Immobilization of Phenylalanine Ammonia-lyase via EDTA Based Metal Chelate Complexes – Optimization and Prospects

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Abstract

Immobilized metal ion affinity chromatography principles were applied for selective immobilization of recombinant polyhistidine tag fused phenylalanine ammonia-lyase from parsley (*Pc*PAL) on porous polymeric support with aminoalkyl moieties modified with an EDTA dianhydride (EDTADa)-derived chelator and charged with transition metal ions. Out of the five investigated metal ions - Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} - the best biocatalytic activity of *Pc*PAL was achieved when the enzyme was immobilized on the Co^{2+} ion-charged support (31.8 ± 1.2 U/g). To explore the features this *Pc*PAL obtained by selective immobilization, the thermostability and reusability of this PAL biocatalyst were investigated. To maximize the activity of the immobilized *Pc*PAL the surface functionalization of the aminoalkylated polymeric carrier was fine-tuned with using glycidol as a thinning group beside EDTADa. The maximal activity yield (Y_A=103 %) was earned when the EDTADa and glycidol were used in 1 to 24 ratio. The reversibility of the immobilization method allowed the development of a support regeneration protocol which enables easy reuse of the functionalized support in case of enzyme inactivation.

Keywords

IMAC, selective enzyme immobilization, phenylalanine ammonia-lyase

1 Introduction

The expansion of employing enzymes in food and pharmaceutical industry, development of novel biosensors and synthesis of small organic compounds spawned the need of creating more stable and easily recyclable biocatalysts [1–3]. Enzyme immobilization can enhance resistance against the conditions of use (pH, temperature, organic solvents) and give an opportunity for reuse and application in continuous flow mode [4–9]. Nowadays there are several well-known methods for enzyme immobilization: covalent immobilization [6, 7, 10–14], adsorption [15, 16], embedding in polymer [17–19] or silica-based matrices [20, 21]. New methods have emerged as well over the last decades, in which specifically tagged enzymes can be immobilized via specific interactions [22–25]. The appropriate immobilization method and a suitable carrier for it can significantly improve the properties of the biocatalyst [26–28].

In the late 1970s, Porath and coworkers discovered that histidine, cysteine and tryptophan amino acids can coordinate with transitional metal ions [29, 30]. Originally, this phenomenon called immobilized metal ion affinity chromatography (IMAC), is used consciously for protein purification [31]. Histidine tags artificially attached to the recombinant target proteins make them suitable to form stable metal-protein complexes, hence facilitating a simple and effective way for protein purification.

In addition to preparing pure enzyme solutions, the principle of IMAC can also be applied in selective enzyme immobilization. Applying this technique, immobilized biocatalyst can be produced in a one-step operation that involves the purification and the immobilization of the enzyme as well [25, 32–35]. The most used metal complexing agents in this IMAC based selective enzyme immobilization methods are the iminodiacetic acid [34–38] and nitrilotriacetic acid [32, 39–43].

The interaction between the immobilized metal ion and the protein provides a reversible immobilization. Opposed

to multipoint covalent attachment, this approach enables more conformational changes of the enzyme which may be advantageous to achieve maximal biocatalytic activity. In case of enzyme inactivation, the reversibility of this method enables elution of the inactivated form of the immobilized enzyme from the surface and the support can be reused in a new immobilization cycle.

Phenylalanine ammonia-lyases (PALs), a member of the MIO-enzymes family, catalyzes the nonoxidative ammonia elimination reaction of ʟ-phenylalanine in plants, fungi and bacteria [44, 45]. Nowadays the properties, structure and catalytic activity of phenylalanine ammonia-lyases (PALs) are intensively investigated because of the importance of PALs in the production of enantiomerically pure amino acids (phenylalanine and its derivatives) [45–51], and their application in the enzyme replacement therapy of phenylketonuria [52].

In this study our aim is to demonstrate the usefulness of the metal affinity-based immobilization method not on a simple monomeric but on a more demanding multimeric enzyme. Thus, phenylalanine ammonia-lyase from *Petroselinum crispum* (parsley) (*Pc*PAL) having a tetrameric structure and promising biocatalytic activity has been selected as target enzyme for this study. So far mostly covalent immobilization methods [47, 48, 53–57] and encapsulation techniques [58, 59] have been studied for PAL enzymes, another affinity-based method have already been described in our previous work [24].

Our major goal was to find an appropriate metal ion for the selective complexation of the *Pc*PAL from crude lysate using EDTA dianhydride derived chelator, explore the properties of the biocatalyst and demonstrate the advantage of a reversible His-tag based immobilization.

