Immobilization of Old Yellow Enzymes via Covalent or Coordination Bonds

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Abstract: Ene-reductases (ERs) belonging to the old yellow enzyme (OYE) family have been thoroughly investigated for the stereospecific reduction of activated prochiral C=C double bonds. In this work, OYE3 was immobilized both by covalent binding on glyoxyl-agarose (OYE3-GA), and by affinity-based adsorption on EziG^{TM} particles (OYE3-EziG). The immobilized OYE3-GA was demonstrated to be active (activity recovery = 52%) and to retain almost 100% of its activity under the enzymatic assay conditions (50 mM phosphate buffer pH 7, 28 °C) for six days, whereas the activity of the non-immobilized enzyme dropped to 50% after two days. In the case of EziG^{TM}, the highest activity recovery (54%) was achieved by using the most hydrophilic carrier (EziG^{TM} Opal) that was selected for the full characterization of this type of enzyme preparation (stability, recycling, re-use, enzyme leakage). OYE3-EziG was slightly less stable than OYE3-GA under the same experimental conditions. OYE3-GA could be recycled and re-used for up to 12 reaction cycles in the bioreduction of α-methyl-*trans*-cinnamaldehyde; after 12 runs, the highest conversion achieved was 40%. In the case of the co-immobilized OYE3/GDH-EziG, the conversion dropped to 56% after two reaction cycles. No enzyme leakage was detected over 48 h for both OYE3-GA and OYE3/GDH-EziG (50 mM phosphate buffer pH 7, 28 °C). These seed results pave the way for a true optimization of the immobilization of OYE3, as well as for the use of immobilized OYE3 for preparative applications both in batch and continuous flow conditions.

Keywords: biocatalysis; enzyme immobilization; old yellow enzymes; glyoxyl-agarose; affinity-based immobilization

1. Introduction

Ene-reductases (ERs) belonging to the old yellow enzyme (OYE) family (EC 1.6.99.1) are flavin mononucleotide (FMN)-containing oxidoreductases, which are able to catalyze the stereoselective reduction of C=C double bonds activated by the presence of a suitable electron-withdrawing group (EWG) as a substituent of the alkene moiety [1,2]. The reaction occurs through a stereospecific anti hydrogen addition to the C=C double bond, according to the following steps [3]. The enzyme-bound flavin (reduced by NAD(P)H cofactor to FMNH$_2$) transfers a hydride to the olefin carbon atom in β position with respect to the EWG. A proton is delivered by a tyrosine side-chain (Tyr196 for OYE1-3) of the enzyme active site to the α-carbon atom (with respect to EWG) on the opposite face of the alkene. For the application of OYE-mediated reductions in synthetic sequences of practical interest, it

is advantageous to promote the in situ regeneration of the reduced nicotinamide cofactor by using a NAD(P)H-dependent glucose dehydrogenase (GDH) with glucose as a sacrificial co-substrate.

The OYE-mediated bioreduction has been widely investigated in the last decade to establish substrate scope and stereoselectivity of these enzymes [4–6], and to study the combination of this reaction with other chemo- and/or biocatalyzed transformations, both in concomitant and subsequent cascade procedures [7,8]. Several works showed the advantages of using OYE-catalyzed hydrogenation to prepare chiral building blocks for the synthesis of APIs (Active Pharmaceutical Ingredients) [9–12], flavors and fragrances [13–15].

The synthetic potential of the OYE-mediated bioreduction can be further expanded by OYE immobilization on solid carriers. If OYEs are converted into stable, readily-recoverable and recyclable heterogeneous catalysts, then their suitability for large-scale applications can be enhanced, in agreement with one of the key green chemistry research areas established by the ACS Green Chemistry Institute® Pharmaceutical Roundtable (catalyst immobilization without significant loss in kinetics) [16].

Studies on the immobilization of oxidoreductases are gradually increasing, but still only a few examples of ER immobilization have been described to date. Recently, a recombinant ER from the OYE family and its cofactor-recycling partner enzyme GDH (commercially available from Amano Enzyme Inc.) were co-immobilized by using two immobilization methods (i.e., cross-linked enzyme aggregates (CLEAs) and a so-called "biomimetic" immobilization (BI)) by entrapment within a network of fused silica particles. Activity recovery of both enzyme preparations were about 45%. Immobilization enhanced the thermal stability (50 °C) of both enzymes and markedly increased their resistance to acidic pH (5-6) in comparison with the non-immobilized enzyme. Moreover, both immobilized enzymes were successfully recycled and re-used for up to 14 reaction cycles. The catalytic "performance" of the CLEA preparation in the reduction of 4-(4-methoxyphenyl)-3-buten-2-one resulted to be superior, both to the non-immobilized enzymes and the entrapped enzymes by BI [17].

