Heterofunctional Magnetic Metal-Chelate-Epoxy Supports for the Purification and Covalent Immobilization of Benzoylformate Decarboxylase From *Pseudomonas Putida* and Its Carboligation Reactivity

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ABSTRACT In this study, the combined use of the selectivity of metal chelate affinity chromatography with the capacity of epoxy supports to immobilize poly-His-tagged recombinant benzoylformate decarboxylase from *Pseudomonas putida* (BFD, E.C. 4.1.1.7) via covalent attachment is shown. This was achieved by designing tailor-made magnetic chelate–epoxy supports. In order to selectively adsorb and then covalently immobilize the poly-His-tagged BFD, the epoxy groups (300 μmol epoxy groups/g support) and a very small density of Co^{2+}-chelate groups (38 μmol Co^{2+}/g support) was introduced onto magnetic supports. That is, it was possible to accomplish, in a simple manner, the purification and covalent immobilization of a histidine-tagged recombinant BFD. The magnetically responsive biocatalyst was tested to catalyze the carboligation reactions. The benzoin condensation reactions were performed with this simple and convenient heterogeneous biocatalyst and were comparable to that of a free-enzyme-catalyzed reaction. The enantiomeric excess (*ee*) of (*R*)-benzoin was obtained at 99 ± 2% for the free enzyme and 96 ± 3% for the immobilized enzyme. To test the stability of the covalently immobilized enzyme, the immobilized enzyme was reused in five reaction cycles for the formation of chiral 2-hydroxypropiophenone (2-HPP) from benzaldehyde and acetaldehyde, and it retained 96% of its original activity after five reaction cycles. *Chirality 27:635–642, 2015.*

KEY WORDS: immobilized benzoylformate decarboxylase; metal-chelate-epoxy magnetic support; metal-affinity magnetic nanoparticles; selective immobilization; covalent attachment; one step purification

Since the second half of the last century, numerous efforts have been devoted to the development of insoluble immobilized enzymes for a variety of applications.[1,2] These applications can clearly benefit from the use of the immobilized enzymes rather than the soluble counterparts, for instance, as reusable heterogeneous biocatalysts, with the aim of reducing production costs by efficient recycling and control of the process.[1,2]

Magnetic metal nanoparticles have been used in protein/enzyme immobilization due to their unique properties such as superparamagnetism, high surface area, large surface-to-volume ratio, and easy separation under external magnetic fields.[3] Another key factor to take full advantages of nanoparticles such as magnetic nanoparticles is how to ideally regulate the orientation of the proteins/enzymes on the supports. Compared to porous supports, such nonporous nanoparticles have no external diffusion problems, making them more competitive especially for large-scale industrial usage in solid–liquid systems (e.g., precipitated protein).[4] However, as drawbacks compared to porous supports, proteins/enzymes immobilized on nonporous nanoparticles may suffer from inactivation for soluble proteins/enzymes, especially through interaction with gas bubbles generated by strong stirring or bubbling of oxygen.[5] Such inactivation by interfaces might proceed and finally result in an irreversible activity loss due to sustained effects such as destabilization of the electrostatic, hydrophobic, and hydrogen bonds.[6] The frequently utilized magnetic nanoparticles are iron oxides, among which superparamagnetic Fe_3O_4 nanoparticles are the most prevalent materials because they have low toxicity and good biocompatibility.[7]

Magnetic nanoparticles can be easily recovered from media by applying a magnetic field. When they are used for enzyme immobilization, particularly in batch reactors and continuous-flow stirred-tank reactors, this method facilitates the separation of enzymes from the product after the completion of the reaction in order to permit the reuse and recycling of the enzyme.[8,9]

One of the most suitable methods for both laboratory and industrial-scale immobilization of proteins is based on epoxy-activated supports.[10–13] Epoxy supports present many advantages; for example, they are very stable during storage and during suspension in neutral aqueous media, allowing for long-term storage, prolonged transport from manufacturer to consumer, and extended enzyme-support reaction periods. In addition, epoxy-activated supports are reactive to directly form very stable covalent linkages with nucleophilic groups

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present on the protein surface (amine, thiol, hydroxyl groups). Moreover, epoxy-activated supports may also react with carboxylic groups producing an ester bond, although this reaction proceeds in a much slower fashion.[14] The immobilization of proteins follows a two-step mechanism: 1) the enzyme is hydrophobically adsorbed on a fairly hydrophobic support at very high ionic strength; a rapid mild physical adsorption between the protein and the support is produced; and 2) a covalent bonding between the adsorbed enzyme and the epoxy groups proceeds.[10–19] However, the immobilization onto conventional epoxy supports has several problems: the use of high ionic strength, which can affect the stability of certain enzymes; the enzymes require a hydrophobic area to be immobilized on the support; the enzymes will be immobilized by their hydrophobic area, which may be not the most convenient to stabilize the enzyme; hydrophobic supports are required for this immobilization, with a likely negative effect on enzyme stability.