2 Results and discussion
In this work, we wanted to test the activity and stability of *Pc*PAL enzyme with an *N*-terminal His-tag [60] in its immobilized form as a metal chelate complex. To find an appropriate metal ion for the IMAC-type immobilization, the different forms of His-tagged *Pc*PAL anchored to EDTA-based IMAC support charged with transition metal ions–iron(III), cobalt(II), nickel(II), copper(II), zinc(II)– were characterized by thermal stability and reusability tests.

First, the surface of an appropriate aminoalkyl polymeric resin was modified with proper functions enabling the metal ion complexation. In the previous IMAC applications, mainly iminodiacetic acid or nitrilotriacetic acid-based functions were applied as metal-complexing agent. Iminodiacetic acid is linked to the support via epoxy groups, and nitrilotriacetic

acid is usually reacted in a C-C coupling reaction [35] at the alpha carbon or in a reaction with a lysine derivative (N_α,N_α-bis(carboxymethyl)-ʟ-lysine) [32, 40, 41, 43]. The disadvantage of the epoxy functionalized surface is that the remaining epoxy groups must be blocked to avoid subsequent covalent enzyme immobilization.

In this research, a commercially available polymeric resin with aminoalkyl functions at the surface was modified and applied as solid support. To create the properly modified surface capable of complexing transition metal ions, dianhydride of ethylenediaminetetraacetic acid (EDTADa) was used. After the simple reaction between an amino group and an anhydride and no blocking steps were required prior to the enzyme immobilization (Fig. 1). This type of surface modification is feasible with any other complexing agent with multiple carboxylate functions turned to anhydride(s).

2.1 Selection of metal ions for the affinity-based support
Three immobilization protocols differing in the washing steps and storage conditions were tested during the metal ion selection for *Pc*PAL immobilization. In the first case (A), the biocatalysts were washed only with buffers containing KCl after the enzyme immobilization and stored at 4 °C under TRIS buffer (100 mM, pH 8.8). In the second case (B), a washing step with low concentration of imidazole buffer was added to the washing process. These biocatalysts were stored at 4 °C under TRIS buffer (100 mM, pH 8.8) after the immobilization as well. In the third case (C), the previous washing protocol (B) was repeated, but after washing with imidazole buffer the biocatalysts were dried in a vacuum chamber and was also stored at 4 °C. For each metal ion charged support, maximal enzyme loading was achieved for the biocatalyst. To determine their capacity the activity of supernatant was measured after the immobilization process and compared to the activity of the original *Pc*PAL containing cell lysate.

During the *Pc*PAL immobilization we found that the Fe^{3+} and Cu^{2+} ions leak from the polymer support (the color of supernatant turned into light orange or blue). In case of Cu^{2+} ion this effect is not surprising due to the known good complexing properties of this ion with amino acids.