In a very recent study, an ER from *Thermus scotoductus* SA-01 (*Ts*OYE) was encapsulated with a light-harvesting dye in an alginate hydrogel for NADH-free, photobiocatalytic asymmetric hydrogenation of 2-methylcyclohexenone. The *Ts*OYE encapsulated in alginate hydrogel exhibited enhanced stability against external stresses (e.g., heat, organic solvents), also on a repeated use. However, the long-term reusability of *Ts*OYE alginate hydrogel was hampered by photobleaching and leakage of the dye used [18].

OYE immobilization is still under-investigated and, to the best of our knowledge, no attempt of immobilization has ever been reported for OYE3, one of the most performing members of this enzyme class. The impressive results obtained in OYE3-catalyzed biotransformations in the past years and the benefits envisaged for ER immobilization urged us to undergo a "seed-study" in this frame.

In this work, OYE3 was immobilized both by covalent binding on glyoxyl-agarose (OYE3-GA), and by metal affinity interaction on EziG™ particles (OYE3-EziG). The GA carrier [19,20], widely explored for the irreversible immobilization of enzymes by multipoint attachment, has proven its efficiency and versatility as immobilization support by affording robust biocatalysts, both for batch and flow applications [21,22]. On the other hand, EziG™ is a novel carrier made of controlled porosity glass (CPG) particles containing chelated Fe(III) for His-tag binding. This carrier can specifically immobilize tagged enzymes from a crude mixture by affinity interaction and is, thus, frequently used to combine immobilization and purification steps [23,24]. In this context, EziG™ was studied to explore a milder immobilization technique based on non-covalent interaction.

2. Results and Discussion

2.1. Enzyme Activity Assays

In order to characterize OYE3, the enzyme activity was assessed spectrophotometrically, as described for other ERs [25–27], by measuring the consumption of NADH at 340 nm in the reduction of cyclohex-2-enone (1) (see Scheme 1 and Materials and Methods, Section 3.3.1). This assay was found to

be suitable for the soluble enzyme, but not for assessing the activity of the immobilized enzyme (and, indeed, to calculate the activity recovery after immobilization) because of the interference of the solid carrier with the spectrophotometric readouts. Therefore, a GC-MS assay (see Scheme 2 and Materials and Methods, Section 3.3.2) based on the reduction of (*R*)-carvone (2) in the presence of stoichiometric NADH was used. Unless otherwise stated, this latter assay was used to determine the activity of both soluble and immobilized OYE3.
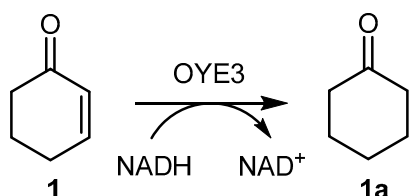

Scheme 1. Reduction of cyclohex-2-enone (1) to cyclohexanone (1a) catalyzed by OYE3 (spectrophotometric activity assay). Experimental conditions: 50 mM phosphate buffer pH 7 (0.5% DMSO), [substrate] = 0.05 mM, [NADH] = 0.2 mM, 28 °C, reaction time = 80 s, volume = 1 mL.
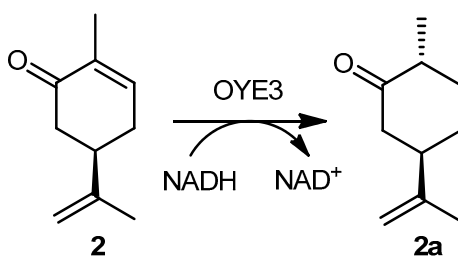
Scheme 2. Reduction of (*R*)-carvone (2) to (2*S*,3*R*)-dihydrocarvone (2a) catalyzed by OYE3 (GC-MS activity assay). Experimental conditions: 50 mM phosphate buffer pH 7 (1% DMSO), [substrate] = 1 mM, [NADH] = 1 mM, 28 °C, reaction time = 30 min, volume = 1 mL.

A spectrophotometric assay was also used to assess the activity of soluble GDH by measuring the formation of NADH over time at 340 nm as a result of glucose oxidation (experimental conditions: 50 mM phosphate buffer pH 7, [substrate] = 20 mM, [NAD$^+$] = 0.2 mM, 28 °C, reaction time = 2 min, volume = 1 mL). Glucose dehydrogenase was coupled to OYE3 for cofactor regeneration in the reduction of α-methyl-*trans*-cinnamaldehyde (3) (see Scheme 3 and Materials and Methods, Section 3.4).
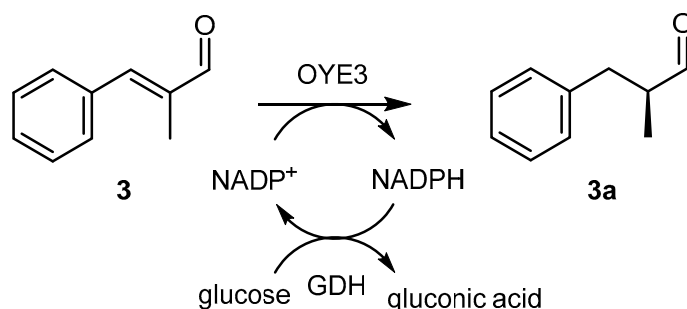

