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His-tagged Horse Liver Alcohol Dehydrogenase: Immobilization and application in the bio-based enantioselective synthesis of (*S*)-arylpropanols

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Dedicated to Prof. Gianfranco Cainelli on the occasion of his 80th birthday.

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ABSTRACT

The novel histidine-tagged Horse Liver Alcohol Dehydrogenase (His-HLADH-EE) was successfully purified and covalently immobilized onto a solid support in a one-step procedure through a metal-directed technique. A full characterization of the immobilized enzyme was carried out. Effects of pH, temperature and organic co-solvents were deeply investigated and they showed a shift in the optimum pH with respect to the free form as well as increased stability to temperature and solvents. The immobilized His-HLADH-EE proved to be effective as catalyst in the reduction of aliphatic and aromatic aldehydes. Application of the free and immobilized His-HLADH-EE to the chemo-enzymatic synthesis of (*S*)-Profenols demonstrated enhanced enantioselectivity and high reusability of the immobilized form. The achievement of a robust and effective immobilization of an alcohol dehydrogenase substantiated the use of biocatalytic reduction in the synthesis of primary alcohols and valuable chiral intermediates especially for pharmaceutical industries.

1. Introduction

Dehydrogenases represent an important class of co-factor dependent redox enzymes that have been successfully employed for the synthesis of chiral alcohols, hydroxy-acids, or aminoacids [1,2]. Alcohol dehydrogenases (ADH) belong to that class and reversibly catalyze the reduction of aldehydes or ketones to the corresponding primary or secondary alcohols, respectively. Despite the dramatic increase in production of optically active compounds integrating biocatalysis, redox enzymes [3–5] are less used for industrial processes than hydrolytic enzymes, such as Lipases for instance [6].

Hurdles in industrial applications of dehydrogenases concern the need for expensive cofactors, low stability and susceptibility to organic solvents, thus limiting their effectiveness on lipophilic substrates poorly soluble in aqueous media. With the introduction of isolated and purified enzymes, recombinant DNA technology and the development of efficient cofactor regeneration techniques [7], some of those hurdles can be overcome. Protein immobilization has been shown as a further step in addressing other important issues that enzymes must face with in order to be used on an industrial scale, namely re-using of the biocatalyst and stability [8,9]. Interestingly, immobilization has also been found to concurrently enhance enzyme properties [10]. Where the enzyme is immobilized within a porous solid (such as a protein aggregate or crystal, an inert porous support) some factors contributing to enzyme inactivation, such as aggregation, adsorption onto hydrophobic surfaces and auto-proteolysis are minimized [11].

While immobilization is widely reported for enzymes such as hydrolases, significantly fewer cases are documented for redox enzymes [12,13]. However, a general strategy applicable to the immobilization of every enzyme is not yet available and the selection of a method strongly depends on the nature of the enzyme and of the carrier of choice [14]. A very reliable procedure has been reported by the group of Guisan which exploits both ionic interaction and covalent immobilization [15–17].

Among the variety of immobilization strategies, methods based on affinity ligands or bio-specific recognitions are quite attractive because the activity of the immobilized biomolecule is generally preserved, the efficiency of the specific immobilization can be high,

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making it possible to work with low biomolecule concentrations, the nonspecific adsorptions are limited, and the stability of the biomolecule is usually improved. Recent examples, specifically on alcohol dehydrogenases, are those reported by Bolivar et al. [13,18] and Rocha-Martin et al. [19].

Horse Liver Alcohol Dehydrogenase (HLADH) is a zinc-containing enzyme, successfully used in biocatalysis [20–22]. Native HLADH is found in two isoforms, E and S, which leads *in vivo* to the formation of a dimeric enzyme of mixed composition (EE, ES and SS) [23]. While the E subunit is specific for ethanol, the S subunit recognizes steroidal substrates. Following our interest in the use of enzymes for the synthesis of bioactive molecules [24–28] we applied HLADH to the enantioselective synthesis of (2*S*)-2-arylpropanols starting from the parent racemic aldehydes *via* the activation of an efficient dynamic kinetic resolution (DKR) process [29–31]. A DKR process combines a selective kinetic resolution and *in situ* fast racemization of the unreacted enantiomer. This approach allows for the conversion of both enantiomers of the racemic substrate into a single enantiomer of the product and overcomes the 50% yield limitation in classical kinetic resolution whenever the racemization rate successfully competes with that of the resolution reaction [32]. In this chemo-enzymatic reaction the commercial HLADH preparation and a recombinant EE-enzyme were used with similar results in enantioselectivities and yields. The products (2*S*)-arylpropanols are useful intermediates in flavour manufacture and were oxidized to (2*S*)-2-arylpropionic acids [33], active ingredients of the Profen class of non-steroidal anti-inflammatory agents (NSAIDs).