To overcome these drawbacks, multifunctional epoxy supports have been reported as a second generation of activated supports that are able to covalently immobilize enzymes, antibodies, or other molecules under very mild experimental conditions.[20–22] These multifunctional supports should contain two types of functional groups: 1) the groups that are able to physically adsorb proteins (such as ionic exchangers or by metal-chelate adsorption); 2) a dense layer of epoxy groups that are able to covalently bind the enzyme.[23–28] The partial modification of the epoxy supports with ethylenediamine, iminodiacetic acid, metal chelates, or *m*-amino-phenyl-boronic acid may be good alternatives for reaching this goal. When an oligopeptide of six or seven histidine residues is linked to the N- or C-terminus of an enzyme, the resulting His-tagged enzyme can be strongly immobilized onto the support material through metal chelates, due to the cooperative bonding of the histidines to a metal ion such as Co^{2+}.[29]

The investigated enzyme benzoylformate decarboxylase from *Pseudomonas putida* (BFD, E.C. 4.1.1.7) is a homotetrameric thiamine diphosphate dependent (ThDP) enzyme. The cofactor ThDP has three distinctive units, which include a pyrophosphate part, a thiazolium core, and a pyrimidine unit. It acts by a covalent interaction with the substrate. ThDP and is mainly engaged in a variety of carbon–carbon bond-forming reactions, in which each unit has a special role in enzymatic catalysis.[30,31] BFD accepts a broad range of aldehydes as substrates to catalyze the enantioselective synthesis of 2-hydroxyketones, which are useful as synthons in organic chemistry.[32–36] These synthons are important intermediates in the production of antifungals.[36,37] as well as of pharmaceuticals like Wellbutrin (treatment of depression) and Zyban (smoking cessation).[36,38]

In this work, the synthesis of (*R*)-benzoin starting from benzaldehyde and (*S*)-2-hydroxy-1-phenylpropanone [(*S*)-2-HPP] starting from benzaldehyde and acetaldehyde was catalyzed by covalently immobilized BFD on M-chelate-epoxy supports. The M-chelate-epoxy supports were optimized to achieve the one-step purification, covalent immobilization, and stable 6xHis-tagged BFD via multipoint covalent attachment.

MATERIALS AND METHODS

Chemicals and Materials

All of the chemicals that were used in the immobilization studies were purchased from Sigma-Aldrich (St. Louis, MO) including 3-glycidoxypropyltrimethoxysilane (GPTMS), iminodiacetic acid (IDA), ferric chloride hexahydrate ($FeCl_3.6H_2O$), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$), ammonium hydroxide (25% [w/w]), tetraethyl ortho silicate (TEOS), and 2-propanol.

The recombinant *E. coli* SG13009 strain carrying the histidine tagged BFD gene was a kind gift from Dr. Martina Pohl (CLIB-Graduate Cluster Industrial Biotechnology, Germany).[34] Chemicals used in the production of BFD such as isopropyl-β-D-galactopyranoside (IPTG) and antibiotics were all of molecular biology grade and obtained from AppliChem (Omaha, NE). Enzyme production was performed in a New Brunswick (Edison, NJ) BioFlo110 Fermenter equipped with pH and temperature probes as well stirring rate controls.

Characterization

Fourier-transformed infrared (FT-IR) spectra were measured on a Thermo Scientific Nicolet IS10 FT-IR spectrometer (Pittsburgh, PA). Sixteen scans were collected at a resolution of 4 cm^{-1}.

Magnetization measurements as a function of magnetic field were carried out using a vibrating sample magnetometer (VSM) (Q-3398, Cryogenic).

BFD-catalyzed reactions were monitored by thin-layer chromatography (TLC) on silica gel (E. Merck, Darmstadt, Germany). Spots were detected by both UV-absorption and phosphomolybdicacid (PMA) staining. The products were identified by ^{1}H-NMR and ^{13}C-NMR spectra (Bruker DPX 400 MHz, Billerica, MA) using tetramethylsilane (TMS) as an internal standard and deutero-chloroform as the solvent. Enantiomeric excess values were determined via HPLC analysis (Agilent 1100 series, Chiralpak AD).

Synthesis of Epoxy-Functionalized Magnetic Particles

Synthesis of magnetite nanoparticles and coating with silica were previously reported by our group[30,39] and silica-coated magnetite was named SMP. One gram of wet SMPs were reacted with 10 mL of 5% GPTMS in toluene at room temperature overnight. After the coupling reaction, the modified magnetic nanoparticles were removed from the solution with the help of a permanent magnet and rinsed thoroughly with toluene and ethanol to remove the physically adsorbed silane. Finally, they were freeze-dried. When prepared in this way, the surfaces of the magnetic nanoparticles had exposed active epoxy groups.[30,39]

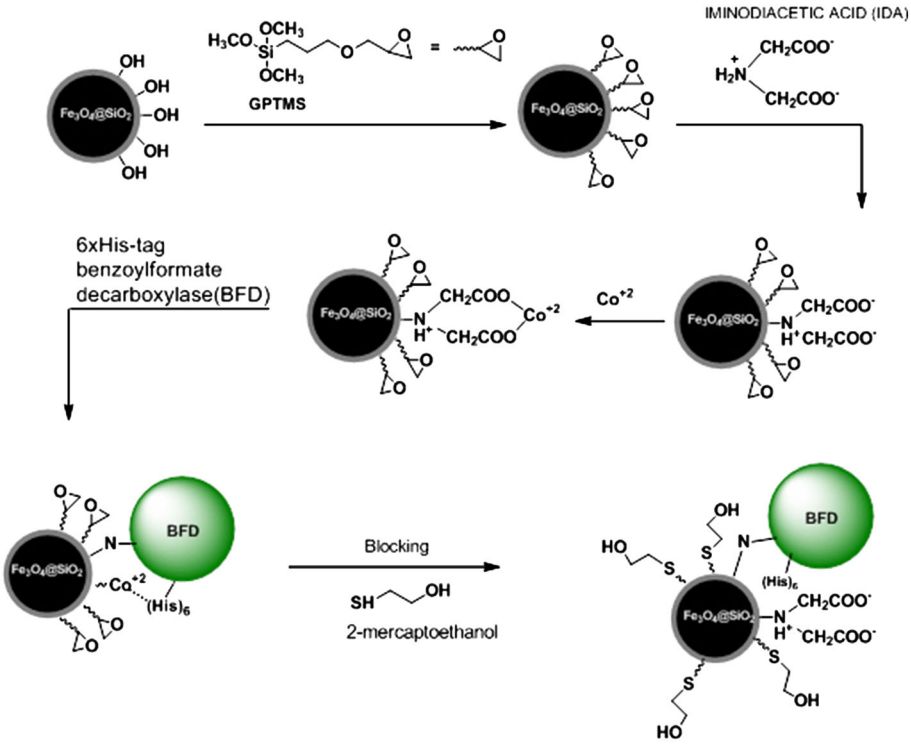
The schematic illustration for the preparation steps of the epoxy-functionalized SMPs is shown in Scheme 1. The epoxy content of the SMPs was determined using the pyridine–HCl method given in the literatüre.[29] This analysis indicated that 300 μmol epoxy groups were attached to per g SMPs.

