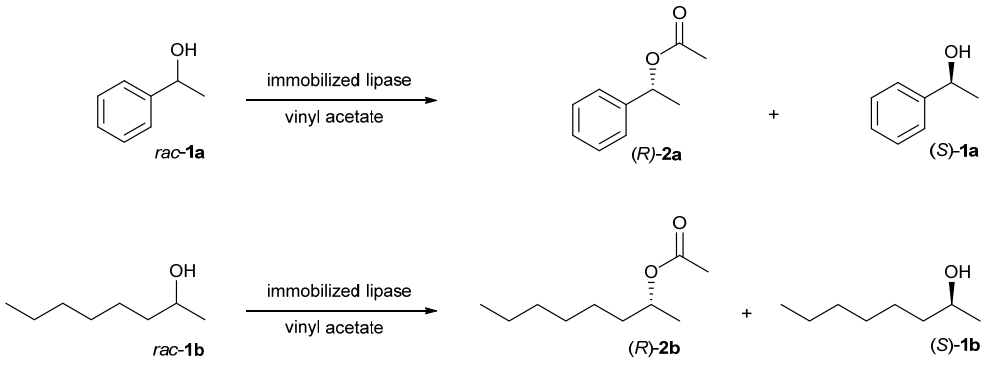

Figure 3. Kinetic resolution of alcohols *rac*-1a,b catalyzed by the novel biocatalysts.

Conversion (*c*) and enantiomeric excess (*ee*) in the kinetic resolution of *rac*-**1a,b** were determined by gas chromatography (GC) on enantioselective stationary phase. To characterize the degree of enantiomer selectivity, the enantiomeric ratio (*E*) for the reactions was calculated from the values of *c* and $ee_{(R)\text{-}\mathbf{2a,b}}$. The activity of the biocatalysts in the acylation of *rac*-**1** could be described by their specific biocatalyst activity (U_B) which was calculated using the equation $U_B = n_P/(t \times m_B)$ (where n_P [mol] is the amount of the product (*R*)-**2a,b**, *t* [min] is the reaction time and m_B [g] is the mass of the applied biocatalyst).

Studies with the lipases immobilized on the activated M540 supports

We have found that lipase AK, lipase PS and CrL can be efficiently immobilized by covalent linkages onto the surface of bisepoxide-activated M540 supports. The nature of the M540 activation significantly influenced not just the productivity but also the selectivity of each immobilized lipase biocatalyst in kinetic resolution of racemic 1-phenylethanol *rac*-**1a** (Table 1) and octan-2-ol *rac*-**1b** (Table 2).

Table 1. Kinetic resolution of *rac*-1a with vinyl acetate catalyzed by native and immobilized forms of lipases (30 °C, after 1 h)

Enzyme	Cross linker	*c* %	$ee_{(R)\text{-}2a}$ %	*E* -	U_B U/g
PS[a]	free	17.0	98.7	58	8.7
	AE	21.9	98.9	>200	30.2
	DE	19.5	99.1	>200	26.9
	FE	33.3	99.9	»200	45.6
	GE	23.5	99.5	»200	32.4
AK	free	20.4	46.6	3.1	28.1
	AE	33.3	99.0	>200	45.9
	DE	42.5	98.7	>200	58.6
	FE	39.2	99.2	>200	54.1
	GE	32.6	99.2	>200	44.9
CrL[b]	free	7.4	83.1	11.7	10.2
	AE	9.7	93.8	34.8	13.3
	DE	38.1	89.7	32.9	52.4
	FE	38.2	98.7	>200	52.6
	GE	4.3	63.5	4.7	5.9

[a] Results agreed with our previous study on immobilization of lipase PS [30]; [b] Results after 24 h

Table 2. Kinetic resolution of *rac*-1b with vinyl acetate catalyzed by native and immobilized forms of several Lipases at 30 °C after 2 h

Enzyme	Cross linker	*c* %	$ee_{(R)\text{-}2a}$ %	*E* -	U_B U/g
PS	free	14.1	96.4	>100	19.4
	AE	41.6	72.1	10.3	43.7
	DE	40.3	69.4	14.4	42.5
	FE	45.7	96.5	>100	57.8
	GE	22.6	78.5	10.4	23.7
AK	free	6.7	57.8	3.9	6.4
	AE	9.0	62.2	4.6	9.4
	DE	14.0	62.6	4.8	14.7
	FE	17.7	69.2	6.3	18.6
	GE	49.8	99.4	»200	52.3
CrL[a]	free	9.2	99.1	»200	12.6
	AE	12.4	85.6	14.7	17.1
	DE	32.8	89.4	28.2	45.2
	FE	40.6	99.7	»200	55.9
	GE	47.9	98.8	»200	66.1

