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One-Step Purification and Immobilization of Recombinant Enzymes Using a Bifunctional Epoxy Support: A Case Study with Phenylalanine Ammonia-Lyase and Transaminase

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Abstract

This work investigates efficient immobilization strategy for recombinant biocatalysts which does not require preliminary enzyme purification, thereby avoiding the costly and time-consuming downstream processes associated with traditional chromatographic isolation. Our approach utilizes a macroporous poly(methyl methacrylate) resin as a solid carrier decorated with mixed functions of both coordinative binding ability (*via* metal ion chelation for affinity capture) and covalent bond-forming ability (*via* epoxide groups for stabilization). This heterofunctional surface enabled the single-operation enrichment and immobilization of two structurally and functionally distinct enzymes: the homotetrameric phenylalanine ammonia-lyase and the dimeric, pyridoxal-5'-phosphate-dependent transaminase. The results demonstrate that a specific surface architecture—characterized by a rigid trisepoxide linker and a defined complexing group-to-epoxy ratio—proved to be optimal for the two diverse histidine-tagged enzymes, governed primarily by the physicochemical properties of the carrier.

Keywords

immobilized metal ion affinity chromatography, selective enzyme immobilization, phenylalanine ammonia-lyase, transaminase

1 Introduction

The growing integration of biotechnology into reaction catalysis is driven by its significant advantages, particularly in pharmaceutical manufacturing. Biocatalysts derived from living organisms offer a safer and often more sustainable alternative to traditional chemical routes, frequently providing superior process economics and enantioselectivity [1].

While yeasts have historically played a fundamental role in food production [2], advances in molecular biology have facilitated the targeted industrial application of microorganisms. This encompasses both whole-cell bioconversions (e.g., citric acid production [3]) and the use of isolated enzymes (e.g., high-fructose corn syrup production [4]). Recombinant DNA technology has further expanded the scope of biocatalysis, enabling the adaptation of enzymes to specific process conditions and the introduction of tags for purification or immobilization.

Whole-cell biocatalysis remains a widespread method due to its ease of handling and the provision of a natural intracellular environment that supports enzyme stability and cofactor regeneration. However, it presents notable drawbacks, including potential side reactions caused by other intracellular enzymes and mass transfer limitations across the cell wall, which can reduce overall activity [5].

To overcome these challenges, the use of isolated enzymes allows for precise control over reaction conditions. Nevertheless, the downstream processing required to purify intracellular enzymes is time-consuming and costly, and recovering the native enzyme from the reaction mixture is often difficult [6].

Enzyme immobilization on solid carriers addresses these issues by facilitating biocatalyst recovery and reuse, increasing operational stability, and often enhancing catalytic efficiency. Furthermore, immobilized enzymes typically exhibit greater resistance to environmental stressors such as pH, temperature, and organic solvents [7]. While entrapment in polymer matrices is a simple and effective

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method to prevent leaching, it suffers from diffusion limitations like those observed in whole-cell systems [8].

Alternatively, enzymes can be immobilized on solid carriers via physical adsorption, ionic binding, affinity interactions, or covalent bonding. A variety of materials—such as porous polymers, nanofibers, nanotubes, and nanoparticles—can serve as carriers, with tunable structural and surface properties [9].

Among these techniques, covalent binding offers distinct advantages: the stable bonds formed between the enzyme and the carrier prevent desorption and can mitigate diffusion limitations [10]. Epoxy-functionalized supports are widely used for this purpose due to their versatility and stability, utilizing carriers ranging from porous polymers [11], silicates [12], magnetic nanoparticles [13], and even nanorods [14] or nanotubes [15].

A major limitation of conventional covalent immobilization is the lack of selectivity, often resulting in low specifity, random orientation or active site blockage. While prior purification of the enzyme can improve specific activity, it is an expensive step for industrial applications. A promising solution is selective enzyme immobilization using heterofunctional supports that contain both groups for covalent binding and a function responsible for enhanced selectivity [16].

Affinity-based methods, such as immobilized metal ion affinity chromatography (IMAC), are particularly effective for selectivity. In this approach, transition metal ions are coordinated to the surface via complexing agents, allowing the selective binding of His-tagged proteins [17].

Such bifunctional surfaces can be prepared by reacting epoxy-carriers with iminodiacetic acid (IDA) [18] or nitrilotriacetic acid (NTA) [19]. A more advanced "one-step" functionalization strategy involves the simultaneous application of the complexing agent (e.g., derivatives of ethylenediaminetetraacetic acid (EDTA), NTA, or DTPA) and di-/tri-epoxy compounds onto amino-functionalized carriers, creating a tunable surface for controlled selective immobilization.

This study is built upon previous work by our research group [20], in which amino-functionalized magnetic nanoparticles (MNPs) were modified with varying ratios of EDTA-dianhydride (EDTADa) and epoxy crosslinkers to immobilize *Petroselinum crispum* phenylalanine ammonia-lyase (*Pc*PAL) [21] a tetrameric enzyme that catalyzes the reversible oxidative deamination of L-phenylalanine (L-Phe), resulting (*E*)-cinnamic acid [22].

