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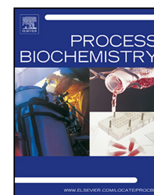

Hydrophobic adsorption and covalent immobilization of *Candida antarctica* lipase B on mixed-function-grafted silica gel supports for continuous-flow biotransformations

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ABSTRACT

Adsorption onto solid supports has proven to be an easy and effective way to improve the mechanical and catalytic properties of lipases. Covalent binding of lipases onto the support surface enhances the active lifetime of the immobilized biocatalysts. Our study indicates that mesoporous silica gels grafted with various functions are ideal supports for both adsorptive and covalent binding for lipase B from *Candida antarctica* (CaLB). Adsorption of CaLB on phenyl-functionalized silica gels improved in particular its specific activity, whereas adsorption on aminoalkyl-modified silica gels enabling covalent binding with the proper reagents resulted in only moderate specific activity. In addition, adsorption on silica gels modified by mixtures of phenyl- and aminoalkyl silanes significantly increased the productivity of CaLB. Furthermore, CaLB adsorbed onto a phenyl/aminoalkyl-modified surface and then treated with glutardialdehyde (GDA) as cross-linking agent provided a biocatalyst of enhanced durability. Adsorbed and cross-linked CaLB was resistant to detergent washing that would otherwise physically deactivate adsorbed CaLB preparations. The catalytic properties of our best immobilized CaLB variants, including temperature-dependent behavior were compared between 0 and 70 °C with those of two commercial CaLB biocatalysts in the continuous-flow kinetic resolutions of racemic 1-phenylethanol *rac*-1a and 1-phenylethanamine *rac*-1b.

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1. Introduction

Enzyme-catalyzed transformations have gained ever increasing importance in modern synthetic processes [1–4]. The application of enzymes in their native forms, however, suffers several drawbacks. In aqueous solutions, enzymes are relatively unstable, and their recovery may be difficult due to their water solubility. Immobilization resulting in a reusable and stable heterogeneous phase form of the enzyme greatly extends the scope of their application in industrial processes [5,6]. Moreover, immobilized enzymes are recyclable, easy to store and handle and may show enhanced stability, activity and selectivity compared to their native forms [1,7–12].

It was found that optimum performance of the immobilized enzymes in a given transformation may be attained by applying different enzyme immobilization strategies [13,14]. Therefore, the nature of the solid support in enzyme immobilization is particularly important [6,11,15,16]. Porous silica gels [15,17–19] and particularly mesoporous silica gels (MPSs) [20,21] have been shown to be useful carriers for enzyme immobilization due to their large surface area, tunable porosity, low cytotoxicity, favorable mechanical properties and functionalizable large surface. Modification by surface functionalization can widen their applicability as carriers for proteins and enzymes [20,21]. Cholesterol esterase (CE) combined with five different types of MPSs differing in structural properties such as pore diameter, pore volume, and particle morphology exhibited various catalytic activities [22]. Grafting the surface of MPSs of large pore size (22.5 nm) with various functions (such as decyl)

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resulted in improved immobilized CE biocatalysts with enhanced recyclability and thermal stability [22].

MPSs with amino functions on their surfaces enable either adsorptive or covalent immobilization. For example, an ordered MPS functionalized by post-synthesis grafting with (3-aminopropyl)triethoxysilane (APTEOS) was used for adsorptive immobilization of lipase from porcine pancreas (PPL), resulting in a higher hydrolytic activity and better reusability than when the enzyme was adsorbed on non-grafted MPS [23].

Amino functions on the surface of enzymes treated with suitable cross-linking agents such as glutardialdehyde (GDA) in the presence of a precipitant enable supportless immobilization by the preparation of cross-linked enzyme aggregates (CLEAs) which are efficient immobilized biocatalysts [24] even on an industrial scale [25].

In addition to immobilization, reactor technology may offer further means of upgrading of the efficacy of biotransformations. In recent years continuous-flow technology has received increasing attention and is becoming a promising alternative to batch processes [26,27]. Most of the continuous-mode biocatalytic syntheses of optically active chiral intermediates on a relatively large scale are performed using immobilized lipases in packed-bed reactors [28]. Stainless steel continuous-flow packed-bed bioreactors can be effectively used to study the effects of temperature, pressure and flow rate on stereoselective biotransformations such as lipase-catalyzed kinetic resolutions [29–32].