[a] Results after 24 h

In case of Lipase PS, all the bisepoxide-activated M540-based biocatalysts had better selectivity and specific activity in kinetic resolution of *rac*-1a and *rac*-1b than the native Lipase PS powder form. The best biocatalytic properties could be achieved by the Lipase PS immobilized on the M540-FE support, resulting in 33.3% conversion from *rac*-1a with excellent enantiomer selectivity (*E* »200, $ee_{(R)\text{-}2a}$= 99.9%) in 1 h reaction time and 45.7% conversion from *rac*-1b with good enantiomer selectivity (*E* >100, $ee_{(R)\text{-}2b}$= 96.5%) in 2 h reaction time.

In case of Lipase AK, all the bisepoxide-activated M540-based biocatalysts surpassed the biocatalytic ability of the native Lipase AK powder form in kinetic resolution of *rac*-1a,b as well. The most active form was the M540-DE-AK biocatalyst resulting in 42.5% conversion from *rac*-1a in 1 h reaction time, whereas the highest enantiomer selectivity (*E* >200, $ee_{(R)\text{-}2a}$= 99.2%) was observed with the M540-FE-AK biocatalyst with this substrate. The effect of the linker properties was quite remarkable in case of kinetic resolutions from *rac*-1b with the immobilized AK biocatalysts. Although the AK biocatalysts on AE-, DE-, or FE-activated M540 supports were all more active and selective than the native AK form, the M540-GE-AK biocatalyst was far the most active and selective biocatalyst resulting in almost complete KR (*c*= 49.8%) with remarkable enantiomer selectivity (*E* »200, $ee_{(R)\text{-}2b}$= 99.2%). This example indicated that the quite moderate enantiomer selectivity of a lipase in KR of *rac*-1b (*E*= 4-6) can be significantly enhanced (up to *E* »200) just by fine-tuning the properties of activation agent of the support for immobilization.

The immobilized biocatalysts containing lipase from *Candida rugosa* (CrL) followed the general trend, as all bisepoxide-activated M540-based biocatalysts turned out to be more active in KRs from *rac*-**1a,b** than the native lipase powder. Although the immobilized CrL biocatalysts were less active than the PS or AK preparations (the suitable conversions required 24 h), there were suitable bisepoxide activating agents for KRs of both substrates *rac*-**1a,b**. The most active and most selective form was the M540-FE-CrL biocatalyst resulting in 38.2% conversion from *rac*-**1a** with remarkable high enantiomer selectivity (*E* >200, $ee_{(R)\text{-}\mathbf{2a}}$= 98.7%) in 24 h reaction time. The importance of fine-tuning the properties of the immobilization support is also indicated by the fact that in case of the KRs from the aliphatic alcohol *rac*-**1b** the M540-GE-CrL form proved to be the most suitable biocatalyst resulting in almost perfect KR with 47.9% conversion and high enantiomer selectivity (*E* »200, $ee_{(R)\text{-}\mathbf{2a}}$= 98.8%) in 24 h reaction time.

Besides the improvement in activity and enantiomer selectivity, the immobilization of lipases enabled also convenient handling, easier product separation, and offered reusability in batch reactions, thus making the lipase-catalyzed synthetic processes more cost-effective. In order to evaluate the

reusability and operational stability of the three types of lipase biocatalysts, the M540-FE forms of each lipase were investigated in five repeated cycles of kinetic resolution of *rac*-**1a** (Figure 4). All samples proved to be very durable when recycled up to 5 times, indicating that the M540-based lipase biocatalysts were mechanically and functionally stable.