Preparation of IDA-Epoxy-Functionalized SMPs

Epoxy functionalized SMPs (1 g) was incubated in 15 mL of 0.1 M sodium borate/2 M iminodiacetic acid (pH 8.0) at room temperature overnight under very gentle stirring. The support was washed with an excess of distilled water and stored at 4°C. The IDA-epoxy-functionalized SMPs support was then incubated with Co^{+2} salt[35,30,39] to obtain the M-chelate-epoxy-supports. Briefly, IDA-epoxy-functionalized SMPs support (1 g) was incubated in 50 mL of distilled water containing 2M of Co^{+2} under very gentle stirring. After 2 h, the M-chelate-epoxy-supports were washed with an excess of distilled water. This treatment should modify 100% of the IDA groups in the support (Scheme 1). Atomic absorption spectroscopy was used to quantify the degree of modification of the epoxy groups with IDA, after releasing the Co^{2+} from a sample of the M-chelate-epoxy-supports by treatment with 100 mM ethylene-diamine tetraacetic acid (EDTA) at pH 7. The M-chelate-epoxy-supports contained 38 μmol of Co^{2+} (to adsorb the His-tagged BFD) and 300 μmol of epoxy groups per gram of support.

Preparation of Recombinant BFD

The recombinant *E. coli* SG13009 strain carrying pKK233-2 plasmid with hexahistidine-tagged BFD gene was used in this study. Cells were grown in 100 mL LB (Luria broth) medium at 37°C until the optical density reached 0.5 at 600 nm, and 1 mM isopropyl-β-D-thiogalactopyranosid (IPTG) was added for induction. The cells were grown for an additional 6 h at 25°C prior to harvesting by centrifugation. Then the cells were resuspended in 10 mL 50 mM potassium phosphate buffer (pH: 7.8, 10 mM NaCl and 20 mM imidazole), disrupted by sonication, and the cell debris was removed by centrifugation. Enzyme was either used as the crude



Scheme 1. Preparation of M-chelate-epoxy-support and their use for immobilization of benzoylformate decarboxylase (BFD). Epoxy supports are incubated in the presence of iminodiacetic acid to introduce a few IDA groups in the support. The IDA supports are incubated with cobalt salts to obtain metal chelate epoxy supports.

form without purification or used as purified enzyme, which was obtained by Ni^{2+}-NTA affinity chromatography (Invitrogen, La Jolla, CA) and desalting column (Amersham, Cleveland, OH). The crude and purified enzyme was lyophilized for long-term preservation.

Activity Assay for Free Enzyme

Benzoin formation reaction with free enzyme was carried out as described in the literature.[30] One unit activity of BFD is defined as the amount of enzyme necessary to catalyze the formation of 1 μmol of benzoin per min. Activity analysis for benzoin synthesis was performed with 5 mg/mL benzaldehyde in 50 mM potassium phosphate buffer containing 0.5 mM TPP, 2.0 mM $MgSO_4$, and 25% DMSO at pH 7.5.

Immobilization of BFD

Two hundred mg lyophilized crude enzyme was dissolved in 10 mL immobilization buffer (50 mM phosphate buffer, pH 8.0), and sonicated (5 sec on, 10 sec off pulse cycles for 1 min at 20% amplitude) to disrupt the cells. Two mL of sonicated crude enzyme was diluted to 5 mL with (50 mM pH 8.0) phosphate buffer and mixed with the 200 mg M-chelate-epoxy-support under appropriate conditions (25°C at 120 rpm). Periodically, samples were withdrawn and the enzyme content of the supernatant and BFD activity of supernatant were analyzed as described in what follows.