Among readily available lipases lipase B from *Candida antarctica* (CaLB) exhibits many attractive characteristics and has thus become one of the most widely used biocatalysts in both industrial applications and scientific research [3,33–35]. Recently, it was demonstrated that during the continuous-flow kinetic resolution of various amines using differently immobilized CaLB biocatalysts the character of the temperature effect in the range of 0–70 °C depended significantly both on the substrate and on the mode of immobilization [36].

Immobilization of CaLB on butyl- [37] or octyl-silica [19,38] has indicated the usefulness of surface-modified silica as lipase carrier. Surface-modified silica supports, especially butyl silica, have proven to be suitable carriers for CaLB-catalyzed reactions in ionic liquid/supercritical carbon dioxide biphasic media [39]. A study with a series of grafted silica gels indicated phenyl-silica as ideal support for CaLB securing satisfactory selectivity in the kinetic resolution of racemic 1-phenylethanol *rac*-**1a**, while the octyl-silica-adsorbed CaLB had poor activity with good enantiomer selectivity [40]. However, the aminopropyl-silica adsorption resulted in CaLB biocatalysts of moderate performance [39].

Covalent immobilization of CaLB on amino-silica supports indicated that the thermal stability of such biocatalysts was better than those prepared by physical adsorption only [41]. CaLB adsorbed and cross-linked on a polypropylene carrier maintained its activity when dispersed in ionic liquids [42].

Coating an MPS with a mixture of the grafting reagents 4-aminophenyltrimethoxysilane and phenyltrimethoxysilane at different ratios demonstrated that the density of the amino groups present on the silica surface can be successfully controlled while keeping the overall number of grafts constant [43]. Thus, a simple method can secure tunable and even dispersion of amino functionality on the surface.

In the present work we set out to compare the usefulness of biocatalysts prepared by simple adsorption of CaLB with adsorption combined with cross-linking and covalent binding onto surface-functionalized silica supports with dispersed amino functions as novel biocatalysts in both in batch and continuous-flow mode.

2. Materials and methods

2.1. Materials

Racemic 1-phenylethanol (*rac*-**1a**), racemic 1-phenylethanamine (*rac*-**1b**), glutardialdehyde (GDA) solution (25%, w/v in H_2O), vinyl acetate, sodium chloride, mono- and dibasic sodium phosphate, Trizma® base (2-amino-2-hydroxymethyl-1,3-propanediol; Tris base), hydrochloric acid and Triton™ X-100 (4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol) were purchased from Sigma–Aldrich.

Davisil® 150 [35–70 μm] (Dv150), Davisil® 250 [40–63 μm] (Dv250), Daraclar® 915 (Dc915) and Daraclar® 920 (Dc920) were the products of Grace (Deerfield, USA). Geduran® Si 60 [63–200 μm] (Ged60) was purchased from Merck (Darmstadt, Germany). Silica supports functionalized with phenyltrimethoxysilane (PTMOS), octyltrimethoxysilane (OTMOS), (3-aminopropyl)trimethoxysilane (APTMOS), [3-(2-aminoethylamino)propyl]trimethoxysilane (AEAPTMOS), [3-(2-aminoethylamino)propyl]methlydimethoxysilane (AEAP-MDMOS) and with various mixtures of such organosilanes were produced by SynBiocat Ltd (Budapest, Hungary). The labels for the functionalized silica supports indicate both the nature of the grafted silica gel and the grafting reagents. For example, Geduran® Si 60 grafted with OTMOS is labeled as Ged60O or Davisil® 250 grafted with a PTMOS:AEAP-MDMOS 1:1 mixture is labeled as Dv250PAEAP11.

Novozym® 435 (CaLB N 435, recombinant lipase B from *Candida antarctica* expressed in *Aspergillus niger* and adsorbed on acrylic resin) and Novozym® CaLB L recombinant (lipase from *Candida* sp. expressed in *Aspergillus niger* with a protein content ~4%, ≥5000 LU/g) were obtained from Sigma–Aldrich (Saint Louis, MO, USA). CaLB T2-150 (*Candida antarctica* lipase B covalently attached to dry acrylic beads with a 150–300 μm particle size) was the product of ChiralVision BV (Leiden, The Netherlands).

