Journal of Molecular Catalysis B: Enzymatic 95 (2013) 41–47

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Purification and covalent immobilization of benzaldehyde lyase with heterofunctional chelate-epoxy modified magnetic nanoparticles and its carboligation reactivity

Bilsen Tural[a,*], Servet Tural[a], Erdal Ertaş[a], İbrahim Yalınkılıç[a], Ayhan S. Demir[b]

[a] Department of Chemistry, Faculty of Education, Dicle University, 21280 Diyarbakir, Turkey
[b] Department of Chemistry, Faculty of Arts and Sciences, Middle East Technical University, Ankara 06531, Turkey

ARTICLE INFO

Article history:
Received 19 February 2013
Received in revised form 22 May 2013
Accepted 25 May 2013
Available online 3 June 2013

Keywords:
Immobilized benzaldehyde lyase
Epoxy-chelate magnetic support
Metal-affinity magnetic nanoparticles
Selective immobilization
Multipoint covalent attachment
One step purification

ABSTRACT

In this work, histidine-tagged recombinant benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I (BAL, EC 4.1.2.38) was immobilized on the magnetically responsive epoxy-chelate magnetic support following a two-step mechanism; that is, the protein is physically adsorbed and then the covalent bonding takes place. This mechanism has been exploited to combine the selectivity of metal chelate affinity chromatography with the covalent immobilization capacity of epoxy supports. In this way, it has been possible to accomplish, in a simple manner, the purification, immobilization, and stabilization of a histidine-tagged recombinant benzaldehyde lyase. To fulfill this objective we prepared and characterized a multifunctional Co^{2+}-IDA-epoxy functionalized the silica coated magnetic nanoparticles (SCMPs) which are modified with glycidyloxypropyltrimethoxysilane (GPTMS) and iminodiacetic acid (IDA).

To test immobilized BAL, benzoin condensation reaction was performed with this magnetically responsive biocatalyst. The results obtained from the carboligation reaction that was performed with this simple and convenient heterogeneous biocatalyst were comparable to that of free-enzyme-catalyzed reaction.

Additional advantages are its reusability and it is easy to work with.

1. Introduction

Benzaldehydelyase (BAL, EC 4.1.2.38), a thiamine pyrophosphate (TPP) dependent enzyme, isolated from *Pseudomonas fluorescens* Biovar I, was first reported by Gonzales and Vicuna [1,2]. They showed that this strain can grow on benzoin as a sole carbon and energy source. The structure of BAL at a 2.6 Å resolution has been reported by Schulz and coworkers, in which they found that BAL is a homotetramer consisting of three domains as Dom-α, Dom-β, and Dom-γ for each monomer [3]. Residues from Dom-γ interact with the diphosphate moiety of thiamin diphosphate (ThDP) and magnesium, while those from Dom-α of a neighboring subunit bind to the pyrimidine ring of ThDP. Four ThDP molecules were located within the protein as well as four magnesium ions. The active center is defined by the thiazolium ring of ThDP, which sits in a deep pocket opening to the outer surface of the tetramer [3,4].

Benzaldehydelyase is a rather important biocatalyst for enantioselective carboligation reactions [5]. The enzyme can produce chiral α-hydroxyketones that are indispensable building blocks in the synthesis of several interesting compounds. Many reactions of the native and recombinant enzyme have been reported, in which the applicability of BAL has already been extended through a wide range of substrate spectra [6].

Immobilization is one of the most efficient methods for increasing the cost effectiveness of enzymatic reactions [7,8]. The stability and reusability of the immobilized enzyme bring several advantages. Functionalized magnetic nanoparticles (MPs) have been extensively used in the immobilization of many bioactive substances, such as proteins, peptides, enzymes, and antibodies [9]. MNPs 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 [10].

Metal-chelate affinity chromatography is a well-developed tool for industrial-scale purification of proteins fused to poly-His-tags [11–16]. In some cases, the adsorption of a poly-His-tagged protein on the chelate support is quite strong and could be used for enzyme immobilization [17]. However, the reversibility of the binding process may be a drawback when used to develop an industrial-scale immobilization procedure. Undesired release of the metals to the reaction media may also become a problem in many cases. For these reasons, immobilized-metal affinity chromatography (IMAC)

* Corresponding author. Tel.: +90 412 2488030/8915; fax: +90 412 2488257.
E-mail address: bilsentural@gmail.com (B. Tural).

1381-1177/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molcatb.2013.05.023

is used mainly for enzyme purification and not for protein immobilization.