The complexations and activity measurements were performed in three replicates. The immobilized *Pc*PAL biocatalysts were tested in the ammonia elimination reaction from ʟ-phenylalanine leading to *trans*-cinnamic acid (Fig. 2, Table 1). For reference, no significant immobilization of *Pc*PAL was observed onto a metal ion-free support.
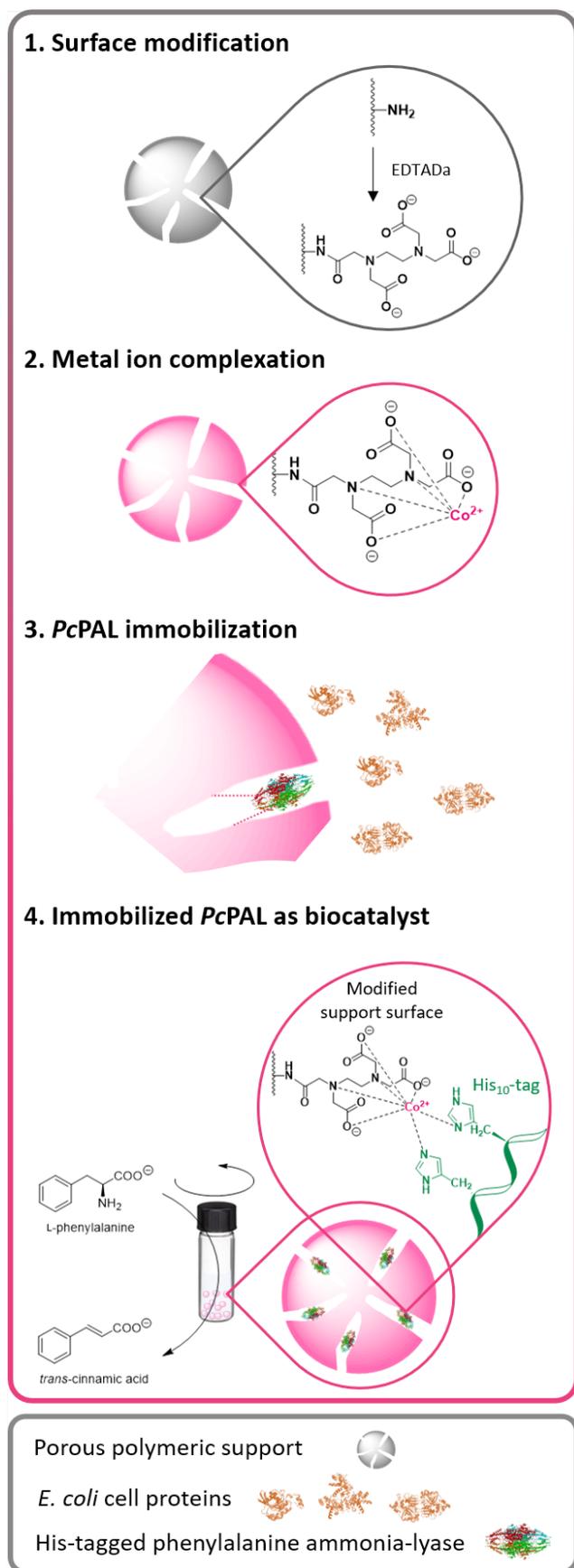
Fig. 1 Surface modification and preparation of selectively immobilized phenylalanine ammonia-lyase biocatalyst.
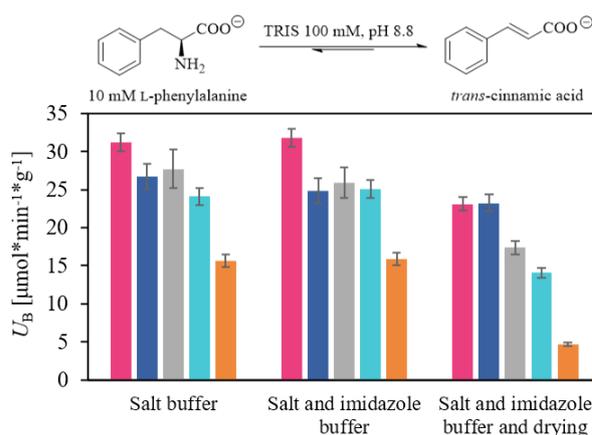
Fig. 2 Specific biocatalytic activity of immobilized *Pc*PAL on chelating support. The complexations were performed with five different metal ions and three different washing protocols were used. Magenta: Co^{2+}, blue: Cu^{2+}, gray: Zn^{2+}, turquoise: Ni^{2+}, orange: Fe^{3+}. The activity was determined in the ammonia elimination reaction of L-phenylalanine (Subsection 3.6).

Independently of the type of the immobilized metal ions, there was no significant difference between the two washing protocols involving no strong drying (methods A and B, Fig. 1, Table 1). In both charging ion test series applying the mild washing methods (A and B), the best activities were observed on the Co^{2+}-charged support. In case of dried biocatalysts (C), the *Pc*PAL on support charged with Co^{2+} ions and Cu^{2+} ions were the most active, but in the latter case Cu^{2+} ion leakage was observed during the test reaction.

In addition to the capacity measurement after the IMAC-type immobilization, the composition of the complexed or adsorbed proteins was analyzed with polyacrylamide gel electrophoresis (Fig. 3). According to the SDS-PAGE analysis, the Fe^{3+} ion charged support could complex the target *Pc*PAL with good selectivity but with quite low capacity.

Using Co^{2+} and Cu^{2+} ions in the complex resulted in much better capacity (Table 1), although in the latter case the selectivity was poor due to the easy complexation of amino acid other than His to Cu^{2+} as mentioned earlier. The Ni^{2+}- and Zn^{2+}-complexed polymers lacked both capacity and selectivity.

Based on the specific biocatalytic activity, capacity and selectivity results and the simplicity of use and storage, the Co^{2+}-based, dried *Pc*PAL biocatalyst (PcPAL$_{Co(II)-C}$) was chosen to characterize further the thermostability, reusability and storage stability measurements.

2.2 Stability tests
The thermal behavior of PcPAL$_{Co(II)-C}$ was measured in 2 hours test reactions between 20 °C and 80 °C (Fig. 4).

Table 1 Capacity, specific biocatalytic activity and activity yield of immobilized *Pc*PAL on chelating support. The complexations were performed with five different metal ions and three different washing protocols were used. The activity was determined as described at Subsection 3.6.