Scheme 3. Reduction of α-methyl-*trans*-cinnamaldehyde (3) to (*S*)-α-methyl-β-phenylpropanal (3a) catalyzed by OYE3. The regeneration of NADP$^+$ was ensured by the GDH glucose system. Experimental conditions: 50 mM phosphate buffer pH 7 (1% DMSO), [substrate] = 5 mM, [NADP$^+$] = 0.1 mM, [glucose] = 20 mM, GDH = 10 μL; 5 mg mL^{-1}, 28 °C, reaction time = 1 h, volume = 1 mL.

2.2. Preliminary Study of OYE3 Stability at Alkaline pH

Covalent immobilization of enzymes on glyoxyl-agarose (GA) relies on the formation of imino bonds between the non-protonated ε-amino groups of enzyme Lys residues and the aldehyde groups

of the carrier [19,20]. This immobilization is required to be carried out at alkaline pH (i.e., pH ≥ 10) and a final chemical reduction step is necessary for the generation of irreversible C-N bonds. Whenever compatible with the enzyme stability, sodium borohydride is used to this aim, since it transforms Schiff's bases into secondary amino bonds and the unreacted aldehyde groups of the carrier into inert hydroxyl moieties. Alternatively, the milder sodium cyanoborohydride or 2-picoline borane can be used for those enzymes which are sensitive to sodium borohydride reduction (but unreacted aldehyde groups of the carrier remain unchanged in this case) [28]. Thus, the first step before immobilization on GA was to assay the stability of OYE3 at alkaline pH. A pH "window" of 8–10 was considered and 3 h was selected as the endpoint (according to the general protocol for this type of immobilization) [19]. Enzyme activity before and after incubation at pH 8–10 (see Section 3.5, Materials and Methods) was assessed spectrophotometrically using the cyclohex-2-enone assay, Scheme 1). Data of the stability assay at pH 8–10 are reported in Figure 1. The residual activity of OYE3 after 3 h at pH 10 was about 70%. This result was considered acceptable for the immobilization on GA.
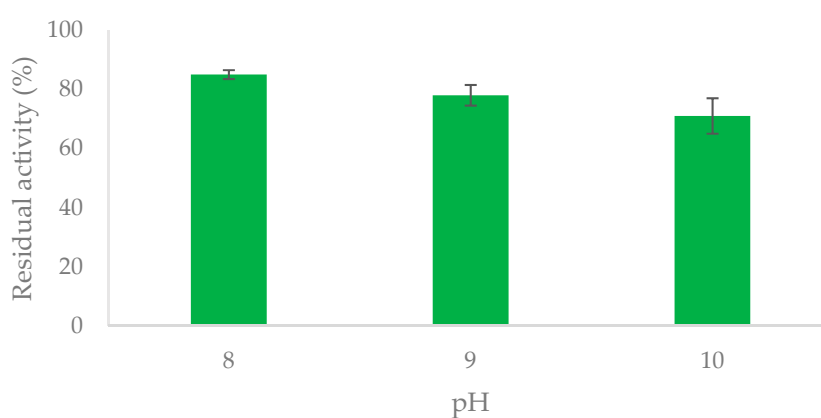
Figure 1. Residual activity (%) of soluble OYE3 upon incubation at pH 8–10 (endpoint—3 h). Experimental conditions: 50 mM phosphate buffer, pH 8; 50 mM Tris-HCl buffer, pH 9; 50 mM NaOH-glycine buffer, pH 10; 20 °C. Enzyme activity was determined as reported in Scheme 1. Residual activity (%) was calculated as reported in Section 3.5 [29].

2.3. Immobilization of OYE3

Immobilization of OYE3 on GA (OYE3-GA) was performed according to a standard protocol by using a 2 mg g^{-1} protein loading [19], as described in Section 3.6 (Materials and Methods). In order to extend the enzyme stability during the immobilization, the reaction was performed at 4 °C. After 3 h, almost all the protein was immobilized (immobilization yield = 95%), as revealed by protein measurements of the supernatant from the immobilization reaction. The activity recovery, after reduction of the immobilized enzyme with NaBH$_4$, was 52% (Table 1).

Table 1. Results of OYE3 immobilization.