To determine the physically adsorbed BFD and its purity, after the enzyme is incubated in immobilization buffer (50 mM pH 8.0 phosphate buffer) with 200 mg M-chelate-epoxy-support at 30 min it is withdrawn by magnetic separation with the help of a permanent magnet. Then the immobilized enzyme is incubated in the presence of desorption solution (100 mM imidazol, 100 mM NaCl in 5.0 mM sodium phosphate buffer, pH 7.0) for 30 min. The protein amount in supernatant and the eluted enzyme solutions from the M-chelate-epoxy-support was determined by the Bradford method using the BSA (bovine serum albumin) standard protein assay.[40] Purity of the eluted enzyme from chelate-epoxy-support was checked by sodium dodecyl sulfate / polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

At the same time, in order to investigate the effect of incubation time on the covalently immobilization efficiency, 200 mg of the M-chelate-epoxy-support was added into incubation medium (2 mL of sonicated crude enzyme was diluted to 5 mL with 50mM phosphate buffer at pH 8.0) from 30-min to 30-h time intervals. The enzyme adsorbed M-chelate-epoxy-supports were withdrawn by magnetic separation with the help of a permanent magnet and washed two times with 50 mM phosphate buffer in order to remove the unbound protein molecules. Then the enzyme immobilized supports were incubated in the presence of desorption solution (100 mM imidazol and 100 mM NaCl in 5.0 mM sodium phosphate buffer, pH 7.0) and the suspension was left under stirring at 25°C for 30 min. The activities of the supernatant were analyzed to study the covalent immobilization (this treatment was enough to desorb the noncovalent adsorbed BFD from the fully modified M-chelate-epoxy-support). If there were no activity releases then covalent attachment was considered.

After the covalently enzyme immobilization, the remaining epoxide groups on the support were blocked with 5 mL of a 5% mercaptoethanol solution (pH 7.5) for 12 h at 20°C to prevent further nonspecific reactions, then the BFD attached to the M-chelate-epoxy-support was separated from the medium by a magnetic separation device and washed three times with 50 mM phosphate buffer pH 8.0, stored at 4°C, and then used for carboligation reaction. The schematic illustration for the preparation steps of immobilized BFD that was used for carboligation reactivity of benzoylformate decarboxylase is shown in Scheme 1.

Carboligation Reactions With Covalently Immobilized BFD

Benzoin condensation reaction. The activity of covalently immobilized BFD on magnetic solid support was determined from the initial formation rate of benzoin. Benzoin formation reaction was carried out as described in the literature[41] and our previous study.[30] 550 mg of the covalently immobilized enzyme on the M-chelate-epoxy-support was equilibrated with 5 mL reaction buffer containing 0.5 mM TPP, 2.0 mM $MgSO_4$, 25% dimethyl sulfoxide (DMSO) in 50 mM potassium phosphate buffer at pH 7.5. The reactions with immobilized enzyme were started under identical conditions with different benzaldehyde concentrations and also performed with free enzyme at 30°C with gentle shaking for 120 min. At 1-min time intervals, a 100 μL sample was withdrawn from the reaction mixture and quenched by addition of 100 μL DMSO. The amount of benzoin formed was analyzed by high-performance liquid chromatography (HPLC) analysis (Agilent 1100 series, Chiralpak AD column). The reaction mixture was extracted with dichloromethane (50 mL) and the

organic layer washed with water (5 mL) and brine (5 mL) and dried over Na_2SO_4. Evaporation of the solvent and purification of the crude product by crystallization afforded (*R*)-2-hydroxy-1,2-diphenylethan-1-one as a colorless solid; yield: 86 ± 3% (131 mg, 96 ± 3% *ee*); mp 135°C [Lit.[41] mp 133-134°C for (*R*)-enantiomer]; $[\alpha]_D^{22} = -112.1$ (c 1.5, CH_3COCH_3); [Lit.[41] $[\alpha]_D^{22} = -113.8$ (c 1.5, CH_3COCH_3). HPLC (Chiralpak AD) R_t(*R*):25.7 min. R_t(*S*):36.1 min. δ_H(400MHz; $CDCl_3/CCl_4$; Me_4Si) 7.8 (2H, d, *J*=7.8 Hz, Ph), 7.4 (1H, t, *J=7.5 Hz*, Ph), 7.3 (2H, t, *J=7.6 Hz*, Ph), 7.1-7.2 (5H, m, Ph), 5.7 (1H, d, *J*=5.9 Hz, CH), 4.42 (1H, *J*=5.9 Hz,, OH). δ_C(100MHz; $CDCl_3/CCl_4$; Me_4Si) 198.7, 139.1, 133.8, 133.6, 129.1, 129.0, 128.6, 128.5, 127.7, 76.2.

Synthesis of (S)-2-hydroxy-1-phenylpropanone [(S)-2-HPP][42]. The reaction with free and covalently immobilized enzyme was carried out according to the procedure described in the literature.[42] 715 mg of the covalently immobilized enzyme-support system was equilibrated with 5 mL reaction buffer (2 mM magnesium sulfate; 0.5 mM thiamin diphosphate, 50 mM pH 7.5 phosphate buffer) two times and supernatant was removed. The immobilized enzyme solution with a volume of 0.5 mL containing 715 mg of dry enzyme immobilized support was added to the 4.5 mL prepared substrate solution (pH 7.5; 50 mM potassium phosphate; 2 mM magnesium sulfate; 0.5 mM thiamin diphosphate; 40 mM benzaldehyde; 400 mM acetaldehyde). The reaction was carried out in a total reaction volume of 5 mL for 30 min at 20°C. Sampling was done after 30 min by stopping the stirring, allowing separation of the enzyme immobilized magnetic M-chelate-epoxy-support from the medium by a magnetic separation device, and taking a volume of 350 μL and analyzed by HPLC.