Here, our aim was to extend this concept to a commercially available macroporous poly(methyl methacrylate) carrier (Resindion ReliZyme™ HA403). The primary objective was to investigate how the optimal epoxy/complexing agent ratio established for MNPs translates to a different carrier morphology and to further evaluate the applicability of the system to a different enzyme class (Fig. 1). For this purpose, we selected the (*S*)-selective

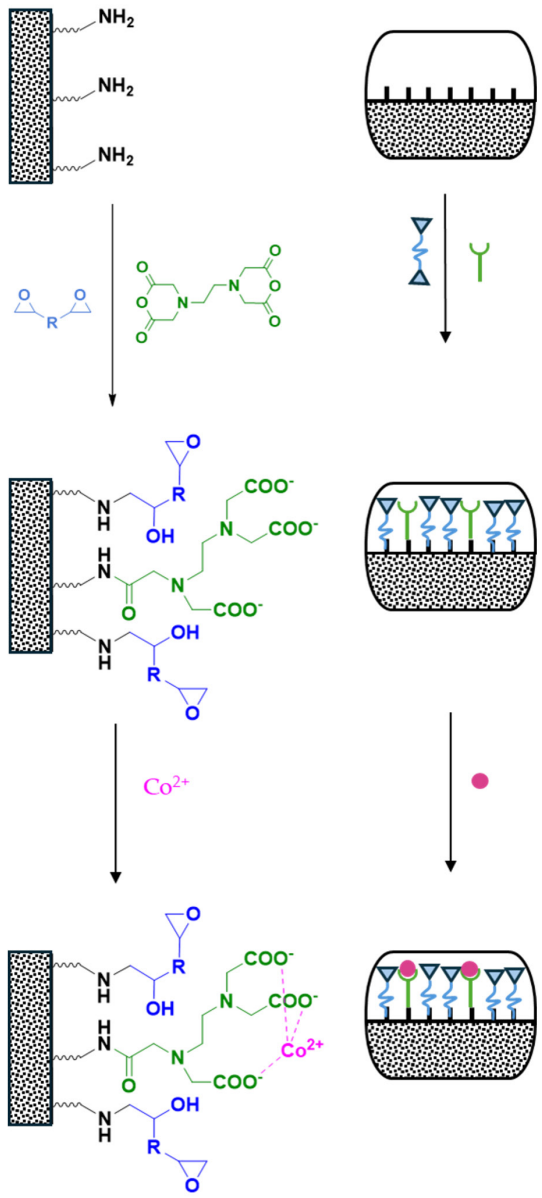


Fig. 1 Preparation of the heterofunctional macroporous support. The amino-functionalized resin is modified in a simultaneous reaction with EDTA-dianhydride (EDTADa; green) and a multiepoxide crosslinker (represented by R; blue), generating both metal-chelating and covalent binding sites. The final step involves the coordination of Co^{2+} ions (pink) to the carboxyl groups of the anchored chelator.

transaminase, the *Vibrio fluvialis* transaminase (*Vf*TA), as a model enzyme [23]. *Vf*TA were applied in the kinetic resolution of racemic 1-phenylethylamine (1-PEA) resulting acetophenone, while Na-pyruvate was used as amino group acceptor. Both enzymes carry a polyhistidine affinity tag (His-tag) and were produced recombinantly in *E. coli*.

Recently, our group reported the selective covalent immobilization of *Pc*PAL on a commercially available epoxy-functionalized resin, where the surface was sequentially modified with diamine linkers followed by the attachment of an EDTADa-derived chelator [24]. In that system, the long linkers provided favorable enzyme orientation, but the structural variation of the applied diamine linkers had no significant impact on the catalytic performance. The present study takes a fundamentally reversed and more streamlined approach. Using an amino-functionalized polymer support, we developed a one-pot surface functionalization strategy where the chelating agent and various multi-epoxide crosslinkers are applied simultaneously. Unlike our previous findings, we demonstrate here that the chemical structure and rigidity of the epoxy crosslinkers have a profound effect on the specific biocatalytic activity. Furthermore, this work validates the universality of the optimized one-pot functionalization method across two structurally and mechanistically distinct enzyme classes.

2 Results and discussion

This study utilized the macroporous, hydrophilic ReliZyme™ HA403 resin as a cost-efficient, commercially available carrier. Due to its hydrophilic surface, this support is ideally suited for enzyme immobilization in aqueous media, while the particle diameter of 100–300 μm renders it applicable in both batch systems and continuous packed-bed reactors.

In our previous study of the metal affinity immobilization of *Pc*PAL [20], the metal ion complexing function was created by reacting to the surface amino groups of the support with EDTA-dianhydride (EDTADa), followed by charging with cobalt(II) ions. In that work using magnetic nanoparticles (MNPs), the optimum in the simultaneous selective complexation and covalent immobilization was achieved at an EDTADa to multiepoxide molar ratio of 1:10 in the amine-modification reaction mixture. Although this ratio provided a robust starting point for the present investigation, the transition from non-porous nanoparticles to the macroporous resin necessitates a re-evaluation of the surface functionalization strategy.

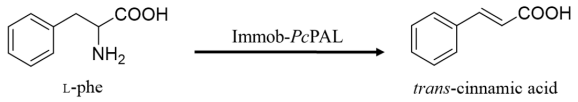
2.1 Immobilization of *Petroselinum crispum* phenylalanine ammonia-lyase on macroporous polymer support

At first, the surface modification was executed in dry DMF using heat-dried glassware to preserve the reactivity of the EDTADa. The amino-functionalized ReliZyme™ beads were treated with different ratios of neopentylglycol diglycidyl ether (NPDGE) and EDTADa for 24 h. Following the cobalt(II) ion charging, the selective immobilization of His-tagged *Pc*PAL was achieved by shaking the supports with the crude cell lysate for 18 h. The specificity and efficacy of the selective covalent binding was confirmed by several consecutive washing steps. First, non-specifically adsorbed proteins were eluted with different buffer solutions. Then an elution step using 500 mM imidazole (in 30 mmol L^{-1} KCl; 50 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5), was applied to disrupt the coordination between metal ion and histidine tagged *Pc*PAL to remove enzyme molecules that were not covalently bound. The immobilization yield was determined from the specific activity measurements of the supernatants after the 18 h immobilization and also the specific activity of the imidazole elution fractions.

