

## NOVEL SOLID SUPPORTS FOR LIPASES IN SOL-GEL IMMOBILIZATION SYSTEMS

DIÁNA WEISER<sup>a</sup>, ANNA TOMIN<sup>a</sup>, LÁSZLÓ POPPE<sup>a,\*</sup>

**ABSTRACT.** Sol-gel encapsulation of lipases proved to be a particularly easy and effective way to enhance the mechanical and catalytic properties of biocatalysts. The sol-gel encapsulated enzymes usually retain their selectivity whereas their heat stability or specific activity may be significantly improved. The aim of our work was to improve the immobilization of lipases in supported sol-gel systems. First, the binding properties of lipase AK on various solid supports were studied. Next, the immobilization properties of the best adsorbent-lipase combinations were tested in sol-gel encapsulation using tetraethoxy-silane/octyltriethoxy-silane/phenyltriethoxy-silane 1/0.7/0.3 silane precursor system.

**Keywords:** lipase, biocatalysis, adsorption, supported sol-gel immobilization

### INTRODUCTION

Lipases (EC 3.1.1.3) are extensively utilized biocatalysts in organic chemistry [1,2]. Lipases are essential in the digestion, transport and processing of lipids (e.g. triglycerides, fats, oils) in most, if not all, living organisms. However, lipases are also being exploited as inexpensive and easy-to-use biocatalysts in more modern applications [3,4,5]. For instance, lipases are used in applications such as baking and laundry detergents and even as biocatalysts in alternative energy strategies to convert vegetable oil into biofuel [6]. Lipases are flexible biocatalysts which can catalyze a wide range of regio- and enantioselective reactions such as hydrolysis, esterifications, transesterifications, aminolysis and ammoniolysis [1,7,8]. These reactions usually proceed with high regio- and/or enantioselectivity, therefore lipases became indispensable biocatalysts in various biotransformations. Development of efficient/economical biotransformations often requires robust technologies for immobilization of biomolecules or microorganisms. Immobilization of enzymes can enhance their activity, thermal and operational stability, and reusability which is important for industrial applications [9,10]. Among many available immobilization methods, including adsorption, covalent attachment to solid supports and entrapment within polymers [9,10], entrapment of enzymes in inorganic/organic hybrid polymer matrices has received a lot of attention in recent years and has provided new possibilities in the field of material science [11,12]. We report here the binding properties of lipases on various solid carriers and further

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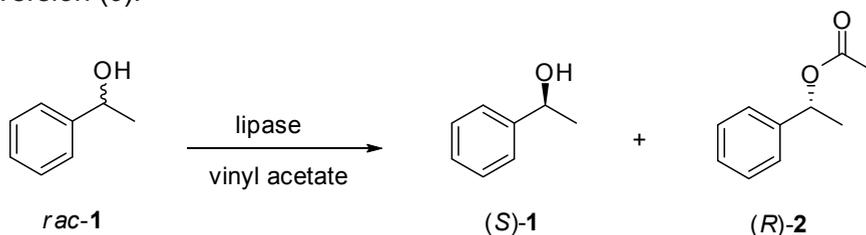
<sup>a</sup> Budapest University of Technology and Economics, Department of Organic Chemistry and Technology, and Research Group for Alkaloid Chemistry of Hungarian Academy of Sciences, Műegyetem rkp 3, H-1111, Budapest, Hungary, \* [poppe@mail.bme.hu](mailto:poppe@mail.bme.hu)

immobilization of the adsorbed lipases in hydrophobic sol-gel materials, which results in the formation of highly active, stable and reusable heterogeneous biocatalysts.

## RESULTS AND DISCUSSION

Adsorption of the lipases at the large specific surface area of porous supports can avoid the aggregation of proteins and thus can result in an increased activity of the biocatalysts. In our study the adsorption behavior of four different lipases – from *Pseudomonas fluorescense* (lipase AK), from *Burkholderia cepacia* (lipase PS), from *Candida cylindracea* (lipase CcL) and BUTE-3 [13] – on various solid supports were investigated. Ten kinds of carriers with different porosity were examined. Most of them were different types of silica gel, but Celite 545 and Filtracel-950 were also studied.

Our recent study indicated that better sol-gel immobilization results can be achieved with lipases adsorbed previously on solid support than with simultaneous addition of the lipase and the supporting material to the silane precursor system [14]. Therefore, our further aim was to use the best supporting materials showing the most pronounced enhancement in the enzyme activity in combined sol-gel encapsulation as well. To test the biocatalytic properties of the resulting biocatalysts, the kinetic resolution of 1-phenylethanol with vinyl acetate in hexan/THF 2:1 was used as model reaction. The immobilization efficiency was characterized by several parameters such as specific biocatalyst activity ( $U_b$ ), specific enzyme activity ( $U_e$ ), enantiomer selectivity ( $E$ ) and conversion ( $c$ ).



**Figure 1.** Lipase-catalyzed kinetic resolution of racemic 1-phenylethanol

Analysis of the above parameters for the free and adsorbed lipases indicated that in many cases the biocatalytic properties of enzymes adsorbed on solid support were superior compared to the native lipase (Table 1.).