On the other hand, one of the most suitable methods for industrial-scale immobilization of proteins is based on epoxy supports [18–20]. Epoxy supports present many advantages; for example, they are very stable, allowing for long-term storage, prolonged transport from manufacturer to consumer, and extended enzyme-support reaction periods. In addition, they are reactive with different moieties of proteins (amine, thiol, hydroxyl groups), yielding very stable protein-support bonds (secondary amine, thioether, ether). Moreover, the remaining epoxy groups may be easily blocked after enzyme immobilization with different compounds, yielding an inert surface.

The mechanism of enzyme immobilization in epoxy supports [21,22] provides new opportunities for coupling immobilization to purification. In fact, it has been described that adsorption of the protein on the epoxy resin is necessary to obtain a significant covalent immobilization because of the extremely low reactivity of the epoxy supports with soluble proteins [21–23]. Therefore, due to the low reactivity of the epoxy groups, immobilization of proteins on epoxy supports follows a two step mechanism: (1) the enzyme is hydrophobically adsorbed on a fairly hydrophobic support at very high ionic strength; and (2) the covalent bonding between the enzyme and the support proceeds. Using this premise, the use of multifunctional epoxy supports to immobilize proteins has been reported [24]. These epoxy supports have different moieties that are able to physically adsorb proteins via different structural features, plus a dense layer of epoxy groups able to covalently bind the enzyme. One of the multifunctional supports that may be easily produced is the metal chelate epoxy support [24–26]. These supports combine the good properties of epoxy supports for enzyme immobilization and stabilization with an increased possibility for IMAC chromatography for purification poly-His-tagged proteins.

In this study, chelate-epoxy modified SCMPs nanoparticles are optimized to achieve the one step purification, covalent immobilization, and stabilization via multipoint covalent attachment of 6XHis-tagged BAL. The chelate-epoxy modified SCMPs (the silica coated magnetite particles) give high yields during acyloin condensation reactions through the covalent immobilization of the BAL on a magnetically responsive epoxy-chelate support system. The epoxy-chelate modified SCMPs were fully characterized. Experimental conditions for surface modification, protein immobilization and purification were investigated and optimized. The BAL immobilized chelate-epoxy modified SCMPs were applied for the ligase activity of the BAL during the self condensation of benzaldehyde.

2. Materials and methods

2.1. Chemicals and materials

Escherichia coli BL21(DE3)pLysS carrying pUC19-BAL_{HIS} construct was used for BAL (EC. 4.1.2.38) production. The *E. coli* BL21 (DE3) PLysS strain (host to produce the recombinant BAL_{HIS}) was purchased from Invitrogen®. Plasmid was a generous gift from Dr. Martina Pohl (Institute of Biotechnology 2, Research center Jülich, Germany) [2,27,28]. Enzyme production was performed in a New Brunswick BioFlo110 Fermenter equipped with pH and temperature probes as well stirring rate controls.

2.2. Characterization

Silica coated magnetic nanoparticles were re-dispersed in pure water by sonication for 10 s. The particles were re-dispersed in pure water by sonication for 10 s and a drop of suspension was placed onto SPI Double Copper Grids 100/200. The particles were detected by transmission electron microscopy (TEM) (JEOL 2100 F, Japan) for particle size and morphology.

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

The particle sizes and distributions of the SCMPs were determined by using Dynamic Light Scattering Technique (Zeta SizerNano-ZS, Malvern). The measurement temperature was kept at 25 °C.

The BAL-catalyzed reaction was monitored by thin layer chromatography (TLC) on silica gel (E. Merck, Darmstad). The detection of spots was performed by both UV-absorption and phosphomolybdicacid (PMA). The product synthesized was identified by ^1H NMR and the ^{13}C NMR spectra were recorded by BRUKER DPX 400 MHz by using tetramethylsilane (TMS) as an internal standard and deutero-chloroform as a solvent. The reaction was followed by HPLC analysis (Agilent 1100 series).

2.3. Synthesis of epoxy-functionalized SCMPs

Magnetite nanoparticles were prepared by the chemical coprecipitation method [29]. A complete precipitation of Fe_3O_4 was achieved under alkaline conditions, while maintaining a molar ratio of Fe^{2+}:Fe^{3+} = 1:2 under a nitrogen gas environment to prevent critical oxidation. To obtain 1 g of Fe_3O_4 nanoparticles, 0.86 g of $FeCl_2{\cdot}4H_2O$ and 2.36 g of $FeCl_3{\cdot}6H_2O$ were dissolved under a N_2 atmosphere in 40 mL of deaerated deionized water with vigorous stirring (1000 rpm). As the solution was being heated to 80 °C, 5 mL of ammonium hydroxide was added. After 30 min, the resulting magnetite nanoparticles were obtained by putting the vessel on a permanent magnet and the supernatant was decanted. The nanoparticles were washed with deionized water six times (50 mL each time) to remove unreacted chemicals.

