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Interfacially activated lipases against hydrophobic supports: Effect of the support nature on the biocatalytic properties

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ABSTRACT

Different lipases (lipase B from *Candida Antarctica*, CAL-B, lipase from *Thermomyces lanuginose*, TLL and lipase from *Bacillus thermocatenulatus*, BTL) and a phospholipase (Lecitase® Ultra) were immobilized by interfacial activation on four different hydrophobic supports (hexyl- and butyl-toyopearl and butyl- and octyl-agarose) and their properties were compared. The results suggested that selection of different supports yielded very different results in terms of recovered activity (ranging from a sevenfold hyperactivation to almost fully inactive biocatalysts), stability, specificity and adsorption strength. Even more interestingly, the enantioselectivity of the enzymes in the hydrolysis of (±)-2-*O*-butyryl-2-phenylacetic acid was strongly dependent on the support utilized. For example, BTL immobilized on octyl-agarose was fully enantiospecific towards the hydrolysis of (*S*)-2-*O*-butyryl-2-phenylacetic acid ($E > 100$), whereas when immobilized on hexyl-toyopearl, the enantiomeric value of the immobilized lipase was only $E = 8$. However, there is not an optimal support; it depends on the lipase and on the studied parameter. In the asymmetric hydrolysis of phenylglutaric acid diethyl diester, BTL immobilized on hexyl-toyopearl was the most enantioselective catalyst with ee > 99% (A factor >100) in the production of *S*-monoester product, whereas the enzyme immobilized on butyl-toyopearl only exhibited an A factor of 3.

Finally, butyl-agarose was chosen as the most specific support on the lipase adsorption – compared to other proteins – at low ionic strength yielding the best purification of BTL from crude preparations.

1. Introduction

Lipases are the most used enzymes in biocatalysis and organic chemistry [1–5]. They recognize a wide variety of substrates while exhibiting high regioselectivity and enantiospecificity in many instances [6,7]. Lipases present a specific catalytic mechanism of action, existing in two structural forms, the closed one, where a polypeptide chain (lid or flat) isolates the active center from the medium, and the open form, where this lid moves and the active center is exposed [8–10]. This equilibrium is shifted towards the open form in the presence of hydrophobic surfaces (e.g., droplets of oils), where the lipase becomes adsorbed by the large hydrophobic pocket around their active center and the internal face of the lid [11,12]. Moreover, lipases may become adsorbed to other hydrophobic surfaces following a similar mechanism: hydrophobic proteins – like hydrophobins – [13], other lipase molecules [14–17] or on the surface of hydrophobic supports [18–20]. This particular mechanism of catalysis has permitted to develop specific protocols for the immobilization of lipases. Thus, the immobilization of lipases *via* interfacial activation on hydrophobic supports at low ionic strength has been reported to be a very simple and efficient method to immobilize and purify lipases [18]. This protocol fixes the open form of lipases *via* interactions between the hydrophobic surroundings of their active centre – the internal face of the lid and the area of the lipase around the active center that interacts with it – and the hydrophobic surface of the support. These immobilized biocatalysts are very active against hydrophobic substrates having small-medium size, even showing higher activity than that of the soluble enzyme when acting on fully soluble substrates [21]. Moreover, these immobilized enzyme preparations are very stable under different experimental conditions [18]. The adsorption of the lipase on the support is quite strong, but it is still reversible, permitting to recover and reuse the support after enzyme inactivation [18].

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Following this immobilization mechanism, the active center of the lipase is in close contact with the support surface, which will generate a hydrophobic environment around the active center. Thus, changes in the support nature (hydrophobicity, internal morphology, etc.) could alter the strength of the lipase adsorption on the support and even the structure of the active center surroundings and, therefore, the final lipase catalytic features. It has been reported that due to the large changes in the lipase conformation during catalysis, it is quite simple to modulate their catalytic properties during immobilization (varying the orientation, rigidity, microenvironment, etc.), greatly altering the enantiospecificity [22–27] or regioselectivity [28,29] of the immobilized enzymes.

Here, the results of a study of the support effects on the properties of lipases (selectivity, specificity, activity, etc.) are presented. In this case, the immobilization of lipases follows the same mechanism: interfacial activation against hydrophobic supports [18]. The lipase active center will be in very close contact with the support surface. However, considering the hydrophilic nature of the lipases out of the active center surroundings, scarce additional influences of the lipase with the support should be expected. The close contact between the support and the active center may produce large differences in the final structure of the enzyme active center and, therefore, on the final enzyme catalytic properties. Thus, the nature of the support that produces that interfacial activation could determine the final properties of the immobilized enzyme.

Moreover, the selectivity in the protein adsorption on the different supports and the adsorption strength of the lipase to the different supports has been analyzed. Two supports with very different internal morphology were used.

Agarose beads are formed by wide trunks [30]. Compared to the lipase size, an almost planar surface will be available to interact with the enzyme. Agarose is very hydrophilic, but if coated with octyl or butyl chains, it offers a hydrophobic surface suitable for the lipase interfacial activation [18]. Toyopearl is an acrylic support formed by a mild crosslink (supplier information), giving quite thin fibers, even smaller than the size of a lipase molecule. The support matrix has a certain hydrophobic character that may be reinforced by coating the fibers surface with butyl or hexyl groups. Previous results suggested that the adsorption of lipases to a hydrophobic support may be driven by reasons more complex that the mere hydrophobicity of the support. Usually, the more hydrophobic the support, the higher the amount of lipases that becomes adsorbed on it [18]. However, in some cases the use of moderately hydrophobic supports permits the adsorption of lipases that did not adsorb on supports with high hydrophobicity, for example pancreas porcine lipase did not adsorb on octadecyl Sepabeads or octyl-agarose but it was strongly adsorbed on phenyl agarose (a support with lower hydrophobicity) [31]. Thus, a more systematic study may help to understand on the reasons for the adsorption of lipases on hydrophobic supports.