The specific biocatalytic activity of the immobilized *Pc*PAL was determined in the ammonia elimination reaction of L-phenylalanine (Scheme 1) in batch reactions.

2.1.1 Optimization of the chelator-to-epoxy ratio with *Petroselinum crispum* phenylalanine ammonia-lyase

Consistent with our previous observations on MNPs, the monofunctional control support demonstrated clear limitations. The support functionalized solely with epoxy groups yielded a negligible specific biocatalytic activity (0.6 ± 0.2 U g^{-1}) which can be attributed to the non-selective, random covalent attachment of contaminating host proteins alongside the target enzyme. The support containing only metal-chelating functions provided a much higher specific biocatalytic activity (4.5 ± 1.2 U g^{-1}) and enzyme content, but the activity was significantly lower than that of the optimized bifunctional support. Consequently, without covalent fixation, a large portion



Scheme 1 The ammonia elimination reaction catalyzed by *Pc*PAL. The reaction was monitored spectrophotometrically to determine the *trans*-cinnamic acid concentration.

of the His-tagged enzymes were eluted. This is also confirmed by the fact that during the immobilization, support was able to complex the entire amount of target enzyme, but only 20% remained on the surface after washing with imidazole solution (data not shown). Because of the carboxylic groups from EDTADa a strong ionic interaction between the surface and the enzyme probably plays a role in this effect.

Most of the supports were able to bind all the target enzyme except for those with the lowest complexing agent content (EC-150 and EC-200) and the support containing only epoxy groups. However, there was a significant difference in the enzyme content of the imidazole elution fractions, and thus in the capacity of the carriers as well.

The specific biocatalytic activity showed a distinct maximum profile (Fig. 2). It increased steadily from the lowest ratios to reach a peak at 1:75 EDTADa:NPDGE ratio (7.5 ± 0.9 U g^{-1}). The capacity also showed a similar trend with decreasing amount of EDTADa.

This means a significant shift in the ideal ratio compared to MNPs supports in which case a higher EDTADa content (1:10 ratio) was optimal during the surface modification [20]. The results clearly show that the morphological characteristics greatly influence the used reagent ratios.

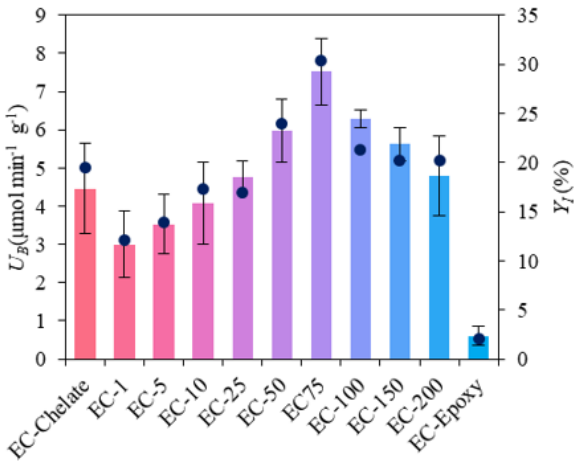


Fig. 2 Optimization of the surface functionalization for *Pc*PAL immobilization. The specific biocatalytic activity (U_B, colored bars, left axis) and protein immobilization yield (Y_I, dark blue dots, right axis) are presented as a function of the chelator-to-epoxy ratio. The notation EC-X refers to an EDTADa:NPDGE molar ratio of 1:X during the surface modification step. EC-Chelate and EC-Epoxy represent the monofunctional control supports. Data represents the mean ± standard deviation of three independent immobilization experiments.

2.1.2 Effect of the multiepoxy crosslinker structure on biocatalytic activity with *Petroselinum crispum* phenylalanine ammonia-lyase

Following the optimization of the metal complex-to-epoxy ratio, we investigated the influence of the chemical structure of the covalent linker on biocatalytic activity. While various pre-activated epoxy supports are commercially available, the post-modification of amino-functionalized carriers offers a distinct advantage: it enables the screening of a diverse library of multiepoxide agents, thereby allowing for the precise customization of the surface properties.

In our experiment, the polymer support was functionalized with ten different multiepoxide using the optimized 1:75 (EDTADa:multiepoxide) stoichiometry, and the resulting heterofunctional supports were applied for the selective immobilization of *Pc*PAL (Fig. 3).

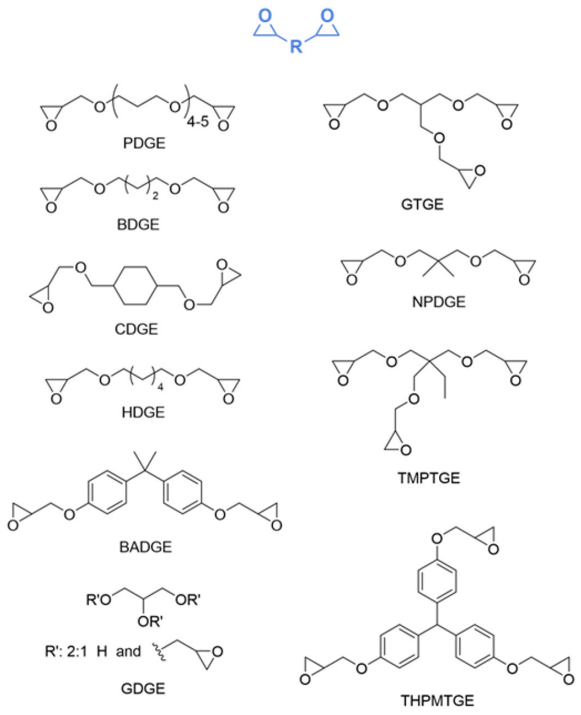


Fig. 3 Chemical structures of the screened bis- and tris-epoxide crosslinkers. The library includes flexible linear chains (polyoxypropyleneglycol diglycidyl ether (PDGE), 1,4-butanediol diglycidyl ether (BDGE), 1,6-hexanediol diglycidyl ether (HDGE), NPDGE, glycerol diglycidyl ether (GDGE)), rigid cyclic or aromatic scaffolds (1,4-cyclohexanedimethanol diglycidyl (CDGE), bisphenol A diglycidyl ether (BADGE), tris(4-hydroxyphenyl)methane triglycidyl ether (THPMTGE)), and trifunctional agents (glycerol triglycidyl ether (GTGE), trimethylolpropan triglycidyl (TMPTGE)) used to modulate the surface properties.