Metal ion	Capacity	Washing protocol A		Washing protocol B		Washing protocol C	
		U_B	Y_A	U_B	Y_A	U_B	Y_A
	µg protein to 5 mg support	µmol min^{-1} g^{-1}	%	µmol min^{-1} g^{-1}	%	µmol min^{-1} g^{-1}	%
Co^{2+}	771 ± 54	31.2 ± 1.2	103	31.8 ± 1.2	104	23.1 ± 0.8	76
Cu^{2+}	656 ± 49	26.7 ± 1.7	103	24.8 ± 1.7	96	23.2 ± 1.1	90
Zn^{2+}	589 ± 119	27.7 ± 2.6	119	25.9 ± 2.2	111	17.3 ± 0.9	75
Ni^{2+}	588 ± 28	24.1 ± 1.1	104	25.1 ± 1.2	108	14.1 ± 0.7	61
Fe^{3+}	505 ± 25	15.7 ± 0.8	78	15.8 ± 0.8	79	4.7 ± 0.2	23
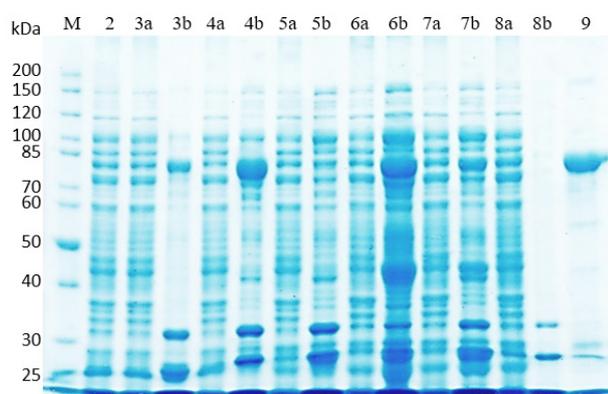
Fig. 3 SDS-PAGE analysis of the immobilized *Pc*PAL biocatalysts. M: Protein ladder, 2: crude lysate of *Pc*PAL expressing *E. coli*, 3-7: *Pc*PAL immobilized on IMAC support charged with various metal ions (3: Fe^{3+}, 4: Co^{2+}, 5: Ni^{2+}, 6: Cu^{2+}, 7: Zn^{2+}, 8: metal free; Series a: supernatant after complexation, series b: polymer after complexation), 9: solution of *Pc*PAL (~81 kDa) purified on NiNTA.
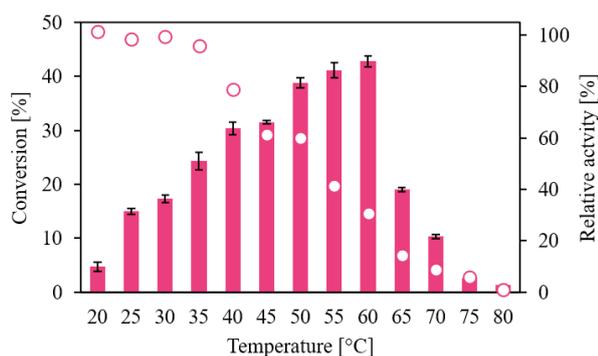
Fig. 4 Effect of temperature on productivity of *Pc*PAL$_{Co(II)-C}$ (columns: conversion in 2 h reactions), and the residual activity of of *Pc*PAL$_{Co(II)-C^-}$ after the 2 h use at different temperatures (dots: relative activity [%] = activity at 30 °C after using the catalyst at a given temperature / activity at 30 °C at first using × 100).

The productivity increased up to 60 °C and then a significant loss of enzyme activity was observed.

After the thermal productivity test, the remaining activity of the *Pc*PAL$_{Co(II)-C}$ was also determined at 30 °C in activity assay to determine the permanent activity loss after the 2 hours reaction at a given temperature.

These experiments clearly showed that longer incubation at 40 °C or higher temperature caused permanent decrease or complete loss of biocatalytic activity. If temperatures above 65 °C were used, a significant decrease in activity was observed even in the first reaction. Previous measurements confirms that at 65 °C protein unfolding already begins, leading to complete denaturation of *Pc*PAL enzyme [48].

To test the *Pc*PAL$_{Co(II)-C}$ operational stability at ambient temperature, the biocatalyst was applied in five consecutive ammonia elimination reactions from L-phenylalanine (2 h at 30 °C, each, Fig. 5). Analyses of small aliquots from the reaction mixtures by SDS-PAGE indicated no detectable enzyme leakage in course of the reusability study.

Between the reaction cycles, the biocatalysts were washed with buffer and were dried out in vacuum chamber to measure the amount of the biocatalyst precisely in the next cycle. Although the activity decreased from cycle to cycle, after the fourth cycle 58 % of the initial enzyme activity could be retained.