Solvents (toluene, ethyl acetate, acetone, *n*-hexane, methyl *tert*-butyl ether, dichloromethane and 2-propanol) from Molar Chemicals (Budapest, Hungary) were dried and/or freshly distilled prior to use.

2.2. Methods

Thin-layer chromatography was carried out using Kieselgel 60 F_{254} (Merck) sheets. Spots were visualized under UV light (Vilber Lourmat VL-6.LC, 254 nm and 365 nm) or by treatment with 5% ethanolic phosphomolybdic acid solution and heating of the dried plates.

Reactions yielding **2a** from **1a** were analyzed by gas chromatography (GC) on Agilent 4890 equipment [FID: 250 °C, injector: 250 °C, carrier gas: H_2 (12 psi), split ratio: 1:50] using a Hydrodex β-6TBDM column (Machery-Nagel, 25 m × 0.25 mm × 0.25 μm, heptakis-(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin); GC data (oven program: 120 °C, 8 min^{-1}; molar response factor for **2a/1a**: 1.23): t_r (min): 4.0 [(*S*)-**2a**], 4.4 [(*R*)-**2a**], 5.8 [(*R*)-**1a**], 6.0 [(*S*)-**1a**. Reactions yielding **2b** from **1b** were analyzed on Agilent 5890 equipment [FID: 250 °C, injector: 250 °C, carrier gas: H_2 (12 psi), split ratio: 1:50] using a Hydrodex β-TBDAc column (Machery-Nagel; 25 m × 0.25 mm × 0.25 μm, heptakis-(2,3-di-O-acetyl-6-O-*t*-butyl-dimethylsilyl)-β-cyclodextrin); GC data (oven program: 100–180 °C, 8 °C min^{-1}, 5 min at 180 °C; molar response factor for **2b/1b**: 1.17): t_r (min): 2.9 [(*S*)-**1b**], 3.1 [(*R*)-**1b**], 9.8 [(*R*)-**2b**], 10.0 [(*S*)-**2b**]. All data in Tables and Figures arose from a precise integration of chromatograms in which both enantiomers of the substrates **1a**, **b** and the products **2a**, **b** were clearly visible. When peaks for the minor enantiomers were indistinguishable from noise, solid curves were replaced by dashed lines in Figs. 4 and 5.

Conversion (*c*), enantiomeric excess (*ee*) and enantiomeric ratio (*E*) were determined by GC. Enantiomeric ratio (*E*) was calculated from *c* and enantiomeric excess of the product (ee_P) using the equation $E = \ln[1 - c(1 + ee_P)]/\ln[1 - c(1 - ee_P)]$ [44]. Due to its sensitivity to experimental error *E* values in the 100–200 range are given as >100, in the range of 200–500 as >200 and above 500 as ≫200.

In batch reactions, the specific activity of the biocatalyst (U_B) was determined using the equation $U_B = (n_{rac} \times c)/(t \times m_B)$ [32]. To characterize the productivity of the biocatalysts, the specific reaction rates in the batch reactions (r_{batch}) were calculated using the equation $r_{batch} = n_P/(t \times m_B)$ (where n_P [μmol] is the amount of the product, *t* [min] is the reaction time and m_B [g] is the mass of the biocatalyst) [44]. Specific reaction rates in continuous-flow systems (r_{flow}) were calculated using the equation $r_{flow} = [P] \times v/m_B$ (where [*P*] [μmol mL^{-1}] is the molar concentration of the product, v [mL min^{-1}] is the flow rate and m_B [g] is the mass of the biocatalyst) [44]. Because the rate of product formation is not a linear function of *c*, rigorous comparisons by the *r* values between the productivity of a continuous-flow reaction and its batch mode counterpart can only be made at similar degrees of conversions [44].

2.3. General procedure for lipase adsorption to silica supports

CaLB L (1.25 mL, ≥6250 LU/g, ~5 mg protein) was dissolved in Tris buffer (11.25 mL, 100 mM, pH = 7.5, ionic strength controlled with NaCl to 175 mM), an then the support (250 mg) was added to the solution. The enzyme-support suspension was incubated at 400 rpm and 4 °C for 18 h. The immobilized CaLB was filtered off on glass filter (G4), washed with 2-propanol (2 × 5 mL) and hexane (5 mL), dried for 2 h at room temperature and stored at 4 °C. The properties of CaLBs immobilized

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