The results obtained with the different linkers reveal a strong correlation between the rigidity and functionality of the crosslinker and the measured biocatalytic activity (Fig. 4). The highest activity value (14.8 ± 0.9 U g^{-1}) was achieved with the bulky, aromatic trisepoxide, THPMTGE. This represents a significant improvement over the flexible, linear bisepoxides, such as PDGE (7.8 ± 0.3 U g^{-1}) or NPDGE (8.4 ± 0.5 U g^{-1}).

Notably, the observed trends are consistent with those reported for the MNP-based system, confirming that the preference for epoxy compounds slightly depends on the quality and properties of the support [20]. Trisepoxides (THPMTGE, GTGE, TMPTGE) consistently outperformed the bisepoxides. The presence of a third reactive group likely facilitates more effective multipoint covalent attachment, stabilizing the enzyme, preventing it from leaching and increasing capacity as well. Linkers containing coplanar aromatic rings (THPMTGE, BADGE) or short, rigid structures (GDGE, GTGE) resulted in higher activities compared to those with long, flexible aliphatic chains or saturated ring (e.g. PDGE, HDGE).

Although structural preferences were conserved, the magnitude of the effect differs between the two carriers (MNPs and polymer), the difference in the specific biocatalytic activity between the THPMTGE modified support and the others was much smaller in case of polymer beads. This suggests that the extra stabilizing effect and higher capacity provided by the trisepoxy is more effective on the non-porous nanoparticle surface.

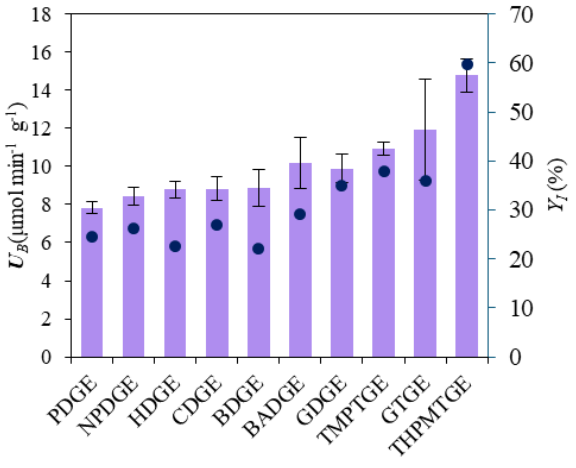


Fig. 4 Effect of the multiepoxide crosslinker structure on the specific biocatalytic activity of immobilized *Pc*PAL. The specific biocatalytic activity (U_B, purple bars, left axis) and protein binding yield (Y_I, dark blue dots, right axis) are compared across a library of bis- and tris-epoxide agents. All supports were prepared using the optimized 1:75 (EDTADa:multiepoxide) molar ratio. Data represents the mean ± standard deviation of three independent immobilization experiments.

2.1.3 Operational stability of the immobilized *Petroselinum crispum* phenylalanine ammonia-lyase

To evaluate the operational stability and industrial potential of the developed biocatalysts, reusability studies were conducted with the immobilized *Pc*PAL preparations. The relative activity of the optimized bifunctional biocatalyst (EC-75) was compared to the monofunctional control supports (EC-Chelate and EC-Epoxy) over five consecutive batch reaction cycles in the ammonia elimination of L-phenylalanine (Fig. 5). The specific biocatalytic activity measured in the first cycle was defined as 100% for each respective support. Between the cycles, the macroscopic polymer resins were easily separated by simple filtration and washed. The results clearly demonstrate the stabilizing effect of the covalent attachment. Both the bifunctional EC-75 and the purely covalent EC-Epoxy supports maintained their full initial catalytic performance (fluctuating around 100%) across all five cycles. In contrast, the biocatalyst based solely on metal-affinity coordination (EC-Chelate) showed a noticeable decrease in relative activity, dropping to approximately 85% by the fifth cycle.

This activity loss started from the fourth cycle is likely due to the progressive leaching of the non-covalently bound enzyme during the repeated washing steps. Considering that the absolute specific biocatalytic activity of the EC-Epoxy support is negligible, the bifunctional

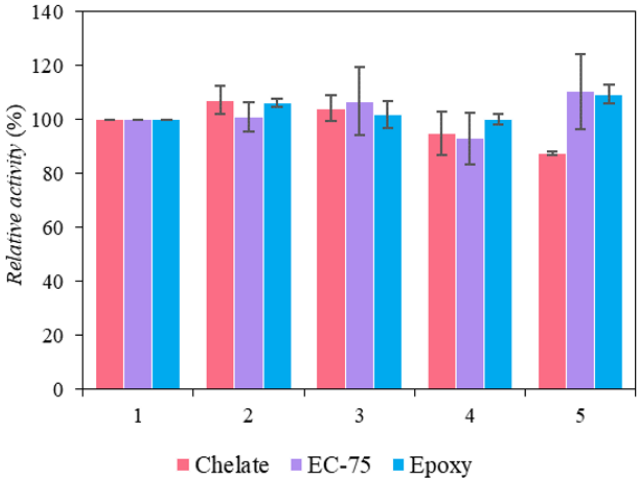


Fig. 5 Reusability of the immobilized *Pc*PAL biocatalysts over five consecutive reaction cycles. The relative activity of the optimized bifunctional support (EC-75, purple) is compared to the monofunctional controls (Chelate, pink; Epoxy, blue). The activity in the first cycle was set to 100% for all preparations. Data represents the mean ± standard deviation of three independent measurements.

carrier (EC-75) provides the optimal solution, successfully combining high initial catalytic efficiency with excellent operational stability.