Carrier	Enzyme Loading (mg g^{-1})	Immobilization Yield [1] (%)	Activity [2] (U g^{-1})	Activity Recovery [3] (%)
Glyoxyl-agarose	2.0	95	0.023	52
EziG^{TM} Opal	4.2	100	0.037	54
EziG^{TM} Coral	4.2	100	0.015	23
EziG^{TM} Amber	4.2	100	0.014	19

[1] Calculated as reported in Section 3.6 [29]. [2] Determined by the (*R*)-carvone (2) assay (see Scheme 2). [3] Calculated as reported in Section 3.6 [29]. Activity measurements were performed at least in duplicate. Deviation from mean values was below 5%.

For the immobilization by metal-coordination, three EziG^TM resins were tested (namely Opal or EziG 1, Coral or EziG 2, and Amber or EziG 3). According to the indications of the supplier, Opal is a hydrophilic carrier (glass), whereas Coral and Amber are hydrophobic and semi-hydrophilic, respectively. The lower hydrophilicity of Coral and Amber in comparison with Opal is due to the presence of different polymeric coatings on the particle silica surface [30]. Specifically, EziG^TM Coral is made from hybrid CPG with polyvinyl benzyl chloride, whereas EziG^TM Amber is produced from hybrid CPG with a blended co-polymer. Pore size distribution of the carriers ranges from ~500 Å as standard (Opal) to ~300 Å (Coral and Amber, due to the polymer coating). According to the indications of the supplier, a higher protein loading (4.2 mg g^{-1}) was applied to EziG^TM carriers. In all cases, the immobilization was complete. The activity recovery for EziG^TM Opal was 54%, thus resulting comparable to that previously obtained with GA. In the case of the hybrid CPG Coral and Amber, the activity recovery was less than half with respect to EziG^TM Opal. Under the same binding chemistry, it is plausible that this result may be ascribed to the effect of the polymer coating in tuning the hydrophilicity–hydrophobicity balance of the carrier. However, a systematic study on these carriers was not performed at this stage. Due to the positive result achieved with EziG^TM Opal, this carrier was selected for the full characterization of this type of enzyme preparation (stability, recycling, re-use, enzyme leakage).

2.4. Stability of Immobilized OYE3

Increased stability is recognized as one of the most important advantages of immobilized enzymes [31,32]. To verify whether this "general rule" might be applied also to immobilized OYE3 preparations, the stability of both OYE3-GA and OYE3-EziG (Opal) was tested under the experimental conditions used for the reduction of (*R*)-carvone (2) (Scheme 2). The stability assay was carried on for six days (Figure 2). From the stability time course, it clearly emerged that immobilization exerted a stabilizing effect on the soluble enzyme. The OYE3-GA retained almost 100% of its activity, whereas the activity of the non-immobilized enzyme dropped to 50% after two days. On the other hand, OYE3-EziG was slightly less stable than OYE3-GA, but it still retained about 90% of its starting activity after 144 h.
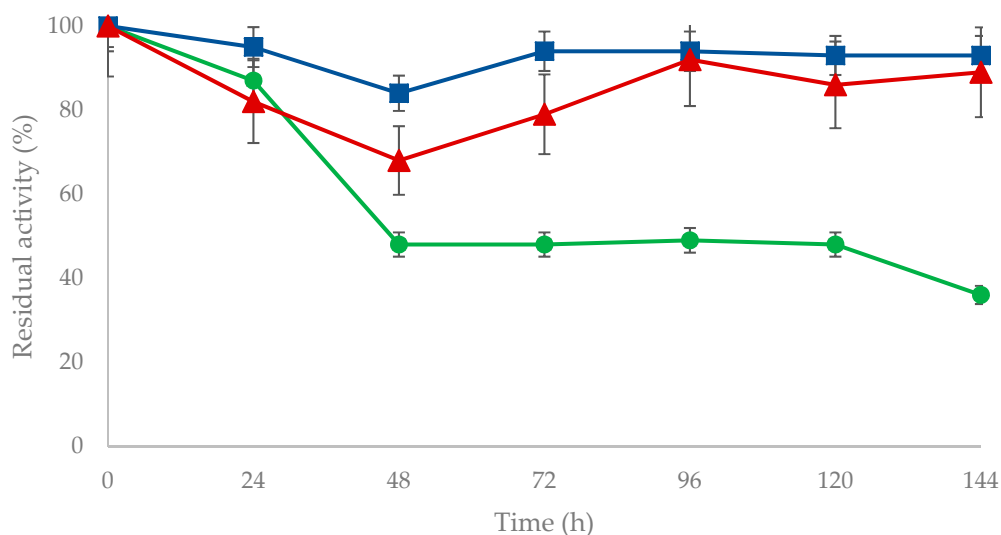

Figure 2. Stability of soluble OYE3 (green circles), OYE3-GA (blue squares) and OYE3-EziG (red triangles). Experimental conditions: 50 mM phosphate buffer pH 7 (1% DMSO), (*R*)-carvone and NADH = 1 mM, 28 °C, volume = 1 mL.