Native HLADH has been immobilized in the past onto different supports with various degree of success by co-polymerization [34] adsorption [35] and more recently by covalent linkage [36,37].

Recently, we reported on the production and characterization of His(6)-tagged Horse Liver Alcohol Dehydrogenase (His-HLADH-EE) [38].

Here we describe a method for its immobilization, which took advantage of the increased metal affinity due to the poly-histidine tag. The immobilized His-HLADH-EE (imHis-HLADH-EE) thus obtained showed enhanced properties in terms of stability, pH and temperature profiles, presence of organic co-solvents, and reusability. Finally we report its application in the bio-reduction of aliphatic and aromatic aldehydes; in particular it showed excellent results in the enantioselective reduction of some racemic Profenals yielding, with a complete unprecedented enantioselectivity, enantiopure (*S*)-Profenols such as (*S*)-ibuprofenol, and (*S*)-naproxenol, valuable intermediates in the synthesis of (*S*)-Ibuprofen and (*S*)-Naproxen, benchmark drugs in the class of NSAIDs.

2. Materials and methods

2.1. Materials, strains, vectors and culture conditions

All chemical reagents, unless otherwise stated, were purchased as analytical grade from Sigma–Aldrich or TCI and used without further purification. Aldehydes were commercial or prepared as reported in ref. [31]. Alcohols were commercial or prepared from the corresponding aldehydes by reduction with $NaBH_4$ in methanol. All spectra were consistent with reported data. NAD^+ was purchased from Apollo Scientific Ltd, Stockport, U.K. Staining and de-staining was performed using the Stain/DeStain-Xpress protein detection kit (Enzolve Technologies, Ltd., Ireland). *Escherichia coli* BL21 competent cells were purchased from Novagen (Germany). Transformed *E. coli* strains were generally cultured in Luria–Bertani (LB) agar and in LB broth, both containing ampicillin (100 μg/mL) at 37 °C, shaking at 250 rpm. *E. coli* strains harbouring a pRSETb-EqADH-E vector were cultured in PG (minimal media) agar and in Auto Induction broth, both containing ampicillin (100 μg/mL) at 37 °C, shaking at 250 rpm. Sepabeads EC-EP/S were kindly donated by Resindion SRL (Binasco, Milan, Italy). Morphological investigation was carried out with a Philips XL-20 Scanning Electron Microscope operating at 15 kV coupled with Energy-Dispersive X-ray Spectrometer. Samples were air-dried, then sputter-coated with carbon 60 s prior to examination.

2.2. Expression and purification of His-HLADH-EE

His-HLADH-EE gene inserted in vector pRSETb was expressed and the protein purified as previously reported [38]. Crude protein concentration was determined by Bradford protein assay dye reagent (Bio-Rad Laboratories GmbH, Germany) with bovine serum albumin as a standard. Pure protein concentrations were determined by UV absorption at 280 nm using the absorbitivity reported in the literature (0.441 mL/(mg cm) for His-HLADH-EE) [39].

2.3. Immobilization of His-HLADH-EE

Sepabeads EC-EP/S (a commercially available rigid methacrylic polymer matrix with diameter ranging between 100 to 300 μm, activated with epoxy groups in a ratio of 100 μmol/g of wet resin) were derivatized with iminodiacetic acid (IDA) and $CoCl_2$ following the procedure reported in literature by Guisan, allowing a modification of around 5% of the epoxy-groups in the support [17], 1 g of beads was shaken at room temperature for 2 h in 2 mL of support modification buffer (0.1 M sodium borate, 2 M iminodiacetic acid, pH 8.5). The derivatized resin, rinsed with double distilled water, was then re-suspended in 5 mL of metal containing solution (0.05 M sodium phosphate buffer pH 6.0, 1.0 M NaCl and 5 mg/mL of $CoCl_2$) and shaken at room temperature for 2 h. The resin, rinsed again with double distilled water, was then put in contact with His-HLADH-EE (1 mg of enzyme per 1 g of resin, in storing buffer pH 8.5, room temperature if not otherwise stated) and the mixture was gently shaken at room temperature over 24 h. The resin was then thoroughly washed using a desorption buffer (20 mM Na_2HPO_4-NaH_2PO_4, 50 mM EDTA, 0.5 M NaCl, pH 7.4, as per IMAC purification procedure) [38] and water to achieve complete removal of the cobalt first and of the residual EDTA after. The un-reacted epoxides were neutralized using a blocking buffer (3 M glycine, pH 8.5, 4 mL per g of beads) over 20 hrs at room temperature, with gentle shaking. The immobilized enzyme was thoroughly washed and routinely stored in buffer (Tris–HCl, pH 8.5). To achieve immobilization using different metals the following salts were used: $NiCl_2 \times 6H_2O$, $CuSO_4 \times 5H_2O$ (5 mg/mL, 5 mL for 1 g of beads, allowing a 2 hrs contact with the derivatized beads). The metal solutions were prepared using buffer 50 mM Na_2HPO_4-NaH_2PO_4, pH 6.0. Immobilization has been performed on purified enzyme, if not otherwise stated.

