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ENTRAPMENT OF LIPASES IN NOVEL SOL-GEL SYSTEMS

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ABSTRACT. Lipases are the most widely used enzymes in synthetic organic chemistry. The search for efficient methods for immobilization of enzymes has emerged an important field of interest in order to increase their activity, stability and to facilitate their recovery because of the industrial application of lipases. In this study, the lipase immobilization by entrapment in sol-gel matrices was investigated to study the effect of enzymes, supports and enzyme/support ratios.

Keywords: lipase, sol-gel, biocatalysis, secondary alcohol, immobilization

INTRODUCTION

Lipases are amongst the most widely used enzymes in organic chemistry [1,2]. Recently, many biological materials such as enzymes [3,4], proteins [5], microbes [6], and mammalian and plant cells [7,8,9] have been immobilized into silica matrices prepared by the sol–gel method. Because the immobilization of biomolecules into organic–inorganic hybrid silica gels prepared by this method brings about an enhancement of activity, thermal and operational stabilities. Commercial applications of these immobilized biomolecules have been widely studied [10,11,12,13,14,15,16].

We report here on the entrapment of lipases in hydrophobic sol-gel materials, which results in the formation of highly active, stable and reusable heterogeneous biocatalysts.

RESULTS AND DISCUSSION

In our recent study the sol-gel encapsulation of two different lipases from *Pseudomonas fluorescence* (lipase AK) and *Pseudomonas cepacia* (lipase PS) combined with adsorption on a solid support were investigated.

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The high specific surface of the support leads to avoiding aggregation and resulting an increased stability and better thermal properties of the obtained biocatalysts. Native lipases and lipases immobilized by simple sol-gel entrapment were used as references. Moreover, the influence of the porosity of the supports (Celite 545 or Silica gel) and the enzyme/support ratio were also studied. The corresponding enzymes were immobilized using octyltriethoxy- (OcTEOS) and tetraethoxy (TEOS) silane precursors in 1:1 molar ratio. The model reaction was the kinetic resolution of 1-phenylethanol with vinyl acetate in hexan/THF 2:1 as solvent. The immobilization efficiency was characterized by several parameters measured at 24 h reaction time such as specific biocatalyst activity (U_b), specific enzyme activity (U_e), enantiomer selectivities (*E*) and enantiomeric excess (*ee*).

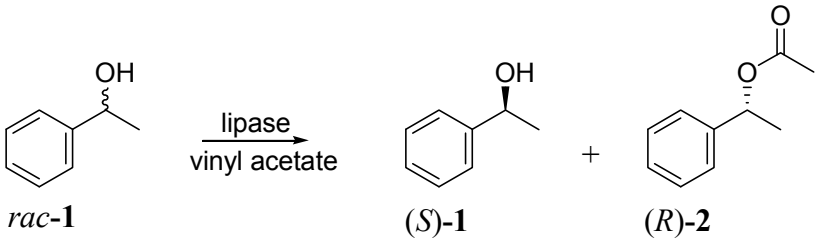

Figure 1. Lipase-catalyzed kinetic resolution of racemic 1-phenylethanol

Comparing the results of sol-gel entrapment and combined method, it is obvious that deposition of the enzymes on Celite or on silica gel resulted in increased specific enzyme activities. Analysis of the investigated parameters like enantioselectivities, conversions, specific biocatalyst and specific biocatalyst/enzyme activities indicated that the immobilized enzymes adsorbed previously on solid support were superior compared to the native enzyme or to the sol-gel immobilized enzyme. Because the enantiomeric excess (*ee*) values of the forming (*R*)-**2** were >98 in all cases, *ee* of the acetates were not applied in the further comparisons.

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 comparison of two silica based supports indicated that with Celite 545 (low porosity) the enzyme immobilization occurred only at the external surface of the support. Using chromatographic grade silica gel (high-porosity adsorbent) support, adsorption and gelation could also occur within the pores. Therefore − indicated by the acylation reaction of the racemic secondary alcohol *rac*-**1** − enhanced specific enzyme activities were observed for both lipases by the combined sol-gel immobilization using silica gel support (Tables 1 and 2).