2.5. Recycling of Immobilized OYE3

Biocatalyst recycling and re-use is a further strength point of immobilized enzymes [31–33]. Thus, both OYE3-GA and OYE3-EziG were used in the bioreduction of α-methyl-*trans*-cinnamaldehyde (3, Scheme 3), then recovered and added to a newly-prepared reaction mixture.

A catalytic amount of the cofactor (NADP$^+$) was used since a GDH glucose regeneration system was coupled to the main biotransformation. In the case of OYE3-GA, GDH was added as a soluble enzyme to each reaction cycle. In the case of OYE3-EziG, a co-immobilized preparation OYE3/GDH was used, taking advantage of the His-tag displayed by both enzymes. The enzymes (OYE3 and GDH) were incubated with EziG^{TM}. Activity recovery for OYE3 was 55%. OYE3-GA could be recycled and re-used for up to 12 reaction cycles. The highest conversion achieved after 12 runs was 40% (Figure 3). Moreover, it is worth noting that OYE3-GA retained completely its activity up to the fifth reaction cycle. In the case of OYE3/GDH-EziG, the initial conversion dropped to 56% after two reaction cycles. Interestingly, when the conversion % was almost negligible (reaction 11) and the biotransformation was supplemented with a fresh aliquot of soluble GDH, the reaction afforded a 30% conversion, thus suggesting that the gradual drop of the conversions registered for OYE3/GDH-EziG might depend either on the loss of activity of immobilized GDH or on its leakage from the carrier and/or deactivation, thereof.
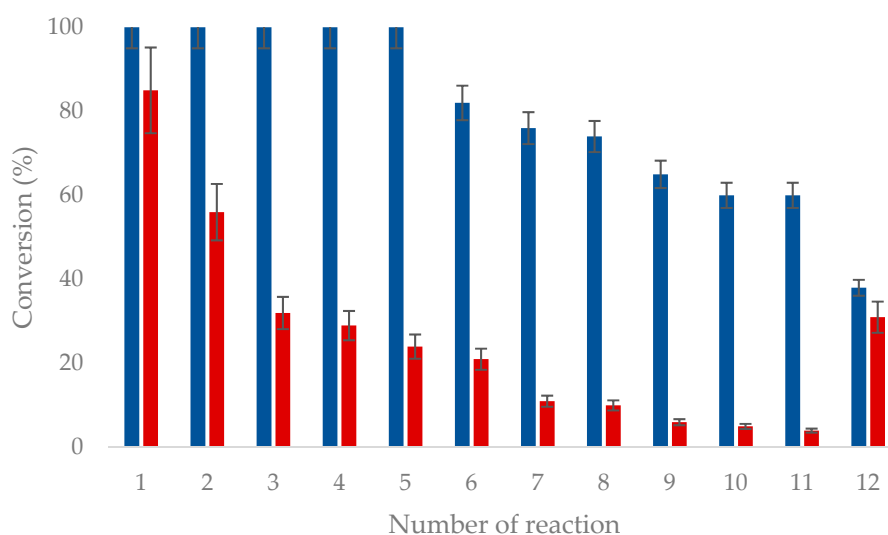
Figure 3. Recycling of immobilized OYE3-GA (blue) and OYE3/GDH-EziG (red). Experimental conditions as reported in Scheme 3.

2.6. Protein Leakage Assay of Immobilized OYE3

Protein leakage from the immobilization carrier is undesirable for several reasons: Loss of activity of the biocatalyst, lack of data reproducibility in the investigated biotransformation, costs and product contamination, to name a few [29]. Covalent immobilization of the enzyme to the carrier generally prevents the protein from leaching from the support surface. In this context, glyoxyl-agarose and other surface-functionalized carriers (i.e., epoxy carriers) are frequently preferred because of the "irreversible" nature of the bonds generated between the enzyme and the carrier. On the other hand, the high affinity and the specificity of the binding interaction based on the His-tag as in EziG^{TM}-type carriers is plausible to generate a strong enzyme-support interaction which might prevent enzyme leakage. However, predictions about how an enzyme will respond to an immobilization process are often hard to make. Enzyme leakage is routinely verified by protein measurements [34] of the supernatant in which the immobilized preparation is incubated under specific conditions dictated by the type of information needed (e.g., reaction or storage conditions). Both OYE3-GA and OYE3/GDH-EziG were, thus, incubated in the reaction buffer (50 mM phosphate buffer pH 7) at 28 °C under stirring for 48 h.

At scheduled times, samples of the incubation buffer were collected and submitted to SDS-PAGE analysis; silver staining, due to its high sensitivity in the low nanogram range, was used to detect protein traces after electrophoretic separation [35]. From the inspection of the SDS-PAGE gel (data not reported), no enzyme traces were detected for either OYE3-GA or OYE3/GDH-EziG. This suggests that the loss of activity of both immobilized enzymes registered during the recycling and re-use study, although to a different extent, cannot be ascribed to protein leakage phenomena.