Work-up was performed as previously described.[35] Viscous oil, ee 96 ± 4%; $[\alpha]_D^{22} = -85.1$ (*c* 2.0, $CHCl_3$), HPLC (Chiralpak AD): 90:10 Hexane/ 2-propanol, 0.8 mL/min, UV detection at 250 nm, Rt (*S*) =12.5, Rt (*R*) =15.1; δ_H (400MHz; $CDCl_3/CCl_4$; Me_4Si) 7.9 (2H, m, Ph), 7.40-7.60 (3H, m, Ph), 5.1 (1H, q, *J*=6.0 Hz, CH), 3.8 (1H, br s, OH). δ_C(100MHz; $CDCl_3/CCl_4$; Me_4Si) 201.7, 134.3, 134.0, 128.9, 128.7, 69.2, 22.0.

Stability and reusability of immobilized BFD. To assess the stability and the reusability of the covalently immobilized BFD on to M-chelate-epoxy-support, repetitive batch experiments were carried out. Briefly, 715 mg enzyme immobilized magnetic support was incubated with 5 mL of the reaction solution containing 40 mM benzaldehyde and 400 mM acetaldehyde. Each batch was conducted for 65 min with four washing steps in between, which again took 15 min. For each period, the immobilized enzyme was separated with the help of a magnet and washed two times with potassium phosphate buffer at pH 7.5. Each washing step of collected immobilized enzyme was done with 1 mL of fresh buffer and incubated with fresh substrate for the next batch reaction. In total, five repetitive batches were performed and the whole experiment was done in 6.5 h. The reaction temperature was maintained at 20°C. Moreover, washed-out solutions were analyzed by the Bradford assay.

RESULTS AND DISCUSSION

Characterization

The FT-IR spectra of the pure IDA molecules, SMPs, and the SMPs reacted with GPTMS are shown in Figure 1. Although the characteristic band of the epoxy group at around 1150 cm^{-1} overlapped with the strong absorption of the bare silica, the alkyl C-H stretching vibration band at 2984 cm^{-1} and its bending vibration bands at 1370 cm^{-1} and 1452 cm^{-1}43–45 were clearly visible in the spectra of the modified nanoparticle (Fig. 1B). The characteristic peak of the N-H group in iminodiacetic acid was observed at 3090 cm^{-1}.[46] The results indicate that epoxysilane and IDA were attached successfully to the surface of SMPs.

The magnetic properties of the Fe_3O_4 and GMCNs were investigated by VSM analysis at room temperature. The saturation magnetization of pure magnetite nanoparticles (Fig. 2A) and the M-chelate-epoxy-support (Fig. 2B) were about 67.0 emu g^{-1} and 23.0 emu g^{-}1, respectively. The decrease in the saturation magnetization of nanoparticles after coating can be explained by the decrease in the amount of the magnetic moments per unit weight due to the diamagnetic contribution of silica shell.

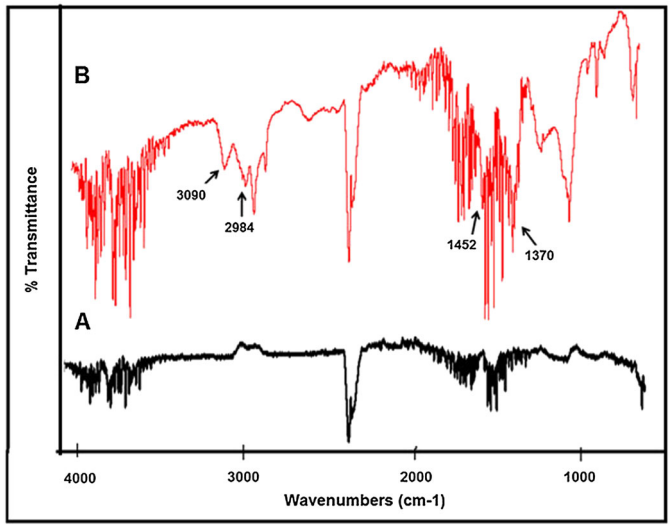


Fig. 1. FT-IR spectra for: (**A**) SMPs (**B**) IDA-Epoxy-functionalized SMPs.

The solid phase was separated from the mixture by magnetic separation with the help of a permanent magnet. As can be seen in Figure 2C, the M-chelate-epoxy-supports were highly responsive to a magnetic field, where the slurry was clarified in 20 s by using a permanent magnet.

Immobilization of BFD

Crude 6Xhis tagged benzoylformate decarboxylase (BFD) was immobilized on the M-chelate-epoxy-support in order to achieve one-step purification and covalent immobilization. The M-chelate-epoxy-support (200 mg) and IDA-epoxy-magnetic support (200 mg) was suspended in 5 mL phosphate buffer (50 mM pH 8.0) containing 200 mg crude enzyme and the M-chelate-epoxy-support (200 mg) was also incubated in the presence of 100 mM imidazol. The activities of the enzyme that remains in the supernatant during incubation with different supports under different conditions were analyzed and shown in Figure 3. The 6Xhis tagged BFD was immobilized quantitatively on the M-chelate-epoxy-support after 1 h of incubation time at pH 8.0 and low ionic strength (Fig. 3). Under the same conditions, we did not observe significant immobilization of the enzyme on an IDA-epoxy-magnetic support without Co^{2+} (Fig. 3). Similarly, in the presence of 100 mM imidazol, 6Xhis tagged BFD remained in the supernatant even when using the M-chelate-epoxy-support (Fig. 3). As shown in Figure 3, the enzyme loading onto the M-chelate-epoxy-support increased as the enzyme loading time increased up to 60 min, and remained constant in subsequent periods. These results suggest that previous physical adsorption of the enzyme is necessary to obtain covalent immobilization of proteins in epoxy supports (Scheme 1).[13,20,47,48]