Because the enantiomeric excess ( $ee$ ) of the forming acetate (*R*)-2 alone is not characteristic for the selectivity, the degree of enantiomer selectivity was characterized by the  $E$  value calculated from the conversion ( $c$ ) and enantiomeric excess of the forming acetate (*R*)-2 ( $ee_{(R),2}$ ) [15]. The activity yield [ $Y_A$  (%)] can be calculated from the effective specific activity of the immobilized biocatalyst ( $U_{e,imm-LAK}$ ) compared to the effective specific activity of the free Lipase AK ( $U_{e,LAK}$ ) [14].

**Table 1.** Behavior of free and adsorbed lipases in kinetic resolution of racemic 1-phenylethanol *rac*-1 with vinyl acetate.

Lipase	Adsorbent <sup>a</sup>	c <sup>b</sup> %	E <sup>b</sup>	U <sub>e</sub> ( $\mu\text{mol min}^{-1}\text{g}^{-1}$ )	Y <sub>A</sub> <sup>c</sup> %
Lipase AK <sup>d</sup>	-	49	>100	10	100
Lipase AK	Filtracel-950	22	>200	82	818
Lipase AK	Davisil 150	18	>100	69	687
Lipase AK	Grace 915	22	72	85	848
Lipase AK	Grace 920	30	>200	113	1124
Lipase AK	PQ 300	22	>200	85	847
Lipase PS <sup>d</sup>	-	29	»200	14	14
Lipase PS	Filtracel-950	48	>200	104	725
CcL <sup>d</sup>	-	7.6	3	2.6	100
CcL	Geduran Si 60	0.9	2,9	3.3	127
BUTE-3 <sup>d</sup>	-	50	»200	17	100
BUTE-3	Grace 920	7.9	62.1	30	174

<sup>a</sup> The enzyme / adsorbent mass ratio was 1 / 10;

<sup>b</sup> Results after 4 h reaction time. The enantiomer selectivity (*E*) was calculated from *c* and *ee*<sub>(R),2</sub> [15] and *ee*<sub>(S),1</sub> and *ee*<sub>(R),2</sub> [16] simultaneously. Due to sensitivity to experimental errors, *E* values calculated in the 100-200 range are reported as >100, values in the 200-500 range are reported as »200 and values calculated above 500 are given as »200;

<sup>c</sup> Activity yield; <sup>d</sup> Free lipase without immobilization

By entrapment the lipases adsorbed on a large surface in a sol-gel matrix the diffusional limitations can be decreased leading to immobilized lipases with enhanced catalytic properties. The study of adsorption of the lipase AK on four silica supports revealed that the activity yield (*Y<sub>A</sub>*) could be increased more than 800 % compared to specific activity of the free enzyme. The reason of this large effect can be the large surface area of the silica supports of pore diameters between 60 and 150 Å. This pore size range allows the adsorption of the enzyme inside the pores and thus formation of a thin layer on the total surface of the catalyst with practically no diffusion limitations for the substrate to reach and the product to leave from the catalyst. The same effect can be observed with lipase PS using Filtracel-950. On the other hand, the activity yields (*Y<sub>A</sub>*) for lipase CcL and BUTE-3 were not enhanced significantly by the adsorptive immobilization.

As the activity yield (*Y<sub>A</sub>*) enhancement by adsorption was most pronounced with lipase AK, further investigation was performed with this enzyme using the four most effective solid supports in a combined adsorption / sol-gel immobilization process (Table 2.). In this study, the preadsorption of Lipase AK on silica-gels was followed by sol-gel immobilization using the tetraethoxy-silane/octyltriethoxy-silane/phenyltriethoxy-silane precursors in 1/0.7/0.3 molar ratio which was found optimal in our previous study with Celite as solid support [17]. The best results were achieved with the combined sol-gel entrapment using the Grace 915 support, because the activity yield (*Y<sub>A</sub>*) enhancement (Table 2) was as high as for the simple adsorption without sol-gel (Table 1).

Besides the reusability of the sol-gel immobilized lipases, the formation of sol-gel polymer matrix can increase significantly the stability of the biocatalysts. The long-term stability test of entrapped lipases indicated that full activity of the

sol-gel biocatalysts was retained after storage in refrigerator or at room temperature (1 day, 1 week, 1 month tests) [18]. The full activity was also maintained for our novel sol-gel lipases after 1 month storage at room temperature.

**Table 2.** Behavior of sol-gel immobilized supported Lipase AK in kinetic resolution of racemic 1-phenylethanol *rac*-1 with vinyl acetate.