3. Materials and Methods

3.1. General

Chemicals and solvents were purchased from Merck Sigma-Aldrich® Srl (Milano, Italy) and used without further purification. Agarose gel 6B-CL was purchased from Amersham Biosciences (Uppsala, Sweden) and activated to glyoxyl-agarose (GA), as previously reported [19]. EziG^{TM} carriers were kindly provided by EnginZyme AB (Sweden) as free samples. GC-MS analyses were performed using a HP-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent). The following temperature program was employed: 60 °C (1 min), 6 °C min^{-1}/150 °C (1 min), 12 °C min^{-1}/280 °C (5 min). Bradford assay [33] was performed by using a Shimadzu UV-1601 spectrophotometer and bovine serum albumin (BSA) was used as reference standard protein. All the enzymatic reactions were kept under stirring and controlled temperature by using a thermoshaker New Brunswick™ Excella® E24. Reaction extraction and phase separation were accomplished through a ZX3 Advanced Vortex Mixer from VELP Scientifica® and a Microcentrifuge ScanSpeed Mini (LaboGene™), respectively.

3.2. Enzyme Production

The enzymes OYE3 from *Saccharomyces cerevisiae* and GDH from *Bacillus megaterium* were prepared as His-tagged proteins and purified according to standard methods [36]. Concentration of the purified OYE3 was in the range 2–3 mg mL^{-1}, whereas the concentration of GDH was 3–4 mg mL^{-1}. Activity of OYE3 was measured both by the cyclohex-2-enone assay (see Section 3.3.1 and Scheme 1) and the (*R*)-carvone assay (see Section 3.3.2 and Scheme 2), resulting in 0.09 ± 0.008 U mg^{-1} and 0.13 ± 0.007 U mg^{-1}, respectively. Activity of GDH was measured by a spectrophotometric assay (see Section 3.3.1), resulting in 6.7 ± 0.35 U mg^{-1}.

3.3. Activity Assays

3.3.1. Spectrophotometric Enzymatic Assay

(a) For the OYE activity assay, in a 2 mL cuvette, a solution of cyclohex-2-enone (5 μL; stock solution = 10 mM in DMSO) and a solution of NADH (20 μL; stock solution = 10 mM in water) were added to 50 mM phosphate buffer pH 7 (916 μL). The reaction was carried out at 28 °C and started by adding to the blank solution 59 μL of OYE3 (177 μg, conc. = 3 mg mL^{-1}).

(b) For the GDH activity assay, in a 2 mL cuvette, a solution of glucose (205 μL; stock solution = 1 M in water) and a solution of NAD$^+$ (20 μL; stock solution = 10 mM in water) were added to 50 mM phosphate buffer pH 7 (960 μL). The reaction was carried out at 28 °C and started by adding to the blank solution 2 μL of GDH (6 μg, conc. = 3 mg mL^{-1}).

The enzymatic activity (U mL^{-1}) was calculated from the consumption formation of NADH, for (a) and (b), respectively, at 340 nm ($\varepsilon_{NAD(P)H}$ = 6200 M^{-1} cm^{-1}) according to the following equation:

$$\frac{U}{mL} = \frac{\Delta Abs/min \cdot V_{tot}}{V_E \cdot \varepsilon}$$

ΔAbs/min: slope of Abs vs. time curve (mAU min^{-1}), V_{tot}: total assay volume (mL), V_E: volume of soluble enzyme used in the assay (mL), ε: NAD(P)H molar absorptivity (6200 M^{-1} cm^{-1}).

One unit (U) is defined as the amount of enzyme that converts 1 µmol of substrate in the product in 1 min under specific temperature and pH conditions.

3.3.2. GC-MS Enzymatic Assay

In a 2 mL test-tube, a solution of (*R*)-carvone (10 µL; stock solution = 100 mM in DMSO) and 15 µL of soluble OYE3 (conc. = 3 mg mL^{-1}) or immobilized OYE3 (ca. 20 mg for OYE3-GA or 45 mg for OYE3-EziG) were added to 50 mM phosphate buffer, pH 7 (final volume = 1 mL). The reaction was started by adding NADH (100 µL; stock solution = 10 mM in water) and the mixture was kept at 28 °C under stirring (thermoshaker) for 30 min, then vortexed for 30 s in the presence of CH_2Cl_2 (200 µL). The organic phase was separated by centrifugation (2 min, 13500 rpm), dried over Na_2SO_4 for 5 min, and analyzed by GC-MS (see Section 3.1 for analytical conditions). (*R*)-Carvone: GC-MS (EI) t_R = 12.99 min: mz (%) = 150 (M$^+$, 15), 135 (10), 108 (40), 93 (40), 82 (100). Product: GC-MS (EI) t_R = 11.83 min: mz (%) = 152 (M$^+$, 30), 137 (25), 123 (10), 109 (55), 95 (100). The enzymatic activity (U mL^{-1} for the non-immobilized enzyme, or U g^{-1} for the immobilized enzyme) was calculated from the percentage of substrate conversion after 30 min according to the following equation:

$$\frac{U}{mL \text{ or } g} = \frac{[S] \cdot c}{t_R \cdot E}$$

[S]: substrate concentration (mM), c: conversion (%), t: reaction time (30 min), E: amount of soluble enzyme (mL) or immobilized enzyme (g).