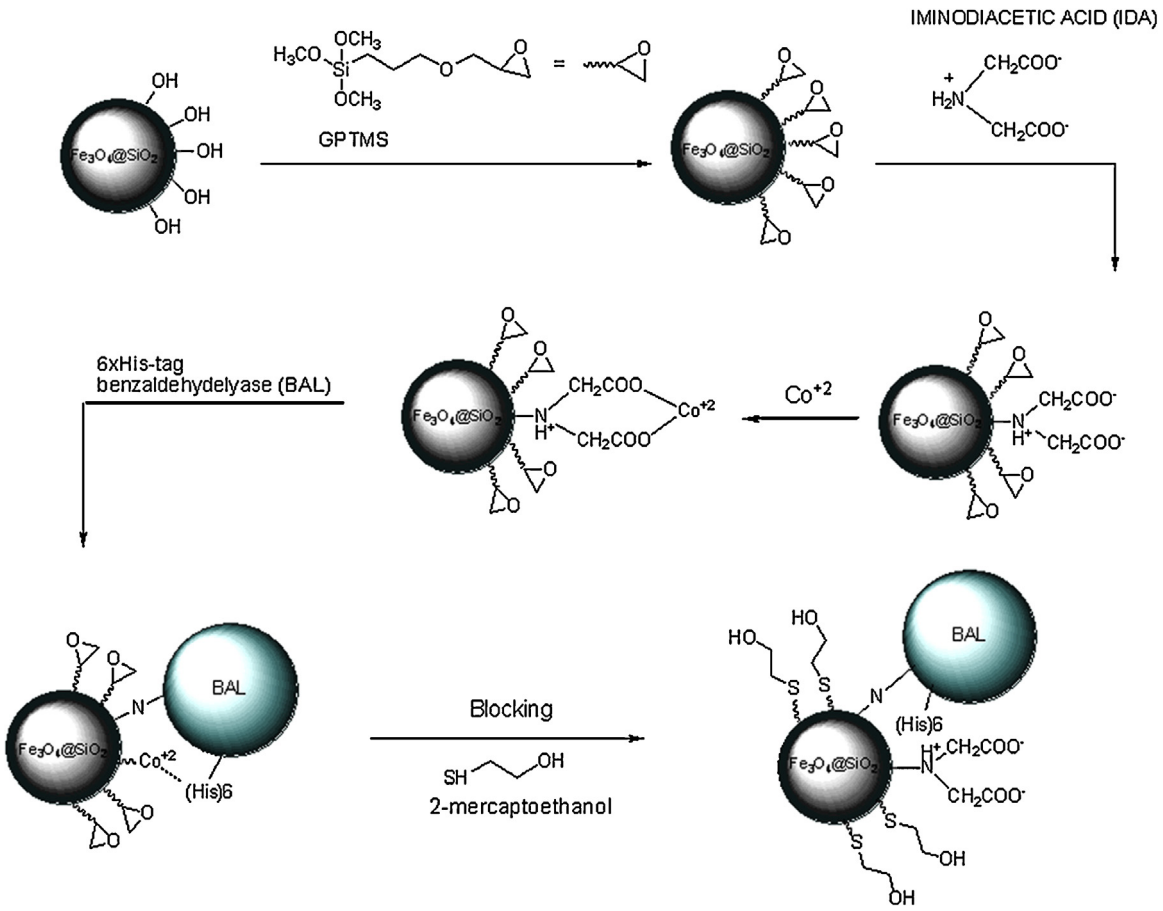
The Fe_3O_4 nanoparticles were coated with silica using the sol–gel method [29]. Typically, 30 mg of superparamagnetic Fe_3O_4 nanoparticles was dispersed in 80 mL of 2-propanol and 6 mL of deionized water by sonication for about 10 min. Then, under continuous mechanical stirring, 7 mL of ammonium hydroxide and 1 mL of TEOS were consecutively added to the reaction mixture. The reaction was allowed to proceed at room temperature for 12 h under continuous stirring. The resultant product was obtained by magnetic separation with the help of the permanent magnet and was thoroughly washed with deionized water six times (50 mL each time).

One gram of wet SCMPs was 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 the 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.