Table 1. Kinetic resolution of racemic 1-phenylethanol *rac*-**1** by acylation with vinyl acetate using free and immobilized lipase AK as catalyst

Biocatalyst	E/S ratio	*c* (%)	*E*[a]	U_b (μmol $min^{-1}mg^{-1}$)	U_e (μmol $min^{-1}mg^{-1}$)
Free L AK[b]	-	49	»200	34.4	34.4
without S[c]	-	51	>100	2.9	5.7
Celite	1/5	33	»200	1.9	12.2
$Celite_{pread}$	1/5	31	»200	1.8	12.2
Silica	1/5	40	>200	2.3	14.0
$Silica_{pread}$	1/5	49	»200	2.9	19.7
without S[d]	-	38	»200	2.2	8.2
Celite	1/10	45	»200	2.6	34.6
$Celite_{pread}$	1/10	31	»200	1.8	24.2
Silica	1/10	37	»200	2.1	29.2
$Silica_{pread}$	1/10	41	>200	2.4	32.3

[a] The enantiomer selectivity (*E*) was calculated from *c* and *ee* [17,18]. 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.
[b] Free lipase AK, without sol-gel entrapment after 2 h reaction time.
[c] Sol-gel immobilized lipase AK without support (S). The amount of enzyme were identical with the applied enzyme at 1/5 molar ratio.
[d] Sol-gel immobilized lipase AK without support (S). The amount of enzyme were identical with the applied enzyme at 1/10 molar ratio.

Table 2. Kinetic resolution of racemic 1-phenylethanol *rac*-**1** by acylation with vinyl acetate using free and immobilized lipase PS as catalyst

Biocatalyst	E/S ratio[b]	*c* (%)	*E*[a]	U_b (μmol $min^{-1}mg^{-1}$)	U_e (μmol $min^{-1}mg^{-1}$)
Free L PS[b]	-	47	»200	32.5	32.5
without S[c]	-	36	»200	2.1	5.4
Celite	1/5	36	»200	2.0	29.0
$Celite_{pread}$	1/5	15	»200	0.9	6.6
Silica	1/5	43	»200	2.5	20.2
$Silica_{pread}$	1/5	32	»200	1.8	12.1
without S[d]	-	36	»200	2.0	10.8
Celite	1/10	14	>200	0.8	10.2
$Celite_{pread}$	1/10	13	>200	0.7	12.4
Silica	1/10	32	»200	1.8	27.8
$Silica_{pread}$	1/10	23	»200	1.3	19.4

[a] The enantiomer selectivity (*E*) was calculated from *c* and *ee* [17,18]. 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.
[b] Free lipase PS, without sol-gel entrapment after 2 h reaction time.
[c] Sol-gel immobilized lipase PS without support (S). The amount of enzyme were identical with the applied enzyme at 1/5 molar ratio.
[d] Sol-gel immobilized lipase PS without support (S). The amount of enzyme were identical with the applied enzyme at 1/10 molar ratio.

In the case of lipase AK, the most efficient immobilization method was the preadsorption on silica gel followed by sol-gel immobilization (Table 1). In contrast, the best immobilization results were achieved with lipase PS without preadsorption on silica gel (Table 2).

Activities of the lipase biocatalysts were investigated at two different enzyme/support ratios by the esterification reaction of 1-phenylethanol *rac*-**1**. Interestingly, the conversions (*c*) and specific biocatalyst activities (U_b) depended only slightly from the amount of lipase AK in sol-gel immobilization. On the other hand, the specific enzyme activities (U_e) were much higher at 1/10 lipase AK/support ratio than at the 1/5 ratio (Table 1). Using lipase PS, the best results were obtained at 1/5 lipase/support ratio with silica gel or Celite without preadsorption (Table 2).

The stability and reusability of the lipase biocatalysts were also studied *via* storage and repeated use. The long-term stability of entrapped lipases proved to be excellent. There were no differences in the activity of the biocatalysts stored in refrigerator or at room temperature (1 day, 1 week, 1 month tests). In the repeated use tests, the catalysts were filtered off after 22h, then washed and reused. The activities in the next reaction remained almost constant as found previously with other sol-gel immobilized enzymes [19].