One unit (U) is defined as the amount of enzyme that converts 1 µmol of substrate in the product in 1 min under specific temperature and pH conditions.

3.4. OYE3-Mediated Reduction of α-Methyl-Trans-Cinnamaldehyde (3)

In a 2 mL test tube, a solution of α-methyl-*trans*-cinnamaldehyde (10 µL; stock solution = 500 mM in DMSO) was added to 50 mM phosphate buffer pH 7 (838 µL) followed by a solution of NADP$^+$ (10 µL; stock solution = 10 mM in water), glucose (20 µL; stock solution = 1 M in water), and GDH (10 µL; 5 mg mL^{-1}). The reaction (final volume = 1 mL) was started by adding 160 µg of OYE3 (non-immobilized enzyme = 53 µL; OYE3-GA = 80 mg; OYE3-EziG = 170 mg) and kept under stirring (thermoshaker) at 28 °C for 1 h. At the endpoint, in the case of the immobilized enzyme, the reaction was stopped by removing the biocatalyst by centrifugation (4 min, 13,200 rpm). The reaction mixture was vortexed for 30 s in the presence of CH_2Cl_2 (400 µL). The organic phase was separated by centrifugation (2 min, 13,200 rpm), dried over Na_2SO_4 for 5 min, and analyzed by GC-MS (see Section 3.1 for analytical conditions). α-Methyl-*trans*-cinnamaldehyde = GC-MS (EI) t_R = 14.60 min = mz (%) = 146 (M$^+$, 70), 145 (100), 117 (80), 115 (70), 91 (50). Product: GC-MS (EI) t_R = 11.68 min = mz (%) = 148 (M$^+$, 45), 133 (25), 105 (25), 91 (100). The conversion was evaluated from the ratio of the peak areas of product and substrate.

3.5. Stability of OYE3 in the pH Range 8-10

The stability of non-immobilized OYE3 was assayed in the pH range 8–10 by incubating 100 µL of the enzyme solution (stored in 20 mM phosphate buffer pH 8.5 containing 200 mM imidazole and 500 mM NaCl) in 100 µL of 50 mM phosphate buffer pH 7.5 (final pH = 8), 50 mM Tris-HCl buffer pH 9.5 (final pH = 9), or 50 mM NaOH-glycine buffer pH 11.5 (final pH = 10) at 20 °C. After 3 h, 100 µL were withdrawn from each sample and the enzymatic activity was measured spectrophotometrically in the reduction of cyclohex-2-enone (see Section 3.3.1). The residual activity was calculated according to the following equation:

$$\text{Residual activity } (\%) = \frac{\text{observed activity at the endpoint}}{\text{starting activity}} \times 100$$

3.6. Covalent Immobilization of OYE3 on Glyoxyl-Agarose (OYE3-GA)

Immobilization of OYE3 on glyoxyl-agarose was performed following the protocol previously reported [19]. Briefly, glyoxyl-agarose (1.8 g = 2.52 mL) was suspended in 50 mM Na_2CO_3 buffer (14.3 mL) at pH 10. After the addition of the desired amount of protein (1.2 mL, loading: 2 mg g^{-1}), the suspension was kept under mechanical stirring for 3 h at 4 °C. Chemical reduction of Schiff bases and unreacted aldehyde groups was carried out over 30 min by adding $NaBH_4$ to the mixture (18 mg; 1 mg mL^{-1} of suspension). The immobilized enzyme was then filtered and washed with 10 mM phosphate buffer pH 7 and deionized water.