To test the long-term stability, the dried biocatalysts were stored at three different temperature (−20 °C, 4 °C and 27 °C)
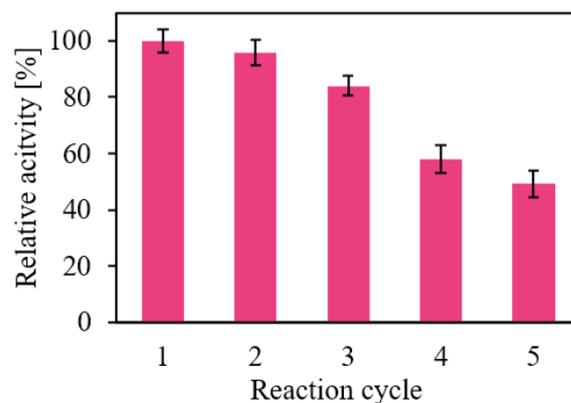
Fig. 5 Operational stability of immobilized *Pc*PAL$_{Co(II)-C}$. Relative activity [%] = activity in given cycle / activity in first cycle × 100.

and the residual activities were tested after 30, 60 and 90 days (Table 2) which is a quiet long interval time compared to previous studies for PAL catalysts [57, 59]. After one month the biocatalysts stored at −20 °C and 4 °C retained more than 80 % of their activity. The residual activity of biocatalyst stored at room temperature after 30 days was a little bit weaker, but it was still comparable to the storage stability of PAL catalysts in the literature [53, 59, 61].

2.3 Regeneration of the support

The advantage of enzyme immobilization via affinity binding to metal complexes is the easy reusability of the support after the enzyme losing its activity. To demonstrate this, we optimized a washing protocol to elute the (exhausted) enzyme from the support and reuse the regenerated IMAC support in a further enzyme immobilization cycle. During the optimization of the support regeneration protocol, the supports and washing fractions were tested with SDS-PAGE.

First, the $PcPAL_{Co(II)-C}$ biocatalyst was washed with imidazole solution (500 mM and 1 M). Surprisingly, even after being washed three times with imidazole solution (1 M), detectable amount of enzyme remained on the support. Furthermore, several chelating agents were tested for elution of the enzyme, such as ethylenediamine (5 % solution in TRIS buffer, 100 mM, pH 8.8), diethylenetriamine (5 % solution in TRIS buffer, 100 mM, pH 8.8), EDTA (at 100 mM and 200 mM, pH 8.0) and diethylenediaminepentaacetic acid (at 100 mM and 200 mM, pH 8.0). Even after elution of the immobilized Co^{2+} ions from the support, a small amount of enzyme remained on its surface. This phenomenon can be explained with a strong ionic adsorption of the enzyme without metal chelate. To elute the remaining enzyme, a two-step elution was used: at first it was washed with diethlyenetriamine solution (three times) and after that the remaining proteins were eluted with NaCl (360 g L^{-1}, three times). During this washing, Co^{2+} ions and enzyme could be detected in the elution fractions, but not on the support after washing.

After the full removal of the enzymes from the previous immobilization, the support was washed with distilled water and then complexed again with Co^{2+} ions. The metal complexed support was used in a new enzyme immobilization cycle. The freshly made and the reused supports were compared in the test reactions as before. Only a very slight difference was observed between the reused and the newly functionalized support (Table 3).

These experiments clearly demonstrated the reusability of the regenerated support after removal of the exhausted enzyme as another advantage of this immobilization technique besides the usual reuse of the biocatalyst. Thus, the tedious production of the IMAC carrier does not need to be repeated for every immobilization process.

2.4 Fine-tuning of the support's surface

Application of inert groups not involved in the complex formation during the surface modification can affect the surface density of the functional groups, the microenvironment around the immobilized enzyme and also stabilizing secondary interactions may occur thereby influencing significantly the specific biocatalytic activity [62–64].

Importantly, these groups are applicable to modulate the surface density of the functional points for enzyme immobilization and thus, the amount of immobilized enzyme in a controllable way. By decreasing the surface density of the functional points – in this case the chelating groups – the amount of the immobilized enzyme can be set properly. There is an optimal space requirement for the individual enzyme molecules. When the binding functions are too dense, the tight packing of the enzyme complexes can result in crowding which may impede conformational changes required for catalytic activity. At the optimal density, packing allows sufficient conformational mobility but there remains no empty surface without enzyme covering. When attachment points are too rare, the low surface coverage results in a poor specific activity.

In addition to modulate the surface density of the functional groups, the inert groups also affect the microenvironment, most importantly the hydrophilic or hydrophobic properties of the surface. The surface properties influence the enzyme adsorption and in case of a porous support have large effect on the diffusion properties also.

Table 2 Long-term stability of PcPAL biocatalysts at three different storage temperature

Storage temperature	Residual activity [%]		
	after 30 days	after 60 days	after 90 days
−20 °C	84	78	69
4 °C	81	71	61
25 °C	65	57	48

Table 3 Comparison of the $PcPAL_{Co(II)-C}$ biocatalysts on freshly prepared and regenerated supports.

