

Influence of Silane Precursors System Composition on the Catalytic Efficiency of Sol-Gel Immobilized Lipases

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Abstract: Sol-gel entrapped lipases gained an increasing attention in the last years, as biocatalysts for several practical applications. In this study, lipases from *Candida antarctica* B (C-Lecta) has been immobilized by sol-gel entrapment using binary and ternary silane precursor systems containing vinyl-trimethoxysilane. The presence of vinyl functional groups in the sol-gel matrix could be important for specific applications that need further functionalization of the sol-gel matrix. The obtained enzymatic preparates were used as biocatalysts for kinetic resolution of racemic 2-hexanol and 2-octanol, by acylation with vinyl acetate in *n*-hexane medium. The immobilization resulted in high activity and enantioselectivity values.

Keywords: lipase, sol-gel, vinyl-trimethoxysilane, kinetic resolution, secondary alcohols.

1. Introduction

Biocatalysis has been increasingly recognized as an efficient and green tool for modern organic synthesis due to the high selectivity and specificity under mild reaction conditions. Thus, the application of enzymes represents a remarkable opportunity for the development of industrial chemical and pharmaceutical processes [1]. In particular, non-aqueous biocatalysis is suitable for synthesis of commercially important pharmaceutical precursors and drugs, such as pure enantiomers, chiral molecules, single isomers and biopolymers [2-4]. However, enzymes often display drastically lower activity in organic solvents than in water. It is believed that the water layer on the molecular surface of enzymes determines their activity in organic media [1].

Lipases are the most used enzymes due to their ability to catalyse a wide range of reactions, such as esterification, transesterification, ammonolysis, polymerization. The catalytic triad of lipases with a α/β -hydrolase fold is composed of serine, histidine and aspartate or glutamate, which is also found in proteases, esterases and thioesterases [5].

Maintenance of the structural stability of enzymes during any biochemical reaction is highly challenging. Consequently, immobilized enzymes with functional efficiency and enhanced reproductibility are used as alternatives [6]. Among the variety of immobilization methods, sol-gel entrapment proved to be a simple technique that allows the synthesis of biocatalysts with excellent stability. The porosity of the gel matrix is controlled to ensure that the structure is tight enough to

prevent leakage of enzyme or cells, yet at the same time allows free movement of substrate and product. This support also acts as a barrier and can be advantageous as it protects the immobilized enzyme from microbial contamination [7].

Vinyl-trimethoxysilane was successfully used as precursor for sol-gel entrapment of subtilisine, leading to a biocatalyst active in peptide synthesis [8].

In this work, we investigated the influence of vinyl non-hydrolyzable groups presence in the matrix on the catalytic properties of sol-gel immobilized lipase. The obtained biocatalysts were tested in the enantioselective acylation of two secondary alcohols.

2. Experimental

2.1. Materials

Lipase from *Candida antarctica* B (CaLB lipase) was produced by C-Lecta (Leipzig, Germany). Silane precursors vinyl- (VTMOS) and phenyl-trimethoxysilane (PhTMOS) were purchased from Merck and tetramethoxysilane (TMOS) from Fluka. Other materials used: tris-(hydroxymethyl)-aminoethane (Loba Chemie), 2-propanol (Merck), sodium fluoride (Fluka), bovine serum albumin BSA (Sigma-Aldrich), Bradford reagent (Merck), 2-hexanol (Merck), 2-octanol (Merck), vinyl acetate (Merck), *n*-hexane (98%, Merck) were of analytical grade and have been used as purchased. Decane (>99%, Aldrich) and *n*-dodecane (Aldrich) were used as internal standards for quantitative gas-chromatographic analysis. The ionic liquid 1-octyl-3-methyl-imidazolium tetrafluoroborate, [Omim]BF₄, was product of Fluka.

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2.2. Immobilization by Sol-Gel Entrapment

A microbial lipase suspension in TRIS/HCl 0.1 M, pH 8.0 buffer was stirred at room temperature for 30 min, centrifuged, and the supernatant used for immobilization. In a 4 mL glass vial, 1 mL of this lipase solution was mixed with 200 μ L ionic liquid, followed by addition of 100 μ L 1M NaF solution, and 200 μ L isopropyl alcohol. This mixture was kept for 30 min under continuous stirring for homogenization, and subsequently a tertiary mixture of silane precursors (total 6 mmoles) was added. The mixture was stirred at room temperature until the gelation started. The obtained gel was kept for 24 h at room temperature to complete polymerization. The bulk gel was washed with isopropyl alcohol (7 mL), distilled water (5 mL), isopropyl alcohol again (5 mL) and finally *n*-hexane (5 mL), filtered, dried at room temperature for 24 h, and in a vacuum oven at room temperature for another 24 h. Finally, it was crushed in a mortar and kept in refrigerator. The amount of immobilized lipase was determined by measuring the protein content of free enzyme and washing solutions. The protein content was determined using the method developed by Bradford [9]. The immobilization yield was calculated as percentage of encapsulated protein and protein subjected to immobilization. The determined protein content of native lipase was 0.11 mg/mg.