In order to determine the amount of physically immobilized BFD, the M-chelate-epoxy-support was incubated with a crude extract of *E. coli* SG13009 containing BFD_{HIS} in the 100 mM phosphate buffer at pH 8.0 for 30 min. It was withdrawn by magnetic separation with the help of a permanent magnet. Then the enzyme immobilized on the M-chelate-epoxy-support was incubated in the presence of desorption solution (100 mM imidazol, 100 mM NaCl in 5.0 mM sodium phosphate buffer, pH 7.0) for

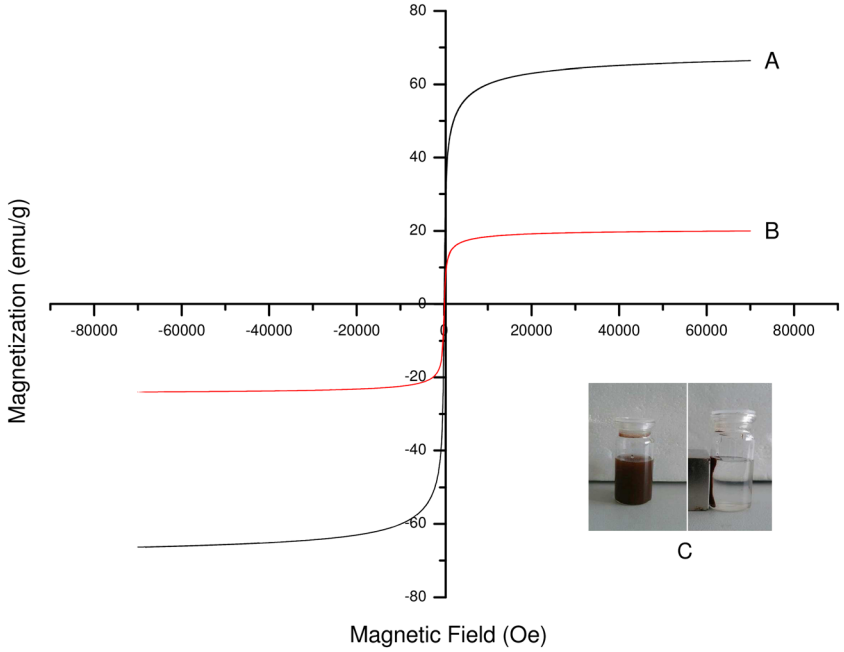


Fig. 2. Magnetization versus magnetic field for nanoparticles. (**A**) Fe_3O_4. (**B**) M-chelate-epoxy-support. (**C**) Response of the M-chelate-epoxy-support to the magnetic field.

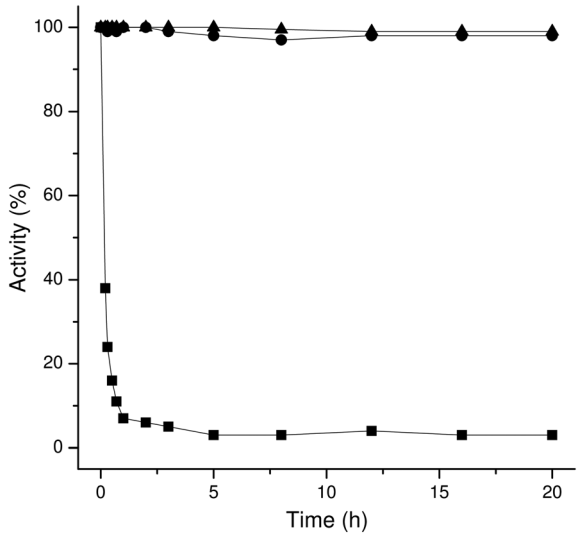


Fig. 3. The activity of the BFD that remains in the supernatant during incubation with different supports is shown under different conditions. (■) Immobilization on M-chelate-epoxy-support in 50 mM of sodium phosphate at pH 8.0; (▲) immobilization on M-chelate-epoxy-support in 50 mM of sodium phosphate at pH 7 in the presence of 100 mM imidazole; (●) immobilization on IDA-epoxy-magnetic support in 50 mM sodium phosphate at pH 7.

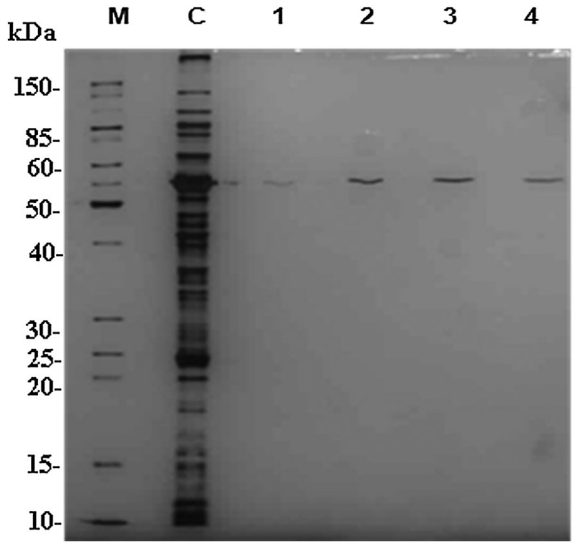


Fig. 4. SDS-PAGE analysis of the purified 6Xhis tagged BFD. M, SDS-PAGE molecular mass markers; lane C, crude extract of *E. coli* SG13009 containing BFD_{HIS}; lane 1–4, enzyme eluted from M-chelate-epoxy-support (100, 250, 700, 1500 μL).