2.4. Activity assays

Spectrophotometric activity measurements were based on the substrate-dependent absorbance change of NADH at 340 nm and routinely done in reaction mixtures (1 mL for the soluble enzyme and 5 mL for the immobilized) at 25 °C, using a Varian Cary 50 Scan UV-visible spectrophotometer equipped with a Cary single cell Peltier temperature controller. For the immobilized enzyme the reaction mixture was shaken at 25 °C, 250 rpm and the absorbance at 340 nm was recorder every minute as single readings. To test the recyclability of the enzyme after each cycle (9 min in total duration) the resin was washed thoroughly with buffer and a new reaction was set up. Unless otherwise stated, the reaction mixture for the oxidative step contained NAD^+ (1 mM), ethanol (4 mM), enzyme sample (appropriate amount) and up to 1 mL of 0.1 M sodium pyrophosphate buffer, pH 8.8. The buffer was equilibrated at 25 °C prior to the assay. One unit of HLADH corresponded to the amount of enzyme required to reduce 1 μmol of NAD^+ per min at 25 °C. For the pH stability test the following buffers were used: 50 mM Na_2HPO_4–NaH_2PO_4 buffer pH 6.5, 50 mM Tris–HCl pH 8.5. To investigate the optimum pH of reaction the following buffers were used: 50 mM Na_2HPO_4–NaH_2PO_4 buffer pH 6.5, 7.5, 50 mM Tris–HCl buffer pH 8.5, 0.1 M pyrophosphate buffer or 50 mM Glycine–NaOH buffer pH 8.8, 50 mM Glycine–NaOH buffer pH 9.5, 10.5, 50 mM Na_2HPO_4–NaOH buffer pH 11.0, 11.5, 50 mM Glycine–NaOH buffer, 50 mM KCl–NaOH buffer pH 12.5.

A pH 9.5 was used to investigate the optimum reaction temperature for the oxidation of EtOH (4 mM) to acetaldehyde and temperatures between 25 °C and 55 °C were tested. The pH values of the buffers were always adjusted at the temperature at which the experiment was carried out. To test the stability at different solvents the enzymes were incubated in 10 (free enzyme) and 20% (free and immobilized enzymes) of solvent (CH_3CN, THF, DMSO and methanol were used) in 50 mM Tris–HCl, pH 8.5 buffer with a total volume of 0.2 mL in all cases. For the immobilized enzyme, 50 mg of resins were used in each sample, while the free enzyme was always tested in a concentration of 0.7 mg/mL. The activity was recorded at time zero and after 24 h in the usual conditions. To test the inactivation upon contact with metals, the free enzyme was incubated at room temperature for 24 h in a 3.5 mM metal solution which is the equivalent ratio of metal to enzyme in the immobilization step. Samples with different metals ($CoCl_2$, $NiCl_2$ and $CuSO_4$) were checked for activity over 24 h and compared to a control sample.

2.5. General procedure for enzymatic reduction of aldehydes

2.5.1. Synthesis

Method A: into a vial stirred on an orbital shaker (140 rpm) at room temperature, all reagents were added in the following order: 0.5 mL of a 5 mM solution of the starting aldehyde in CH_3CN, 0.146 mL of EtOH (0.5 M), 0.5 mL of a 0.1 mM solution of NADH freshly prepared in the appropriate 0.1 M buffer, then 0.1 M buffer to reach a total final volume of 5 mL and the chosen amount of enzyme.