	Support type	U_B [a] [$\mu mol\ min^{-1}\ g^{-1}$]
1	Freshly prepared support	23.1 ± 0.8 [a]
2	Reused support after regeneration process	22.8 ± 1.0 [a]

[a] the activity values are the average of five parallel measurements

To modulate the density of the chelating groups on the aminoalkyl functionalized support, glycidol was used in addition to EDTA dianhydride (EDTADa) in different ratios. In addition to adjust the surface density of chelating groups, glycidol results in formation of hydrophilic diol functions on the surface [65]. Beside the creation of IMAC support solely with EDTADa functionalization, modified IMAC supports were also prepared with eight different EDTADa / glycidol molar ratio from 1:1 to 1:36. There was no difference in the specific biocatalytic activity, however, as the ratio increased the amount of immobilized enzyme was decreased (Table 4). These results are understandable in the view of the large surface area covered by a single *Pc*PAL enzyme (being almost a spheroid with ~ 20 Å length and ~10 Å diameter, see the PDB structure of *Pc*PAL with a substrate analogue: 6HQF).

3 Experimental
3.1 Chemicals and biological reagents
EDTA (ethylenediaminetetraacetic acid, 99.5 %), glycidol (96 %), TRIS [tris(hydroxymethyl)aminomethane] (99.8 %), potassium chloride (99 %), nickel(II) acetate tetrahydrate (98 %), cobalt(II) acetate tetrahydrate (98 %) and copper(II) acetate monohydrate (98 %) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Iron(III) chloride hexahydrate (97 %), zinc(II) acetate dihydrate (97 %), HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (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

Table 4 Effect of glycidol – EDTADa ratio to the during IMAC support formation on the specific biocatalytic activity and activity yield of the resulting *Pc*PAL$_{Co(II)\text{-}c}$.

	Quantity of glycidol to one unit of EDTADa	Specific biocatalytic activity (U_B)[a] [μmol min^{-1} g^{-1}]	Activity yield (Y_A)[a] [%]
1	0	23.0 ± 0.5	76
2	1	23.2 ± 1.0	74
3	3	23.3 ± 1.6	75
4	6	21.9 ± 0.7	83
5	9	23.2 ± 1.1	89
6	12	23.3 ± 1.3	77
7	18	22.6 ± 0.8	81
8	24	23.3 ± 1.0	103
9	36	21.7 ± 1.6	95

[a] Specific biocatalytic activity and activity yield values were calculated as described in Subsection 3.6.

which was dried over 4 Å molecular sieve. Patosolv (~85-90 % EtOH+10-15 % 2-propanol) was obtained from Molar 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).

Expression of recombinant phenylalanine ammonia-lyase from parsley (*Petroselinum crispum*) carrying *N*-terminal His$_{10}$-tag in *E. coli* Rosetta™ host was carried out at Fermentia Ltd. according to the method already described by Dima et al. [60].

All the other chemicals used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma-Aldrich (Saint Louis, MO, USA) (ammonium persulfate, glycerol, sodium dodecyl sulfate) or Merck KGaA (Darmstadt, Germany) (glycine, 40 % acrylamide/bis 37.5:1 solution, DL-dithiothreitol, bromophenol blue). Protein Ladder (10 to 200 kDa) was product of ThermoFisher Scientific (Waltham, Massachusetts, USA), and ProSieve Blue protein staining solution was the product of Lonza (Basel, Switzerland). Brine (360 g L^{-1} NaCl in deionized water) was prepared in our lab.

The UV absorbance and kinetic measurements were carried out Genesys 2 type spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and semi-micro PMMA cuvettes were used.

SDS-PAGE analysis was carried out on Mini-PROTEAN Tetra cell electrophoresis chamber (Bio-Rad Laboratories Inc, Hercules, CA, USA) with an Enduro Power Supplies thickness of 0.75 mm.

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

The centrifugation separation steps were carried out in a Hermle Z 400K universal laboratory centrifuge (Hermle LaborTechnik GmbH, Wehingen, Germany).

3.2 Preparation of ethylenediaminetetraacetic dianhydride (EDTADa)
EDTA (100 g, 0.34 mol) and pyridine (170 mL) were mixed in a 500 mL four necked flask under argon. The suspension was stirred for 1 h at 65 °C followed by dropwise addition of acetic anhydride (130 mL, 1.34 mol) to the solution. The reaction mixture was kept at 65 °C for 16 h. The resulting product was filtered off, washed with pyridine and diethyl ether, subsequently, and dried under vacuum. The white solid product was stored in a sealed container under argon at 4 °C.