The schematic illustration for the preparation steps of the epoxy-functionalized SCMPs is shown in Scheme 1. The epoxy content of the SCMPs was determined using the pyridine–HCl method given in the literature [30]. This analysis indicated that 0.30 mmol epoxy groups were attached to per gram of SCMPs.

2.4. Preparation of IDA-epoxy-functionalized SCMPs

Epoxy functionalized SCMPs (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



Scheme 1. Preparation of Co^{2+}-IDA-epoxy-functionalized SCMPs and their use for immobilization of benzaldehyde lyase (BAL). 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.

was washed with an excess of distilled water and stored at 4 °C. The IDA-epoxy-functionalized SCMPs support was then incubated with Co^{2+} salt [24] to obtain the chelate epoxy supports. Briefly, IDA-epoxy-functionalized SCMPs support (1 g) was incubated in 50 mL of distilled water containing 2 M of Co^{2+} under very gentle stirring. After 2 h, the support was 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 support by treatment with 100 mM ethylene-diamine tetraacetic acid (EDTA) at pH 7. Co^{2+}-IDA-epoxy-functionalized SCMPs, contained 40 μmol of Co^{2+} (to adsorb the His-tagged BAL) and 0.26 mmol of epoxy groups per gram of support.

2.5. Immobilization of BAL

2.5.1. Preparation of recombinant BAL

BAL was grown through culturing *E. coli* BL21(DE3)pLyS strain containing pUC19-BAL [5,27,28] by using 1.5 L Luria Broth (LB) medium that contains the necessary antibiotics (35 μL/mL chloramphenicol and 100 μL/mL ampicillin) in a 2 L fermentor (New Brunswick BioFlo110) at 37 °C. BAL induction was initiated by the addition of Isopropyl-β-D-thiogalactopyronoside (IPTG). 6 h after the induction, the cells are harvested via centrifugation at 4 °C. Broken cells were lyophilized for 36 h. Enzyme was either used as crude form without purification or used as purified enzyme which is obtained by Ni^{2+}-NTA affinity chromatography (Invitrogen®) and desalting column (Amersham).

2.6. Activity assays

One unit activity of BAL is defined as the amount of enzyme necessary to catalyze the formation of 1 μmol of benzoin per minute under standard conditions (30 °C, pH: 7.5). BAL catalyzed benzoin condensation reactions were analyzed with HPLC.

2.7. Immobilization of BAL

200 mg lyophilized crude enzyme was dissolved in 10 mL (50 mM pH 8.0) phosphate buffer, and sonicated (5 s on, 10 s off pulse cycles for 1 min at 20% amplitude) to disrupt the cells. 2 mL of sonicated crude enzyme was diluted to 5 mL with (50 mM pH 8.0) phosphate buffer and mixed with the 200 mg Co^{2+}-IDA-epoxy-magnetic support under appropriate conditions (25 °C at 120 rpm). Periodically, samples were withdrawn and the enzyme content of the supernatant and BAL activity of supernatant were analyzed as described in what follows [31]. The amount of protein loaded to the Co^{2+}-IDA-epoxy-magnetic support was determined from the amount of unbound enzyme by the Bradford method by using bovine serum albumin as the standard [31].

To determine the physically adsorbed BAL and its purity, after the enzyme is incubated in immobilization buffer (50 mM pH 8.0 phosphate buffer) with 200 mg Co^{2+}-IDA-epoxy-magnetic support at 60 min it is withdrawn by magnetic separation with the help of the permanent magnet. Then, the immobilized enzyme is incubated in the presence of desorption solution (100 mM imidazol in 5.0 mM sodium phosphate buffer, pH 7.0) for 30 min and supernatant is checked by 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 epoxy-chelate magnetic sorbent was added into incubation medium (2 mL of sonicated crude enzyme was diluted to 5 mL with 50 mM phosphate buffer at pH 8.0) from 30 min to 24 h time intervals. The enzyme adsorbed magnetic supports were withdrawn by magnetic separation with the help of the 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 non-covalent adsorbed BAL from the fully modified Co^{2+}-IDA-epoxy-magnetic support). If there are 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) during 12 h at 20 °C to prevent further non-specific reactions and then, the BAL attached to chelate-epoxy-magnetic 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 BAL that was used for carboligation reactivity of benzaldehyde lyase is shown in Scheme 1.

2.8. Representative reaction with covalently immobilized BAL

The reaction with free and immobilized enzyme was carried out according to the procedure described in the literature [6].