Support <sup>a</sup>	<i>c</i> <sup>b</sup> %	<i>E</i> <sup>b</sup>	<i>U<sub>e</sub></i> ( $\mu\text{mol min}^{-1} \text{g}^{-1}$ )	<i>Y<sub>A</sub></i> <sup>c</sup> %
-	49	>100	10	100
Grace 920	3,9	38	16	156
Grace 915	16	34	87	868
Davisil 150	8.2	75	40	400
PQ 300	5.1	50	22	218

<sup>a</sup> Lipase AK adsorbed on solid support (Table 1) was added to the tetraethoxy-silane/octyltriethoxy-silane/phenyltriethoxy-silane 1/0.7/0.3 silane precursor system during sol-gel matrix formation;

<sup>b</sup> Results after 4 h reaction time. The enantiomer selectivity (*E*) was calculated from *c* and *ee*<sub>(R),2</sub> [15] and *ee*<sub>(S),1</sub> and *ee*<sub>(R),2</sub> [16], simultaneously. Due to sensitivity to experimental errors, *E* values calculated in the 100-200 range are reported as >100;

<sup>c</sup> Activity yield

## CONCLUSION

The activity and stability of lipases had been increased significantly by applying commercially available solid silica supports of wide pores. The adsorptive or the combined sol-gel immobilization did not influence the selectivities of lipases, whereas the robust combined adsorption / sol-gel encapsulation resulted in biocatalysts which are reusable and thus applicable in various synthetic processes.

## EXPERIMENTAL SECTION

### Chemicals and enzymes

Lipase AK (lipase from *Pseudomonas fluorescens*), Lipase PS (lipase from *Burkholderia cepacia*), CcL (lipase from *Candida cylindracea*), and Davisil 150 were obtained from Sigma-Aldrich. 2-Propanol (IPA), vinylacetate and sodium fluoride (NaF) were products of Aldrich. 1-Phenylethanol, 2-heptanol, polyethyleneglycol 1000 (PEG), Celite® 545, tetraethoxy-silane and phenyltriethoxy-silane were obtained from Fluka. Octyltriethoxy-silane were obtained from Alfa Aesar. Grace 920, Grace 915 were obtained from Grace. PQ 300 was obtained from PQ Corporation. Filtracel EFC-950 was a product of Rettenmaier and Söhne GMBH. Geduran® Si 60 was obtained from Merck KGaA. The lipase BUTE-3 was obtained as described earlier [13].

### Preadsorption of lipases on solid adsorbent

The lipase powder (50 mg) was suspended in TRIS-HCl buffer (0.1 M, pH 7.5, 25 mL) at room temperature. The solid support (500 mg) was added to the solution. After and stirring (at 800 rpm for 15 min), the resulting suspension was kept at 4°C for 24 h. After filtration, the residual solid was washed with buffer (25 mL), dried at room temperature at air overnight and finally dried in vacuum exicator for 3 h.

### ***Immobilization of lipases in sol-gel systems***

The solution of TRIS-HCl buffer (0.1 M, pH 7.5, 390  $\mu$ L), polyethylene glycol (PEG, 4% w/v, 200  $\mu$ L), aqueous sodium fluoride (NaF, 1M, 100  $\mu$ L) and 2-propanol (IPA, 200  $\mu$ L) were shaken at room temperature for 10 minutes in a glass of 20 ml vial. Then the silane precursors [tetraethoxy-silane, octyltriethoxy-silane, phenyltriethoxy-silane precursors in 1/0.7/0.3 molar ratio; total 780  $\mu$ mol] were added to the aqueous solution and the resulting two-phase emulsion was shaken for further 5 minutes until gelation. The lipase powder (22.7 mg free or 250 mg preadsorbed lipase) was added to the gel at intensive shaking. To complete the polymerization, the mixture was shaken for 12 h at room temperature. The forming fine, white powder was washed by 2-propanol (7 ml), distilled water (5 ml), 2-propanol (5 ml) and n-hexane (5 ml). The immobilized biocatalysts were dried in a vacuum exicator for 5 h then stored in air at room temperature. The immobilization efficiency was calculated on the basis of the enzyme supplied to the immobilization process.

### ***Esterification assay***

To a solution of racemic 1-phenylethanol (*rac*-1, 50 mg, mmol) in hexane/THF 2/1 (1 mL) and vinyl acetate (100  $\mu$ L), biocatalyst (50 mg) was added and the mixture was shaken in a sealed glass vial at 1000 rpm at room temperature. For GC analyses, samples were taken directly from the reaction mixture (sample size: 10  $\mu$ L, diluted with CH<sub>2</sub>Cl<sub>2</sub> to 100  $\mu$ L) at 2,4,8 and 24 h. Data on conversion and enantiomeric selectivity of the process with various enzymes are presented in Tables 1 and 2.

### ***Gas chromatographic analysis of the products***

The products of the kinetic resolutions with the various lipase biocatalysts [(*R*)-2 and (*S*)-1] were analyzed by gas chromatography on Acme 6100, equipped with flame ionization detector and Hydrodex  $\beta$ -6TBDM [30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m film of heptakis-(2,3-di-O-methyl-6-O-*t*-butyldimethyl-silyl)- $\beta$ -cyclodextrin] column. The oven temperature, injector and detector temperatures were 135, 250 and 250  $^{\circ}$ C, respectively. Hydrogen was used as carrier gas at constant flow (1.8 mL/min).

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