The immobilization reaction was monitored by measuring the amount of protein in the supernatant before the addition of the carrier and after 1.5 h and 3 h [34]. The immobilization yield (%, endpoint = 3 h) was calculated according to the following equation [29]:

$$\text{Yield } (\%) = \frac{\text{immobilized protein}}{\text{starting protein}} \times 100$$

The activity of the immobilized OYE3 was measured by the GC-MS enzymatic assay reported in Section 3.3.2 (reduction of (*R*)-carvone, 2). The activity recovery (%) was calculated according to the following equation [29]:

$$\text{Activity recovery } (\%) = \frac{\text{observed activity}}{\text{starting activity}} \times 100$$

3.7. Immobilization of OYE3 by Affinity-Based Adsorption (OYE3-EziG)

Immobilization of OYE3 by affinity-based adsorption on EziG^{TM} (Opal, Coral, and Amber) was performed following the protocol of the supplier with minor modifications. EziG^{TM} (100 mg) was added to a 2 mL test tube containing the enzyme (140 μL, loading = 4.2 mg·g^{-1} of dry carrier) diluted in 50 mM phosphate buffer pH 7 (1.5 mL) at r.t. After 30 min, the test tube was centrifuged (5 min, 13,200 rpm) and the supernatant was removed by pipetting. Following supplier's instructions, the wet enzyme preparation was stored as such (when the immobilized enzyme was filtered under vacuum, it showed an almost complete loss of activity. The weight of the wet carrier was estimated to be 4.5-fold higher than the dry carrier. The activity recovery (%) was referred to the wet carrier weight).

The immobilization yield (%, endpoint = 30 min) was calculated as reported above (Section 3.6). The activity of the immobilized OYE3 was measured by the (*R*)-carvone assay (see Section 3.3.2.) and the activity recovery (%) was calculated as described above (Section 3.6).

Co-immobilization of OYE3 and GDH on EziG™ Opal was carried out by following the procedure applied to OYE3 immobilization. Briefly, EziG™ Opal (100 mg) was added to 50 mM phosphate buffer pH 7 (1.5 mL) containing OYE3 (140 μL, U = 0.06) and GDH (45 μL, U = 0.87). Monitoring and work-up of the immobilization mixture was performed as above reported.

3.8. Stability of Non-Immobilized OYE3, OYE3-GA, and OYE3-EziG

For each endpoint, non-immobilized or immobilized OYE3 (42 μg of protein) was incubated in a solution containing 50 mM phosphate buffer pH 7 (890 μL) and (*R*)-carvone (10 μL; stock solution = 100 mM in DMSO) at 28 °C. Each reaction was started by adding NADH (100 μL; stock solution: 10 mM in water). The final volume was 1 mL. The enzyme activity was assessed by the standard activity assay described in the Section 3.3.2 (reduction of (*R*)-carvone). The enzyme activity was compared with the starting activity which was considered as 100%.

3.9. Protein Leakage Assay

For each endpoint (1-3-6-24-48 h), 42 μg of protein (corresponding to 21 mg of OYE3-GA and 45 mg of OYE3/GDH-EziG) were incubated in 50 mM phosphate buffer pH 7 (200 μL). The suspensions

were kept at 28 °C under stirring (thermoshaker). At each endpoint, a sample (15 µL) was withdrawn from the supernatant and analyzed by SDS-PAGE. GDH and OYE3 (450 ng, corresponding to 0.15 µL of each stock solution) were loaded in the gel as reference standards. Precision Plus Protein™ Unstained Protein Standards (10–250 kD) was used as molecular weight proteins size marker. PAGE analysis was performed in a Mini-Protean 3 system (Bio-Rad). SDS-PAGE was carried out on 12% polyacrylamide (running buffer = 50 mM MES, 50 mM Tris base, 0.1 % w/v SDS and 1 mM EDTA). Proteins were stained by means of a silver staining kit (SilverQuest™ kit, Thermofisher) according to supplier's protocol.

3.10. Recycling of Immobilized Enzymes

Recycling of immobilized OYE3-GA and OYE3/GDH-EziG was performed by evaluating the conversion (%) of α-methyl-*trans*-cinnamaldehyde (3) into 3a (see Section 3.4). At the end of the reaction, the immobilized biocatalyst was washed twice with 1 mL of 50 mM phosphate buffer pH 7, separated through centrifugation, and then re-suspended under the conditions above described for the next reaction cycle.

4. Conclusions

Ene-reductases are a powerful tool for the stereoselective reduction of activated C=C double bonds under mild and environmentally friendly conditions. Due to the great interest in these enzymes for preparative applications, the access to stable and reusable heterogeneous biocatalysts is being sought. Immobilization of OYE3 both on the well-known glyoxyl-agarose (GA) by covalent binding and on controlled pore glass EziG™ by affinity-based adsorption was shown to produce active, stable and reusable biocatalysts. Although activity recovery for both enzyme preparations was similar (≈50%), OYE3-GA appeared to be superior about stability and recycling properties. In this latter regard, OYE3-GA could be re-used up to five reaction cycles without any loss of activity. Still, upon a prolonged re-use (up to 12 runs), this immobilized biocatalyst allowed to obtain a 40% conversion in the reduction of α-methyl-*trans*-cinnamaldehyde (3). On the other hand, in the case of the co-immobilized OYE3/GDH-EziG, a drop of conversion was observed after two reaction cycles, presumably due to deactivation of GDH. Interestingly, no evidence of enzyme leaching from both the supports was registered.

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