In this study, we have used three different lipases: those from *Bacillus thermocatenulatus* (BTL) [14], *Candida antarctica* B (CAL-B) [32,33] and from *Thermomyces lanuginose* (TLL) [34]. Moreover, we have included in this study a commercial phospholipase, Lecitase® Ultra, which is used in degumming processes [35] and that has been described to behave as a lipase in many aspects. This enzyme presents a certain interest in fine chemistry [36,37].

2. Materials and methods

2.1. Materials

Lecitase® Ultra, CAL-B and TLL were obtained from Novozymes (Denmark). Butyl- and octyl-agarose 4BCL was purchased from GE healthcare (Uppsala, Sweden). BTL cloned in *E. coli* was produced as previously described [14]. Butyl- and hexyl-toyopearl were from Tosoh Corporation (Tokyo, Japan). *R*/*S* mandelic acid, Triton X-100, hexadecyltrimethylammonium bromide (CTAB), and *p*-nitrophenyl butyrate (*p*NPB), were from Sigma. 2-*O*-Butyryl-2-phenylacetic acid (**1**) was synthesized as previously described [38].

2.2. Lipase activity determination

The standard assay was performed by measuring the increase in absorbance at 348 nm (isoblastic point of *p*-nitrophenol) produced by the releasing of *p*-nitrophenol in the hydrolysis of 0.4 mM *p*-nitrophenyl butyrate (*p*NPB) in 25 mM sodium phosphate at pH 7 and 25 °C, using a thermostatized spectrophotomer with magnetic stirring. To start the reaction, 0.1 mL of lipase solution or suspension was added to 2.5 mL of substrate solution. An international unit of *p*NPB activity is defined as the amount of enzyme necessary to hydrolyze 1 μmol of *p*NPB/min (IU) under the conditions described above.

2.3. Lipase immobilization

Ten grams of support were added to 100 mL of lipase solution (0.01 mg prot/mL or 1 mg/mL) in 5 mM sodium phosphate pH 7 at 25 °C under very mild stirring. Periodically, samples of supernatant and suspension were withdrawn and the activities were determined as described above. After immobilization, the immobilized enzymes were washed with an excess of distilled water and stored at 4 °C. The preparations with low enzyme loading, where diffusion problems were avoided, were used to determine the activity with *p*NPB and inactivation activity. The preparations with high enzyme loading were used in the hydrolysis of 2-*O*-butyryl-2-phenylacetic acid and phenylglutaric acid diethyl diester.

2.4. Lipase desorption

One gram of immobilized lipase preparation was re-suspended in 10 mL of 5 mM sodium phosphate pH7 and 25 °C and detergent (Triton X-100, except for TLL where CTAB was used due to the inhibition caused by Triton X-100 [39]) was progressively added [18]. The immobilized enzymes were incubated under gentle stirring for 30 min before measuring the enzyme activity in the supernatant. Afterwards, whenever necessary, new additions of detergent were performed. References with soluble enzymes submitted to identical treatment were used to determine the effect of the detergent on the enzyme activity.

2.5. Lipase inactivation experiments

Immobilized enzyme suspensions were incubated at the indicated temperature and at pH 7. Samples were periodically withdrawn and the activity was determined as previously described.

2.6. Synthesis of 3-phenylglutaric acid diethyl diester (2)

A solution of ethanol (70 mmol), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (3.6 mmol) with dimethylamino-pyridine (0.72 mmol) in diethyl ether (10 mL) was added drop-wise over a stirred solution of 3-phenylglutaric acid (1.44 mmol) in diethyl ether (25 mL) The mixture was stirred at 25 °C for 5 h and the reaction was followed by HPLC. After that, the mixture was extracted with 100 mM NaCl solution and after re-extraction of the water phase with diethyl ether. The combined organic solvents were dried with $NaSO_4$ and the solvent was evaporated. The crude extract was washed several times with cold ether (5× 2 mL) and dried in vacuum. Yield: 98%. ^{1}H NMR (400 MHz $CDCl_3$): δ = 7.33–7.20 (m, 5H, Ph); 4.52 (m, 1H, CH), 4.13 (m, 4H, 2× CH_2), 2.54 (m, 4H, 2× CH_2), 1.29 (t, 6H, 2× CH_3).

2.7. Enzymatic hydrolysis of substrates 1 and 2

One gram of immobilized lipase (prepared using 10 mg of protein per g of support) was added to 10 mL of 5 mM (**1**) in 25 mM sodium acetate at pH 5 and 25 °C or 1 mM (**2**) in 25 mM sodium phosphate at pH 7 and 25 °C.

During the reaction, the pH value was maintained constant by automatic titration using a pH-stat Mettler Toledo DL50 graphic. The degree of hydrolysis was confirmed by reverse-phase HPLC (Spectra Physic SP 100 coupled with an UV detector Spectra Physic SP 8450) on a Kromasil C18 (25 cm × 0.4 cm) column supplied by Analisis Vinicos (Spain). At least triplicates of each assay were made. The elution was isocratic with a mobile phase of acetonitrile (35%) and 10 mM ammonium phosphate buffer (65%) at pH 2.95 and a flow rate of 1.5 mL/min. The elution was monitored by recording the absorbance at 225 nm (substrate **1**) and 205 nm (substrate **2**). The enzymatic activity was measured in μmol of substrate hydrolyzed per hour per mg of immobilized protein.

2.8. Determination of enantiomeric excess

At different conversion degrees, the enantiomeric excess (ee) of the acid (in the hydrolysis of **1**) or the formed monoester (in the hydrolysis of **2**) was analyzed by