3.3 Surface modification of the hexylamine-functionalized polymer resins

The original water content (~65 %) of the macroporous hexylamine-functionalized resins was removed before further use. In a glass filter wet resins (50 g) were suspended and washed with Patosolv (2 × 100 mL) and hexane (100 mL), including filtration between the washing steps. After the final filtration, the resins were dried at room temperature in a vacuum drying chamber 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 (1.0 g) were suspended in N,N-dimethylformamide (10 mL). To the suspension of resins the proper amounts of EDTADa, glycidol and N,N-diethyl-N-isopropylamine (two equivalents to the EDTADa) were added [the total amounts of EDTADa and glycidol were two equivalents related to the amino group content of the polymer (1750 µmol g⁻¹) but in different molar ratios (EDTADa-glycidol: 1–0; 1–1; 1–3; 1–6; 1–12; 1–18; 1–24; 1–36)]. The resulted mixture was shaken at 450 rpm for 24 h at 60 °C, then distilled water (100 µL) was added and the mixture was shaken further for 1 h at 60 °C. After the hydrolytic quenching, the resins were filtered and washed with acetonitrile (3×20 mL) and 2-propanol (20 mL), dried in a vacuum drying chamber at room temperature for 5 h (until the vacuum level dropped below 10 mbar).

3.4 Charging of the chelator-functionalized resins with metal ions

Charging of the chelator-functionalized resins with metal ion was performed in 15 mL screw cap vials. The modified IMAC resins (100 mg) were suspended in the solution of the corresponding metal salt [5 mL, 50 mmol L⁻¹ of Ni(OAc)$_2$×4 H$_2$O, Co(OAc)$_2$×4 H$_2$O, Cu(OAc)$_2$×H$_2$O, FeCl$_3$×6 H$_2$O, or Zn(OAc)$_2$×2 H$_2$O]. The mixture was shaken at 450 rpm for 30 minutes at room temperature, then the metal-charged resins were filtered off and washed with distilled water (3×10 mL) and 2-propanol (10 mL), dried in vacuum drying chamber at room temperature for 5 h (until the vacuum level dropped below 10 mbar).

3.5 Immobilization of phenylalanine ammonia-lyase from parsley (washing protocol C)

To the metal ion-complexed resins (20 mg) was added the lysate of *E. coli* cells (30 mg/mL total protein in lysis buffer: 50 mmol L⁻¹; TRIS 150 mmol L⁻¹, pH 8.0) expressing His-tagged *Pc*PAL (4 mL, *Pc*PAL concentration 1.4 mg/mL, sufficient for maximal enzyme loading). The suspension was shaken at 450 rpm for 30 min at room temperature to bind the His-tagged enzyme. The nonspecifically adsorbed host

proteins were eluted with sequential addition 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); low concentration imidazole buffer (25 mmol L⁻¹ imidazole, 30 mmol L⁻¹ KCl, 50 mmol L⁻¹ HEPES, pH 7.5). The immobilized biocatalysts were washed with deionized water (3×1 mL) and dried in a vacuum drying chamber at room temperature for 2 h (until the vacuum level dropped below 10 mbar).

A tenfold scaling-up of the immobilization was accomplished with the EDTADa-Co(II) modified resin using the same immobilization method as described above.

3.6 Activity measurements with the *Pc*PAL biocatalysts

To a solution of L-phenylalanine (2 mL, 10 mmol L⁻¹ in 100 mmol L⁻¹ TRIS buffer; pH 8.8) was added the immobilized *Pc*PAL (5 mg) and the mixture was shaken at 750 rpm at 30 °C. After 0.5, 1 and 2 h, samples (50 µL) were taken and diluted with distilled water to a final volume of 1 mL. Conversion of L-phenylalanine to *trans*-cinnamic acid was determined by UV-spectroscopy from the absorption change of cinnamic acid at 290 nm (ε_{290}=9.530 L cm⁻¹ mol⁻¹) by using the Lambert-Beer equation.

To monitor the efficiency of the enzyme immobilization, the *Pc*PAL activity of the crude cell lysate was measured before and after the immobilization process. An aliquot of the centrifuged crude cell lysate (20 µL) was added to L-phenylalanine solution (1 mL, 10 mmol L⁻¹ in TRIS buffer (100 mmol L⁻¹; pH 8.8)) at 30 °C and the absorbance change was detected at 290 nm for 2 min.

To characterize the productivity of the immobilized *Pc*PAL, the specific biocatalytic activity was calculated using Eq. (1):

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

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). Activity yield was calculated using Eq. (2)

$$Y_A = 100 \times U_E \left(\text{cat} \right) / U_E \left(\text{im} \right), \tag{2}$$

where $U_E(\text{cat})$ is the U_E of the biocatalyst and $U_E(\text{im})$ is the difference of the U_E of the lysate between the beginning and the end of the immobilization process). The specific enzyme activity was calculated using Eq. (3):

$$U_E = U_B \times \left(m_B / m_E \right), \tag{3}$$

where m_E (g) is the mass of the *Pc*PAL in the applied biocatalyst; in case of the cell lysate m_E (g) was calculated from the determined *Pc*PAL concentration of the lysate).