2.2 Extension and validation of the method with *Vibrio fluvialis* transaminase

To assess the versatility and robustness of the developed immobilization strategy, the optimized protocol was extended to a catalytically and structurally different enzyme class. For this purpose, the (*S*)-selective transaminase *Vf*TA was selected as a model enzyme.

Unlike the homotetrameric *Pc*PAL (~300 kDa) discussed in the previous section, which utilizes the electrophilic MIO prosthetic group, *Vf*TA is a smaller homodimeric protein (~100 kDa) that strictly depends on the pyridoxal-5'-phosphate (PLP) cofactor (Scheme 2).

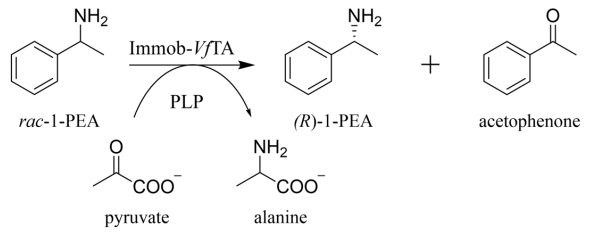
Since the transaminase was applied in the kinetic resolution of racemic 1-phenylethylamine, evaluating the enantiomeric purity of the remaining substrate is of high importance. Therefore, the enantiomeric excess (*ee*) of the unreacted (*R*)-1-phenylethylamine was determined via chiral gaschromatography to confirm the stereoselectivity of the immobilized enzyme. The analysis revealed that the strict (*S*)-selectivity of *Vf*TA was perfectly retained after covalent immobilization, yielding an excellent ee of >98% for the remaining (*R*)-enantiomer after 2 h of reaction at an almost 50% conversion rate.

2.2.1 Optimization of the chelator-to-epoxy ratio with *Vibrio fluvialis* transaminase

To determine if the optimal parameters established for *Pc*PAL are applicable to this cofactor-dependent enzyme, a series of supports with varying EDTADa-to-NPDGE ratios (from 1:25 to 1:150) were prepared and tested with *Vf*TA. The specific biocatalytic activity profile indicates a high degree of consistency with our previous findings (Fig. 6). The specific biocatalytic activity reached its maximum in the 1:50 to 1:75 ratio range, peaking at 19.0 ± 1.1 U g^{-1} for the 1:50 composition. However, considering the standard deviations, the catalytic performance of the 1:50 and 1:75 (18.2 ± 112 U g^{-1}) supports these are statistically comparable.

The experiments confirmed the necessity of the bifunctional surface for *Vf*TA. The support functionalized solely with epoxy groups (EC-Epoxy) yielded the lowest specific biocatalytic activity (7.7 ± 0.3 U g^{-1}). The difference between the non-selective and the best bifunctional support was lower than in case of *Pc*PAL which can be explained by the better expression of *Vf*TA. However, the specific biocatalytic activity achieved with the selective carrier was still almost three times higher, confirming that without the specific metal-affinity coordination, the random covalent immobilization is inefficient. Supports with only metal complexing groups (EC-Chelate) provided a respectable specific biocatalytic activity of 14.9 ± 1.4 U g^{-1}, however, the bifunctional supports (EC-50 and EC-75) significantly outperformed them in this case also.

Comparing these results with our previous model enzyme reveals similarity in the optimal surface. Regardless of fundamental differences between the two



Scheme 2 Kinetic resolution of *rac*-1-phenylethylamine catalyzed by *Vf*TA. The (*S*)-selective ω-transaminase converts the (*S*)-enantiomer into acetophenone using pyruvate as the amino acceptor, leaving the unreacted (*R*)-1-phenylethylamine. The reaction depends strictly on the presence of the PLP cofactor.

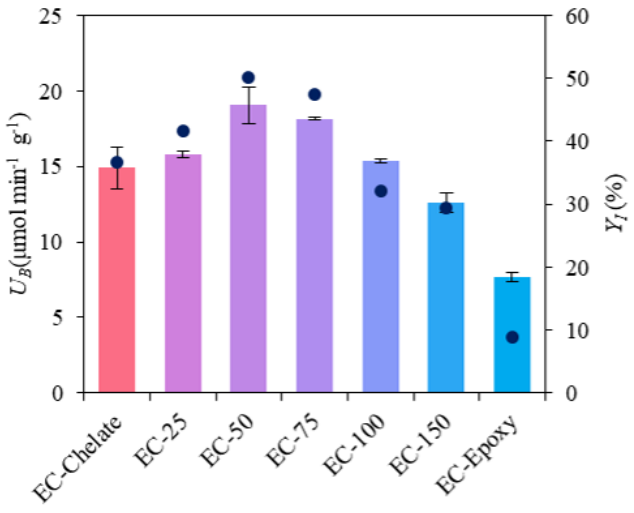


Fig. 6 Optimization of the surface functionalization for the immobilization of *Vf*TA. The specific biocatalytic activity (U_B, purple/blue bars, left axis) and protein binding yield (Y_I, dark blue dots, right axis) are displayed for varying EDTADa-to-epoxy ratios. The notation EC-X corresponds to a 1:X molar ratio during the grafting process. The optimal range was identified between 1:50 and 1:75, demonstrating the robustness of the method for different enzymes. Data represents the mean ± standard deviation of three independent immobilization experiments.

enzymes like size, structure, and catalytic mechanism, each enzyme has the highest specific biocatalytic activity in the same ratio range (1:75 for *Pc*PAL and 1:50–1:75 for *Vf*TA). This strongly suggests that the optimal selective coordination and covalent bonding stoichiometry is governed primarily by the physicochemical properties of the carrier—such as pore size, specific surface area—rather than the structure and properties of the enzyme. Consequently, the 1:50–1:100 ratio range can be considered a robust, starting point for the immobilization of other His-tagged enzymes.