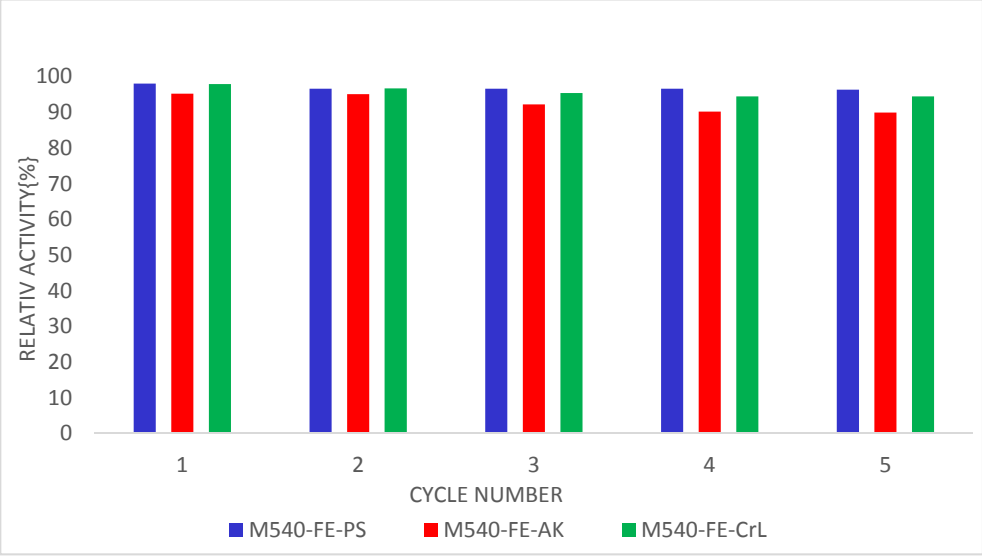

Figure 4. Reusability of the three M540-FE-lipase biocatalysts in 5-cycles of kinetic resolution from racemic 1-phenylethanol *rac*-1a

After the recycling experiments the morphology of the biocatalysts was checked by scanning electron microscope again, proving that the particles were not shattered or mechanically deteriorated.

CONCLUSIONS

Covalent immobilization of three lipases (Lipase AK, from *Pseudomonas fluorescens*; Lipase PS, from *Burkholderia cepacia*; and CrL, from *Candida rugosa*) was successfully carried out on bisepoxide-activated silica microspheres (M540). Our study with four bisepoxides of different properties as activating agents for M540 hollow silica microspheres bearing amine functions at the surface as support proved that immobilization of lipases on bisepoxide-activated M540 could enhance the biocatalytic properties of the lipases as compared to their native powder form. Recycling studies of the resulted preparations

indicated that bisepoxide-activated M540 lipases were suitable and fine-tunable biocatalysts for synthetic biotransformations such as kinetic resolution of racemic secondary alcohols.

EXPERIMENTAL SECTION

Chemicals and enzymes

Racemic 1-phenylethanol, octan-2-ol, propan-2-ol (IPA), 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris), vinyl acetate, hexane, acetone, and ethanol were commercial products of Alfa-Aesar Europe (Karlsruhe, Germany), Sigma–Aldrich (Saint Louis, MO, USA) or Merck (Darmstadt, Germany). M540 (Matspheres 540) was the product of Materium Innovations. M540-AE, M540-DE, M540-FE and M540-GE supports were the products of SynBiocat LLC. (Budapest, Hungary; the bisepoxide-activated derivatives of the M540 hollow silica microspheres were prepared according to a previously published method [31].

Lipase from *Pseudomonas fluorescens* (Lipase AK), from *Burkholderia cepacia* (Lipase PS), and from *Candida rugosa* (CrL) for immobilization experiments were obtained from Sigma–Aldrich (Saint Louis, MO, USA).

Analytical methods

GC analyses were carried out on Agilent 4890 instrument equipped with FID detector and Hydrodex-6TBDM column [25 m × 0.25 mm, 0.25 μm film of heptakis-(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl)-ß-cyclodextrin]; Macherey & Nagel) using H_2 as carrier gas (injector: 250 °C; FID detector: 250 °C; head pressure, 12 psi; split ratio, 50:1, oven: 120 °C; 8 min). GC: t_r (min) for **1** and **2**: 4.0 [(*S*)-**2**], 4.4 [(*R*)-**2**], 5.8 [(*S*)-**1**], 6.0 [(*R*)-**1**].

The structure and morphology of different M540-based lipase preparations were investigated with a JEOL JSM-5500LV scanning electron microscope (SEM). Samples were prepared by placing some silica support on a copper grid coated with carbon and coated with gold layer by a nebuliser prior to analysis. Electron beam energy of 20-22 kV was used.

Enzyme immobilization onto bisepoxide-activated M540 supports

Bisepoxide-activated support (150 mg; either of M540-AE, M540-DE, M540-FE, M540-GE) was suspended in Tris buffer (15 mL, 100 mM, p*H* 7.5) containing the selected lipase (3 mg/mL). The suspension was shaken at room temperature for overnight. The resulted suspension was washed three times with 100 mM Tris buffer (100 mM, p*H* 7.5, 3 × 10 mL) and stored at 4 °C for further experiments.

Enantiomer selective acetylation of racemic 1-phenylethanol *rac*-1a and octan-2-ol *rac*-1b

To a solution of the racemic alcohol *rac*-**1a** or *rac*-**1a** (50 mg) in hexane/*t*-butyl methyl ether/vinyl acetate 6/3/1 (1100 µL) immobilized lipase (25 mg) was added in a sealed amber glass vial and the resulting mixture was shaken (1000 rpm) at 30 °C for 24 h. The reactions were analyzed by GC after the time indicated in Tables 1 and 2 (1 h, 2 h or 24 h).

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