2.3. Acylation of secondary alcohols

Acylation was performed in 4 mL capacity glass vials, charged with a mixture of 2-hexanol or 2-octanol (0.5 mmole), vinyl acetate (1.5 mmole), internal standard (*n*-dodecane or *n*-decane, 15 μ L), reaction medium (*n*-hexane, 1 mL) and free (5 mg) or immobilized lipase (25 mg).

The mixture was incubated using an orbital shaker (MIR-S100, Sanyo, Japan) at 300 strokes/min and 40°C (ILW 115 STD incubator, Pol-Eko-Aparatura, Poland). The conversion and enantiomeric excess of the product were assayed by gas-chromatography, on a Varian 450 instrument (Varian Inc., USA) equipped with flame ionization detector, using a 30 m x 0.25 mm Elite-Cyclosil B chiral column with 0.25 mm film thickness (Perkin-Elmer, USA). The analysis conditions were: oven temperature: 50° to 120°C with 10°C/min heating rate, injector temperature 240°C, detector temperature 280°C, carrier gas (hydrogen) flow 1.2 mL/min. The reactions were usually run for 24 h. Conversions have been calculated based on the internal standard method.

Transesterification activities were calculated at 6 and 24 h reaction time and expressed as the average 2-acetoxy-alcohol amount (in micromole) synthesized per hour by 1 mg of free or immobilized enzyme. The control reaction without enzyme did not give any product in the same conditions.

To characterize the overall efficiency of the immobilization process, relative total activities were calculated as the ratio of total enzymatic activity recovered following immobilization, divided by the total activity of lipase subjected to immobilization. The enantiomeric

excess of the resulted ester product (ee_p) was determined from peak areas of enantiomers, and the enantiomeric ratio (E) values were calculated based on conversion and ee_p values using the relation (1) [10]:

$$E = \frac{\ln[1 - C(1 + ee_p)]}{\ln[1 - C(1 - ee_p)]} \quad (1)$$

where C represent the conversion at 24 h.

All reactions have been run in duplicate and sampling was also made in duplicate. As the differences between the data for the same assay were less than 2%, average values have been calculated and presented in tables and figures.

3. Results and Discussion

Immobilization conditions and the composition of silane precursors system are essential for the final properties of the immobilized biocatalyst. The properties and morphology of the final materials can be easily tuned by the appropriate choice of the silane precursors. The aim of this study was to obtain materials by sol-gel entrapment of lipase from *Candida antarctica* B using a silane precursor with vinyl non-hydrolyzable groups. In this way we obtained a polysiloxane type hybrid matrix. The properties of the sol-gel matrix, its hydrophobicity and interactions with the enzyme are influenced by the vinyl groups.

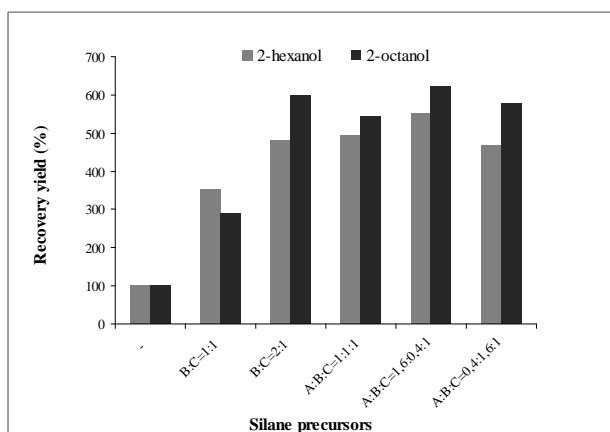
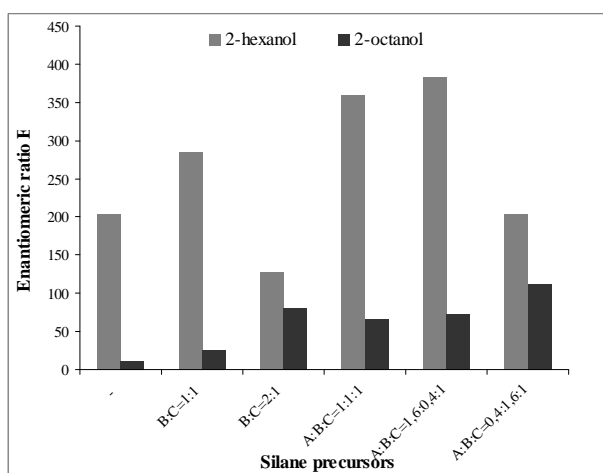
Lipase from *Candida antarctica* B (CaLB) was entrapped as described in the experimental section using binary and ternary precursors systems and ionic liquid [Omim]BF₄ as immobilization additive. Enzyme loading of the matrix was 17.4 mg protein/g xerogel. The catalytic efficiency of the immobilized lipase was studied in the enantioselective acylation of two secondary alcohols (2-hexanol and 2-octanol) in the previously established reaction conditions: vinyl acetate as acylation reagent, *n*-hexane as reaction medium, and 40°C temperature [3]. The results obtained at 6 h reaction time are showed in Table 1.