2.2.2 Effect of the multiepoxy crosslinker structure on biocatalytic activity with *Vibrio fluvialis* transaminase

Following the optimization of the ligand composition, the influence of the crosslinker's chemical structure was investigated to maximize the stability and activity of the immobilized transaminase. Using the optimized 1:50 stoichiometry, the earlier mentioned ten multiepoxide agents were screened.

The results reveal a striking structure-activity relationship that parallels what was found in case of *Pc*PAL (Fig. 7). The bulky, aromatic trisepoxide, THPMTGE, yielded the highest specific biocatalytic activity (25.5 ± 0.1 U g^{-1}), significantly outperforming all other agents. This was followed by the rigid or trifunctional linkers (GTGE, TMPTGE, and BADGE). In contrast, the linear, flexible bisepoxides (such as BDGE, HDGE, and NPDGE) resulted in noticeably lower catalytic performance, ranging below 20 U g^{-1}. However, in the case of *Vf*TA there was no significant difference in the capacity of the different carriers, meaning that the differences in activity were mostly due to how efficiently the enzyme could function after bonded covalently via the different epoxides.

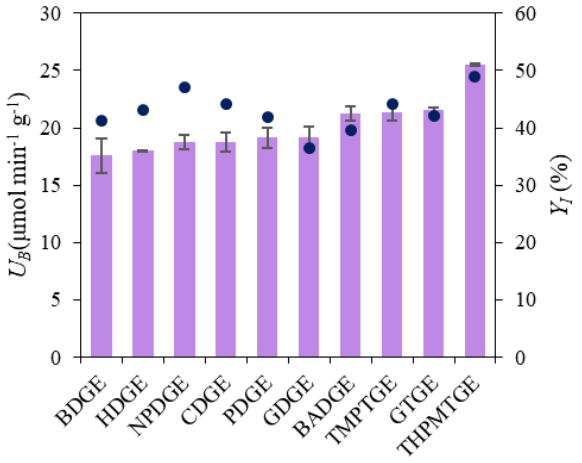


Fig. 7 Impact of the crosslinker structure on the biocatalytic performance of immobilized *Vf*TA. The specific biocatalytic activity (U_B, purple bars, left axis) and protein binding yield (Y_I, dark blue dots, right axis) are presented for the library of multiepoxide agents using the optimized 1:50 (EDTADa:multiepoxide) stoichiometry. The rigid, aromatic THPMTGE provided the highest stabilization for the dimeric enzyme. Data represents the mean ± standard deviation of three independent immobilization experiments.

Based on the results, even in the case of structurally distinct enzymes, the trifunctional epoxy compounds (THPMTGE, TMPTGE, GTGE) and epoxy compound with a more rigid structure (THPMTGE, BADGE) were more effective than crosslinkers with long, flexible aliphatic chains. It suggests that rigidity is a critical factor in the stable multipoint covalent immobilization. Furthermore, the generally better performance of trisepoxides (THPMTGE, TMPTGE, GTGE) over their bisepoxide counterparts (BADGE, NPDGE, GDGE) indicates that the presence of a third reactive group increases the probability of successful covalent binding before the enzyme can desorb.

3 Experimental

3.1 Chemicals and biological reagents

EDTA (99.5%), TRIS [tris(hydroxymethyl)aminomethane] (99.8%), potassium chloride (99%), cobalt(II) acetate tetrahydrate (98%), glycerol diglycidyl ether (GDGE), Bisphenol A diglycidyl ether (BADGE) and tris(4-hydroxyphenyl)methane triglycidyl ether (THPMTGE) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). HEPES (99%) and L-phenylalanine (99%) were purchased from Alfa Aesar Europe (Karlsruhe, Germany).

Acetic anhydride and all solvents were purchased from Merck KGaA (Darmstadt, Germany) and used without further purification except for dimethylformamide which was dried over 4 Å molecular sieve. Patosolv (~85–90% EtOH, 10–15% 2-propanol) was obtained from Molar Chemicals Ltd. (Budapest, Hungary).

Bisepoxides BDGE, NPDGE, HDGE, CHDGE, PEDGE, TMPTGE and GTGE were the products of Ipox Chemicals Ltd. (Budapest, Hungary).

Hexylamine-functionalized methacrylic polymer resin (ReliZyme™ HA 403/S) with a 150–300 μm particle size, and 400–600 Å pore size was product of Resindion S.r.l. (Rome, Italy).

The UV absorbance and kinetic measurements were carried out with a Thermo Scientific Multiscan SkyHigh microplate reader and Greiner UV-Star® 96 well-plate in 300 μL reaction volume.

For the drying processes VDL 23 vacuum drying chamber (Binder GmbH, Tuttlingen, Germany) was used.