To determine m_E, a small amount of the lysate was purified on Ni-sepharose 6 Fast Flow affinity chromatography medium (Cytiva, Marlborough, MA, USA) according to the specification of the manufacturer.

3.7 Tests for thermal effects on productivity and stability of the *Pc*PAL biocatalyst

The $PcPAL_{Co(II)-C}$ biocatalyst (5 mg) was added to a solution of L-phenylalanine (2 mL, 10 mmol L^{-1} in TRIS buffer (100 mmol L^{-1}, pH 8.8)) and the mixture was shaken at 750 rpm at different temperatures between 20 °C and 80 °C with 5 °C increments. After 2 hours reaction time, the conversion and biocatalytic activity (U_B^T, where T is the reaction temperature) was calculated based on UV-spectroscopic method described at Subsection 3.6. Then the biocatalyst was washed with TRIS buffer (100 mmol L^{-1}; pH 8.8) three times. The biocatalyst was separated with centrifugation (3500 rpm, 2 min) in each washing steps.

To measure the thermal stability, fresh solution of L-phenylalanine (2 mL, 10 mmol L^{-1} in TRIS buffer (100 mmol L^{-1} pH 8.8)) was added to the washed biocatalyst and the mixture was shaken at 750 rpm at 30 °C. The conversion and biocatalytic activity ($U_B^{T-30\,°C}$, where T is the reaction temperature at the first reaction) were measured after 2 h reaction. The residual activity for every temperature was calculated from the activity in the second reaction at 30 °C and the activity of the first use at 30 °C:

$$U_{B,rem} = \left(U_B^{T-30°C} / U_B^{30°C} \right) \times 100. \tag{4}$$

3.8 Reusability tests with the *Pc*PAL biocatalyst

The $PcPAL_{Co(II)-C}$ biocatalyst (25 mg) was added to a solution of L-phenylalanine (2 mL/5 mg biocatalyst, 10 mmol L^{-1} in 100 mmol L^{-1} TRIS buffer; pH 8.8) and the mixture was shaken at 750 rpm at 30 °C. After 2 h reaction, the biocatalyst was separated and washed with TRIS buffer (5 mL, 100 mmol L^{-1}, pH 8.8) three times and with deionized water (5 mL). In each washing steps the biocatalyst was separated with centrifugation (3500 rpm, 2 min). After washing, the biocatalyst was dried in a vacuum drying chamber at room temperature for 2 h (until the vacuum level dropped below 10 mbar). The mass of the dried biocatalyst was measured and the same substrate solution – biocatalyst ratio was used in the second reaction cycle. The reactions were performed five times in this way.

3.9 Regeneration of the chelator-functionalized support

The $PcPAL_{Co(II)-C}$ biocatalyst (50 mg) was washed three times with diethlyenetriamine solution (10 ml, 5 % solution in TRIS buffer (100 mM, pH 8.8); each), three times with brine (10 mL; each) and finally with deionized water (3 × 10 mL). The biocatalysts were separated with centrifugation (3500 rpm, 2 min) in each washing steps. After that the support was used in a second metal ion complexation and enzyme immobilization step according to Subsections 3.4 and 3.5.

4 Conclusion

In this study a facile way of selective enzyme immobilization is presented that allows a simple support recycling. This method is based on the IMAC principle, thus provides a one-step purification and immobilization process for polyhistidine-tagged recombinant enzymes. The usefulness of this method was demonstrated with immobilization of His-tagged phenylalanine ammonia-lyase from parsley (*Pc*PAL) on an EDTA-functionalized macroporous polymer resin charged with various metal ions.

The immobilization efficiency and specific biocatalytic activity of *Pc*PAL on the EDTA-based IMAC resins proved to be the better with cobalt(II) ion (23.1 U/g) then with nickel(II) ion (14.1 U/g), which is widespread in commercial protein purification protocols. Additionally, the cobalt(II)-containing *Pc*PAL biocatalyst performed the best capacity (771 µg/5 mg support) with negligible enzyme leakage. Additionally, this immobilization method enables a facile recycling of the valuable IMAC carrier by elution of the exhausted *Pc*PAL when needed.

The surface modification is easy to implement with any amino groups containing support and can be simply fine-tuned by "diluting" the chelating functions on the surface by various inert groups. Setting a proper density of the chelating functions by "dilution" with diol functions deriving from glycidol increased the activity yield of *Pc*PAL immobilization without decreasing the specific biocatalytic activity.

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