30 min. The eluted enzyme solutions from the M-chelate-epoxy-support was determined by the Bradford method using BSA as a standard protein.[40] The amount of immobilized enzyme was ~12 mg per gram of support.

To determine the degree of purity of the immobilized enzyme, the enzyme adsorbed support (incubated in immobilization conditions at 60 min) was incubated in the presence of 100 mM imidazol and 100 mM NaCl in 5.0 mM sodium phosphate buffer, at pH 7.0. In this way, any molecule not covalently attached to the support was released into the desorption medium. Then SDS-PAGE analysis of the supernatant was performed (Fig. 4). Figure 4 shows that our M-chelate-epoxy-support was efficient for the selective binding of HIS-tagged BFD. Overexpressed BFD was observed as a correspondent band in the induced cell lane (56 kDa). As can be seen from Figure 4, a single line was observed in lane 1–4, which belongs to the BFD, although a series of many bands of different sizes was observed for a crude sample in lane C.

In order to analyze the covalent immobilization time for the BFD, the enzyme was incubated with M-chelate-epoxy-support in incubation medium. Periodically, BFD immobilized on M-chelate-epoxy-support was withdrawn, washed several times with 50 mM phosphate buffer (pH 7.0), and incubated in 100 mM imidazol and 100 mM NaCl in 5.0 mM sodium phosphate buffer (pH 7.0) for 30 min, which is enough to desorb all noncovalently attached BFD. The activity of the eluted

enzyme was analyzed as described in the Experimental section. The proper covalent immobilization time for the enzyme-resin system was determined as 21 h (Fig. 5). This result can be verified well with the high reusability of the BFD enzyme, as shown in Figure 5. The amount of desorbed protein was not changed after 21 h. The amount of covalently immobilized enzyme was ~8.5 mg per gram of support. The covalently immobilized enzyme on M-chelate-epoxy-support results in an activity decrease from 1.80 to 0.74 U/mg enzyme. The result given for activity analysis is an average value based on values from independent duplicate assays.

Benzoin Condensation Reaction

In the present study, the benzoin condensation reaction was performed with different benzaldehyde concentrations (Fig. 6). Benzaldehyde amounts were chosen as 30 mM, 50 mM, and 100 mM, and the benzoin concentration was determined by HPLC.[30] The immobilized enzyme activities for 30 mM, 50 mM, and 100 mM benzaldehyde amounts were determined as 2.4U, 3.5U, and 0.6U, respectively. The reaction was started with 3.5 mg free enzyme purified by Ni affinity chromatography and freshly applied for the benzoin condensation reaction. The free enzyme activity was determined as 3.5U with 50 mM benzaldehyde concentration (0.15 mM TPP, 2.5 mM $MgSO_4$, 50 mM pH:7.5 phosphate buffer). The reaction resulted in a 89 ± 4% yield and 99 ± 2% *ee* for free enzyme and 86 ± 3% yield and 96 ± 3% *ee* for immobilized enzyme after 45 min.

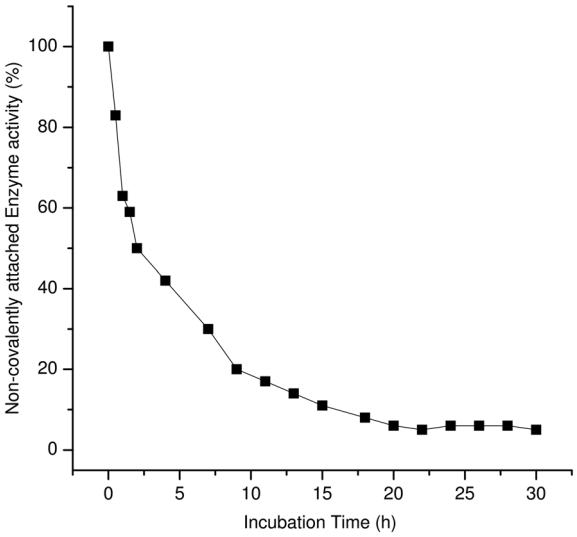
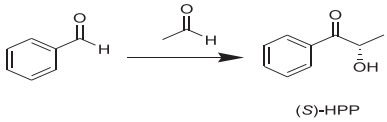
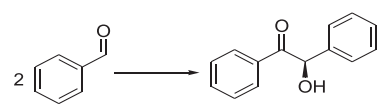


Fig. 5. Effect of incubation time on the covalent immobilization. Specific time intervals immobilized BFD on M-chelate-epoxy-supports were withdrawn and incubated with 100 mM imidazole and 100 mM NaCl for 30 min. The BFD activity of the eluted enzyme was analyzed.

Synthesis of (S)-2-Hydroxy-1-Phenylpropanone [(S)-2-HPP]

Carboligation reactions were performed for the formation of the cross-acyloin product (*S*)-2-HPP with free and immobilized BFD to find the effect of immobilization on enzyme activity. These reactions were screened with 4.5U immobilized and 4.5U free enzyme to obtain maximum product formation (the reaction was monitored by HPLC; Table 1).