3.2 Expression of recombinant enzymes

Production of *Pc*PAL was achieved in *E. coli* Rosetta containing the recombinant pET19b plasmid with the gene of *Pc*PAL and 10 Histidine. LB-CarCA medium (5 mL; LB medium containing carbenicillin [50 mg L^{-1}], and chloramphenicol [30 mg L^{-1}]) was inoculated with one fresh colony from an overnight LB-CarCA agar plate and cells were grown overnight in shake flask (37 °C, at 200 rpm). Autoinduction medium (0.5 L: Na_2HPO_4, 6 g L^{-1}; KH_2PO_4, 3 g L^{-1}; tryptone, 20 g L^{-1}; yeast extract, 5 g L^{-1}; NaCl, 5 g L^{-1}; glycerol, 7.56 g L^{-1}; glucose, 0.5 g L^{-1}; lactose, 2 g L^{-1}) in a 2 L flask was inoculated with seed culture (2 mL) and was shaken for 16 h at 25 °C, 200 rpm. Production of *Vf*TA was achieved in *E. coli* BL21 containing the recombinant pASK-IBA35+ plasmid with the gene of *Vf*TA and 6 Histidine. LB-Car medium (5 mL; LB medium containing carbenicillin [50 mg L^{-1}]) was inoculated with one fresh colony from an overnight LB-Car agar plate and cells were grown overnight in shake flask (37 °C, at 200 rpm). LB medium (0.5 L: tryptone, 5 g L^{-1}; yeast extract, 5 g L^{-1}; NaCl, 2.5 g L^{-1}) in a 2 L flask was inoculated with seed culture (2 mL) and was shaken for 4 h at 25 °C, 200 rpm, until the culture reaches an optical density (OD600) of 0.8. When it reached the required optical density IPTG was added (40 µL; 1,25 mg L^{-1}) and was shaken for 12 h at 25 °C, 200 rpm. After that, for both enzyme production, the cells were harvested by centrifugation (16,000×g, 4 °C, 20 min), then suspended with 40 mL protease inhibitor containing lysis buffer (50 mM tris(hydroxymethyl)aminomethane, 150 mM NaCl, 0.5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 mM tris(2-carboxyethyl)phosphine, 1 mM benzamidine). The cell distruption were done using sonicator (Bandelin, Sonoplus GM4200), then the media were centrifugated (20,000×g, 4 °C, 20 min) resulting the final recombinant *Pc*PAL and the recombinant *Vf*TA containing cell lysate.

3.3 Preparation of ethylenediaminetetraacetic dianhydride

The preparation of EDTADa was carried out as described previously by Sánta-Bell et al. [20].

3.4 Surface modifications of the hexylamine-functionalized polymer resins

Before further use the original water content (~65%) of the macroporous hexylamine-functionalized resins (50 g) was removed via washing with Patosolv (2 × 100 mL) and hexane (100 mL) in a glass filter. After the final filtration, the resins were dried at room temperature in a vacuum drying chamber (Binder GmbH, Tuttlingen, Germany) until the vacuum level dropped below 10 mbar, resulting in 17.4 g of dry resins.

In a dried 20 mL screw cap vial dried resins (0.050 g), variable amounts of a multiepoxide solution (100 µmol mL^{-1} in DMF), EDTADa solution (40 µmol mL^{-1} in DMF) and *N*-ethyl-*N*,*N*-diisopropylamine (DiPEA) solution (60 µmol mL^{-1} in DMF, 2 equivalents to the EDTADa) were added and the total reaction volume was filled up to 8750 µL by addition of the required amount of DMF.

The total amounts of EDTADa and the multiepoxide were two equivalents related to the amino group content of the support (87.5 µmol) but in different molar ratios (EDTADa-multiepoxide: 1-0; 1-1; 1-5; 1-10; 1-25; 1-50; 1-75; 1-100; 1-150; 1-200; 0-1). The reaction mixture was shaken at 450 rpm for 24 h at 60 °C, then 50 µL distilled water was added to the mixture which was shaken for further 1 h at 60 °C. Than the surface-treated polymer support was filtered and washed with acetonitrile (2 × 5.0 mL), 2-propanol (5.0 mL) and distilled water (1 × 5.0 mL). Then without drying the supports the chelator-functionalized resins were charged with cobalt(II) ions. In 20 mL screw cap glass vials 5.0 mL of cobalt(II) acetate solution (in distilled water, 50 mM) was added to the bifunctional chelate-epoxy supports and the mixtures were shaken for 30 min. After the resins were filtrated and washed with distilled water (3 × 5.0 mL) and 2-propanol (5.0 mL). The previously white resins turned pink after complex formation.

The resins were dried in a VDL 23 vacuum drying chamber (Binder GmbH, Tuttlingen, Germany) until the vacuum level dropped below 10 mbar.

3.5 Immobilization of histidine-tagged enzymes

The lysate of *E. coli* cells (1.5 mL in lysis buffer: 50 mmol L^{-1}; TRIS 150 mmol L^{-1}, pH 7.5) was added to the metal ion-complexed resin (5 mg). The suspension was shaken at 450 rpm for 16 h at room temperature to complex and covalently bind the His-tagged enzyme. After the overnight immobilization the nonspecifically adsorbed host proteins were eluted with sequential washing steps of the following solutions (1 mL each): low salt buffer (30 mmol L^{-1} KCl; 50 mmol L^{-1} HEPES, pH 7.5); high salt buffer (300 mmol L^{-1} KCl, 50 mmol L^{-1} HEPES, pH 7.5). Next, the non-covalently bonded His-tagged proteins were removed from the cobalt(II)-charged MNPs by washing with an imidazole solution (1 mL, 500 mM in low salt buffer). Finally, the immobilized biocatalysts were washed

with TRIS buffer (3 × 1 mL, 100 mM, pH 8.8) in case of *Pc*PAL enzyme and HEPES buffer (3 × 1 mL, 50 mM, pH 7.5) in case of transaminase enzyme. The immobilized biocatalysts were tested directly in the proper test reaction.