Table 3. Kinetic resolution of racemic 1-phenylethanol *rac*-**1** by acylation with vinyl acetate using commercial sol-gel lipases at 24 h

Commercial available biocatalyst[a]	*c* (%)	*E*[b]	U_b (μmol $min^{-1}mg^{-1}$)
Sol-gel Lipase AK	27	>100	5
Sol-gel Lipase AK on pumice	7	>200	1
Sol-gel Lipase PS	35	»200	6

[a] Commercial sol-gel entrapped lipases.
[b] The enantiomer selectivity (*E*) was calculated from *c* and *ee* [17,18]. 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.

Finally, commercial sol-gel lipases were tested (Table 3). The results indicated that the sol-gel lipases with support prepared by our methods (Tables 1 and 2) showed higher enantioselectivities and conversions then the commercial sol-gel lipase AK or PS preparations.

CONCLUSION

A robust sol-gel entrapment method involving addition of silica based supports resulted in immobilized lipases with excellent activity and stability. The resulting biocatalysts are easy to reuse and thus they are valuable biocatalysts in various synthetic processes. A particular advantage of deposition enzymes on supports is the large catalytic surface resulting in enhanced biocatalysts. The high-porosity silica gel support proved to be superior over the less porous Celite in the sol-gel lipase immobilization.

EXPERIMENTAL SECTION

Preadsorption of lipases on solid adsorbent

The lipase powder (50 mg) was suspended in TRIS-HCl buffer (0.1 M, pH 7.5, 780 μl) at 4 °C. After 10 min, the solid support (Celite® 545 or silica gel, 500 mg) was added to the solution and the resulting suspension was stirred for further 20 min. To the support-enzyme suspension acetone (10 mL, 10 mL min-1, -18 °C) was added dropwise. The resulting precipitate was filtered off and left air dried at room temperature for 12 hours. The preadsorbed enzyme was encapsulated in sol-gel matrices according to the procedure described below.

Immobilization of lipases in sol-gel systems

In a 20 mL glass vial the solution of TRIS-HCl buffer (0.1 M, pH 7.5, 390 μL), polyethylene glycol (PEG, 4% w/v, 200 μL), aqueous sodium fluoride (NaF, 1M, 100 μL) and 2-propanol (IPA, 200 μL) were shaken at room temperature for 10 minutes. Then the silane precursors [780 μmol; octyltriethoxy-silane (OcTEOS) and tetraethoxy-silane (TEOS) precursors in 1:1 molar ratio] were added and the two phase mixture was shaken for further 5 minutes until gelation. The lipase powder (22.7 mg free or 250 mg preadsorbed lipase; see Tables 1 and 2) was added to the resulting gel at violently shaking. To complete the polymerization, the mixture was stirred for 12 hours at room temperature. To get the product as white powder, the following washing procedure was applied in all cases: 2-propanol (7 ml), distilled water (5 ml), 2-propanol (5 ml) and n-hexane (5 ml). The products were dried in a vacuum exicator for 5 hours then left air dried for further 24 hours. The immobilization efficiency was evaluated by calculating the percentage of the supplied protein encapsulated in the sol-gel.

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 μL) enzyme (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 μL, diluted with CH_2C_{l2} to 100 μL) at 2,4,8 and 24 h. Data on conversion and enantiomeric excess of the products [(*R*)-**2** and (*S*)-**1**] with various enzymes are presented in Tables 1-3.

Gas chromatographic analysis of the products

The esters were analyzed by gas chromatography on Acme 6100, equipped with flame ionization detector and Hydrodex β-6TBDM [30 m × 0.25 mm × 0.25 μm film of heptakis-(2,3-di-O-methyl-6-O-*t*-butyldimethyl-

silyl)-β-cyclodextrin] column. The oven temperature, injector and detector temperatures were 135, 250 and 250 °C, respectively. Hydrogen was used as carrier gas at constant flow (1.8 mL/min).

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