After immobilization we obtained high values of conversion, activity and enantiomeric excess, with no respect to the tested substrate. When sol-gel entrapped lipase was employed as catalyst conversion values were at least double compared to the native enzyme. Highest values of this parameter were obtained at a higher concentration of vinyl groups in the sol-gel matrix. When the sol-gel matrix was obtained using a ternary silane precursors system the transesterification activity was higher. The enantiomeric excess was almost the same with no respect to the composition of the sol-gel system.

The recovery yield of enzymatic prepreparates is a much more accurate measure of activity. For the two substrates, all the tested prepreparates gave higher values of this parameter in comparison with the native lipase. When a ternary silane precursors system was used, the catalytic efficiency was better than in case of the binary system (Figure 1).

TABLE 1. Acylation of 2-hexanol and 2-octanol in *n*-hexane catalyzed by sol-gel immobilized CaLB using [O_{mim}]BF₄ as immobilization additive (A=PhTMOS, B=VTMOS, C=TMOS).

Silane precursors	Conversion (%)		A _{trans.} (μmole·h ⁻¹ ·mg ⁻¹)		Enantiomeric excess (%)	
	2-hexanol	2-octanol	2-hexanol	2-octanol	2-hexanol	2-octanol
Native enzyme (control)	11	10	1.824	1.660	99	81
B:C=1:1	27	20	0.934	0.704	99	91
B:C=2:1	40	42	1.280	1.452	97	95
A:B:C=1:1:1	37	36	1.211	1.218	99	95
A:B:C=1.6:0.4:1	39	39	1.289	1.320	99	95
A:B:C=0.4:1.6:1	41	44	1.304	1.461	98	96

Figure 1. Influence of the silane precursors molar ratio on the catalytic efficiency of sol-gel entrapped biocatalysts in the enantioselective acylation of 2-hexanol and 2-octanol in *n*-hexane at 40°C (A=PhTMOS, B=VTMOS, C=TMOS)Figure 2. Influence of the silane precursors molar ratio on the enantiomeric ratio of sol-gel entrapped biocatalysts in the enantioselective acylation of 2-hexanol and 2-octanol in *n*-hexane at 40°C (A=PhTMOS, B=VTMOS, C=TMOS)

Enantioselectivity is a key issue for practical applications, because it is determined from both conversion and enantiomeric excess values. In this situation, the E ratio values were very different for the tested substrates (Fig. 2). In case of 2-hexanol we obtained the highest enantiomeric ratios. When a ternary precursor system was

employed in the synthesis of sol-gel matrix the enantioselectivity decreased with the increase of vinyl groups in the final material, so the best molar ratio was 1.6:0.4:1.

Same enzymatic materials were tested in the acylation of 2-octanol. In this situation, the enantiomeric ratio had a completely different trend, increasing with the concentration of vinyl groups from the matrix. The highest value (E=146) was obtained when the silane precursors were used in 0.4:1.6:1 molar ratio.

4. Conclusions

The final properties of sol-gel materials are strongly influenced by the immobilization conditions. For every substrate the biocatalyst composite should be appropriately designed in order to fulfill the requested necessities. The presence of the vinyl group in the matrix is not detrimental to the activity and enantioselectivity of the enzyme, but allows grafting of different functional groups by post-modification of the initial composite. Other biomolecules, like as peptides, can also be immobilized using this procedure. The structure and morphology of the sol-gel matrix must be carefully selected for each substrate, in order to maximize the positive effects of immobilization.

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