3.6 Activity of the immobilized *Petroselinum crispum* phenylalanine ammonia-lyase biocatalysts in the ammonia elimination of L-phenylalanine

In 4 mL screw cap glass vial L-phenylalanine solution (2 mL, 10 mM L-PHE in 100 mM TRIS, pH 8.8) was added to the immobilized *Pc*PAL biocatalyst (5 mg) and the resulted suspension was shaken at 450 rpm, 30 °C. After 30 min, 60 min and 120 min samples (20 µL, each) were taken and diluted to 300 µL with distillated water and the absorbance of the samples was measured at 290 nm.

Conversion of L-phenylalanine to *trans*-cinnamic acid was determined from the measured concentration of cinnamic acid at 290 nm by using the Lambert-Beer equation.

3.7 Activity of the immobilized *Vibrio fluvialis* transaminase biocatalysts in the kinetic resolution of 1-phenylethylamine

In 4 mL screw cap glass vial racemic 1-phenylethylamine solution (2 mL, 20 mM *rac*-1-PEA, 0.2 mM PLP, 12 mM Na-pyruvate in 50 mM HEPES, pH 7.5) was added to the immobilized *Vf*TA biocatalyst (5 mg) and the resulted suspension was shaken at 450 rpm, 30 °C. After 30 min, 60 min and 120 min samples (20 µL, each) were taken and diluted to 1980 µL with distillated water and the absorbance of the samples was measured at 245 nm.

Conversion of 1-phenylethylamine to acetophenone was determined from the measured concentration of acetophenone at 245 nm by using the Lambert-Beer equation.

3.8 Characterization of the productivity and immobilization yield

To characterize the productivity of the different biocatalysts, the specific biocatalytic activity was calculated using Eq. (1):

$$U_B = n_P / (t \times m_B) \tag{1}$$

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 applied biocatalyst. To determine the ratio of immobilized target enzyme the activity of the crude cell lysate was measured before and after the immobilization process as well as the imidazole elution fractions during the washing steps. The immobilization yield was calculated using Eq. (2):

$$Y_I = \left(U_{lys} - U_{res} - U_{im}\right) / U_{lys} \times 100 \tag{2}$$

where U is always $U = n_P/t$ specific enzyme activity, U_{lys}: the activity of the lysate before the immobilization; U_{res}: the activity of the lysate after the immobilization; U_{im}: the activity of the imidazole elution fraction).

All immobilization experiments and subsequent activity measurements were performed in triplicate, and the results are presented as the mean ± standard deviation.

3.9 Reusability of the immobilized *Petroselinum crispum* phenylalanine ammonia-lyase

The operational stability of the immobilized *Pc*PAL biocatalysts (EC-75, EC-Chelate, and EC-Epoxy) was investigated in repeated batch reactions. After each 1-h reaction cycle (as described in Section 3.6), the polymer resin was separated from the reaction mixture, washed thoroughly with TRIS buffer (3 × 1 mL, 100 mM, pH 8.8), and resuspended in a fresh substrate solution (2 mL, 10 mM l-PHE in 100 mM TRIS, pH 8.8) to initiate the next cycle. This procedure was repeated for five consecutive cycles, and the specific biocatalytic activity was determined for each step.

3.10 Analytical methods for the determination of enantiomeric excess

The reaction mixtures from the kinetic resolution were analyzed on an Agilent 4890 GC instrument equipped with a flame ionization detector (FID) and a chiral Hydrodex *β*-6 TBDM column (Macherey-Nagel; 25 m × 0.25 mm, 0.25 µm film thickness of heptakis-(2,3-di-O-methyl-6-O-t-butyldimethylsilyl)-*β*-cyclodextrin phase). Operating conditions were as follows: FID (250 °C), injector (250 °C), carrier gas H_2 (head pressure 12 psi, split ratio 1:50). The oven temperature program was: 130 °C for 60 min, increased to 140 °C at 2 °C/min, then increased to 180 °C at 10 °C/min, and finally held at 180 °C for 3 min. The enantiomeric excess (*ee*) of the unreacted (*R*)-1-phenylethylamine was calculated from the peak areas determined by chiral gas chromatography using Eq. (3):

$$ee = \left(A_R - A_S\right) / \left(A_R + A_S\right) \times 100 \tag{3}$$

where A_R and A_S are the GC peak areas of the (*R*)- and (*S*)-enantiomers, respectively.

4 Conclusion

In this study, we successfully developed a selective immobilization strategy on a cost-efficient, commercially available macroporous polymer support (ReliZyme™ HA403),

utilizing a mechanism that combines metal affinity immobilization with covalent stabilization. By systematically optimizing the surface a low chelator-to-epoxy ratio (1:75) was identified as specific ligand architecture and demonstrated that pore confinement effects and high ligand density significantly alter the optimal binding stoichiometry compared to non-porous nanomaterials.

Crucially, the robustness of this protocol was validated by its successful extension to a structurally and mechanistically distinct enzyme, the PLP-dependent transaminase *Vf*TA. Despite fundamental differences in molecular size and catalytic mechanism, the optimal surface parameters remained conserved across both biocatalysts. This finding strongly suggests that the immobilization efficiency is primarily governed by the physicochemical properties of the carrier rather than the specific surface features of the enzyme. Furthermore, the rigid, trifunctional crosslinker (THPMTGE) proved to be universally superior, providing essential stabilization for the quaternary structure of both enzymes. The optimized bifunctional carrier exhibited excellent operational stability, maintaining its high catalytic performance over five consecutive reuse cycles without any significant activity loss.

Collectively, these results indicate that the developed ReliZyme-EDTADa-THPMTGE system functions as a versatile platform technology. It enables the rapid, single-step creation of stable and active biocatalysts directly from crude cell lysates, offering a powerful tool for industrial biotransformations that eliminates the need for costly enzyme purification.

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