TABLE 1. Yields and enantiomeric excess (ee) values for the immobilized BFD catalyzed reactions

Reaction	Reaction mode	Yield (%)	ee%
 (*S*)-HPP	Free (4.5U)	89±5	94±2
	Immobilized (4.5U)	88±3	96±4
 (*R*)- Benzoin	Free (3.5U)	89±4	99 ±2
	Immobilized (3.5U)	86±3	96 ±3

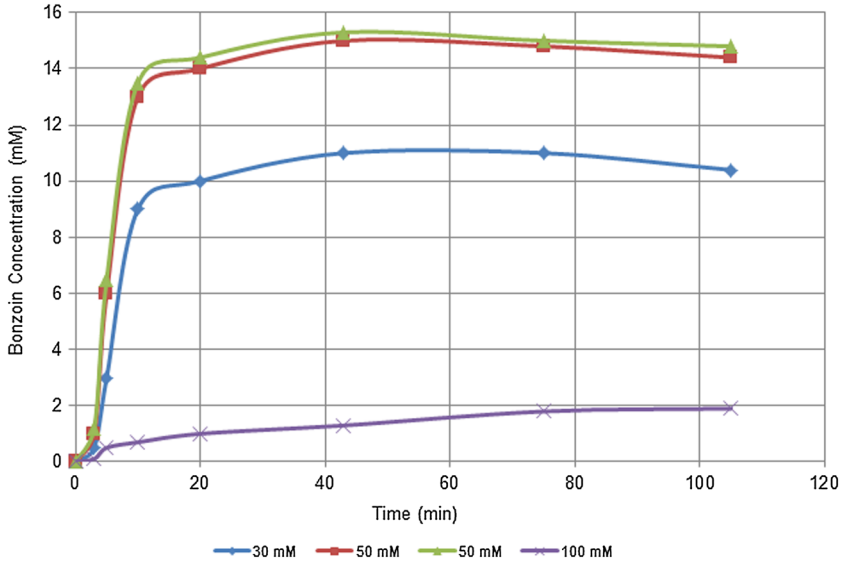


Fig. 6. Time course of the benzoin concentration during the reaction of free (Δ: 50 mM) and immobilized enzyme on M-chelate-epoxy-supports at a different benzaldehyde concentration (:30 mM :50 mM and X:100 mM), all reactions were monitored by HPLC.

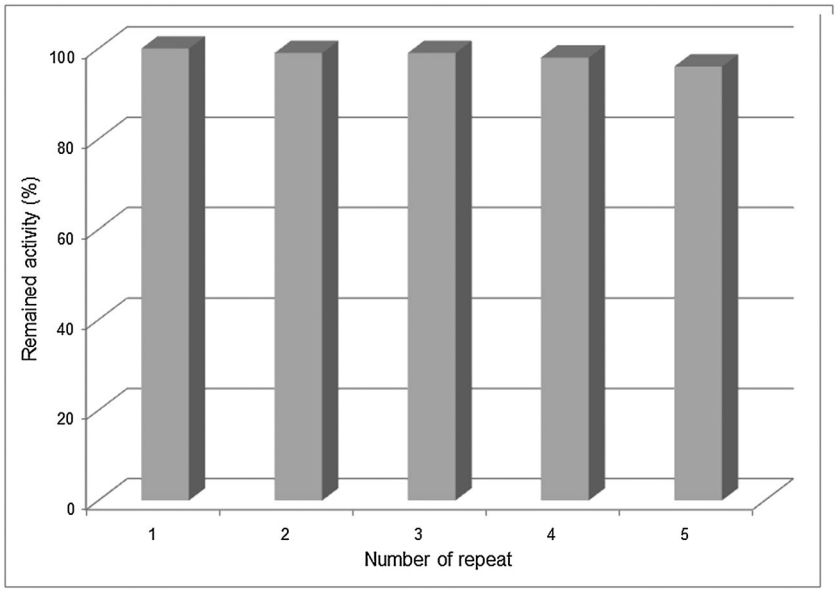


Fig. 7. Activity (%) after a repeated set of experiments with covalently immobilized BFD.

The *ee* of (*S*)-2-HPP synthesized by immobilized enzyme was determined to be 96 ± 4% (*S*)-*HPP*, which is in good agreement with the *ee* yielded by free enzyme [94 ± 2% (S)-HPP]. In Table 1 the results of the carboligation reactions are summarized.

The stability of BFD immobilized on the M-chelate-epoxy-support during batch synthesis of (*S*)-*2-HPP* was determined by the repeated use of the same biocatalyst for five reaction cycles. As shown in Figure 7, the residual activity of the immobilized BFD slightly decreased gradually with the increasing number of reaction cycles, and it retained 96% of initial activity after five batches of repeated use. This result indicated that the immobilized enzyme was very stable under operating conditions, which would be an advantage for industrial applications.

CONCLUSION

In this work, we took advantage of the two-step mechanism of immobilization of histidine-tagged recombinant benzoylformate decarboxylase (BFD, E.C. 4.1.1.7) in M-chelate-epoxy-support to develop a tool that allows the use of IMAC chromatography for protein purification and epoxy support for enzyme immobilization (Scheme 1). Thus, control of both steps (adsorption and immobilization) permits purification and immobilization of BFD to take place in one step. Since this magnetic M-chelate-epoxy-support is easily dispersive in medium, it is convenient and effective for protein binding and elution procedures. These magnetically responsive M-chelate-epoxy-supports can be used for the immobilization and easy separation of enzymes from a crude extract in order to obtain pure immobilized enzyme as well as a direct use for catalytical purposes. In addition, two important representative reactions were performed with this system in which the products were obtained in high chemical and optical yields. Additionally, the reusability of the immobilized enzyme (minimum 5 times without losing considerable activity) is another advantage compared to free enzyme. These results are a good demonstration of the fact that covalent attachment of the enzyme is achieved. To sum up, a heterocatalyst system-mediated acyloin reactions via C-C bond formation reactions with a high optical and chemical yield is presented in this study.

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