Synthesis of (R)-2-hydroxy-1,2-diphenyl-1-one: BAL was immobilized to chelate-epoxy-magnetic support by following the procedure described above. Then, 3.5 mg BAL/650 mg the enzyme-support system was equilibrated with 5 mL reaction buffer (0.15 mM TPP, 2.5 mM $MgSO_4$, 50 mM pH 7.5 phosphate buffer) two times and supernatant was removed. The reaction was initiated with the different amount of benzaldehyde ranging from 20 mM to 100 mM. The mixture was incubated under the appropriate conditions (120 rpm, 37 °C) overnight. The reaction was monitored with TLC and stopped at 110 min. The mixture was extracted with ethyl acetate (3× 50 mL). The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to give desired compound. $[\alpha]_D^{22}$ −112 (c 1.5 in CH_3COCH_3). HPLC (Chiralpak AD) $R_t(R)$: 27.1 min. $R_t(S)$: 34.5 min. δ_H (400 MHz; $CDCl_3/CCl_4$; Me4Si) 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 (100 MHz; $CDCl_3/CCl_4$; Me4Si) 198.7, 139.1, 133.8, 133.6, 129.1, 129.0, 128.6, 128.5, 127.7, 76.2.

2.9. Stability and reusability of immobilized BAL

To assess the stability of the covalently immobilized BAL and their reusability for carboligation processes, benzoin condensation reaction was carried out for 5 consecutive batches with the same immobilized enzyme. Briefly, 650 mg of immobilized BAL was incubated with 5 mL 80 mM of benzaldehyde solution at 30 °C and pH 7.5, (0.15 mM TPP, 2.5 mM $MgSO_4$, 50 mM phosphate buffer, 20% DMSO). For each period, the immobilized enzyme was separated with the help of a magnet, washed two times with potassium phosphate buffer at pH 7.5, and incubated with fresh substrate for the next batch reaction. The residual activity of the immobilized biocatalyst was determined via determination of benzoin concentration by HPLC after each batch of reaction.

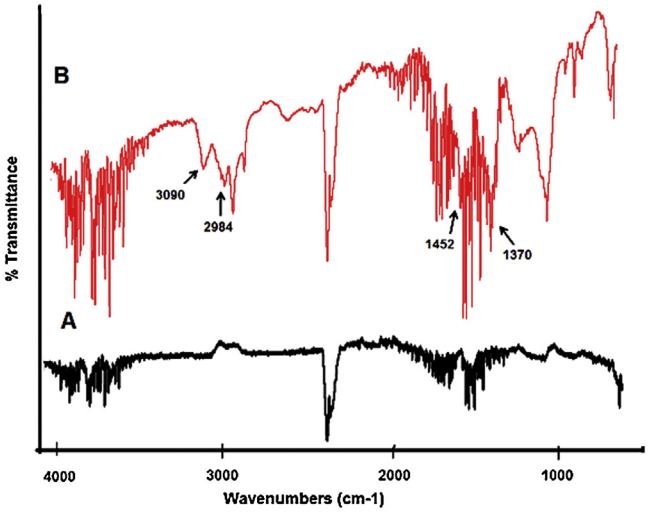


Fig. 1. FT-IR spectra for: (A) SCMPs and (B) IDA-epoxy-functionalized SCMPs.

3. Results and discussion

3.1. Characterization

Epoxy-IDA-functionalized SCMPs were characterized by FT-IR. Fig. 1 shows the FT-IR spectra of SCMPs and the nanoparticles reacted with GPTMS and IDA molecules. Although the characteristic band of 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} [32–35] were clearly visible in the spectra of the modified nanoparticle (B). The N—H absorption band in iminodiacetic acid is observed at 3090 cm^{-1} [36]. The results indicate that epoxysilane and IDA were attached successfully to the surface of SCMPs.

The image in Fig. 2A shows the TEM bright field micrograph for the SCMPs. As can be seen from the TEM image, the average size of the nanoparticles is approximately 11 nm.

The particle size distribution of SCMPs is shown in Fig. 2B. The average particles agglomeration size of SCMPs was ca. ~500 nm.

3.2. Immobilization of BAL

Crude 6Xhis tagged benzaldehyde lyase (BAL) was immobilized on the Co^{2+}-IDA-epoxy-magnetic support in order to achieve one pot purification-covalent immobilization steps. The BAL was immobilized quantitatively on the Co^{2+}-IDA-epoxy-magnetic support after 60 min of incubation time at pH 8.0 and low ionic strength (Fig. 3A). Under the same conditions, we did not observe significant immobilization of the enzyme on an IDA-epoxy-magnetic support without Co^{2+} (Fig. 3A). Similarly, in the presence of 100 mM imidazol, 6Xhis tagged BAL remained in the supernatant even when using the Co^{2+}-IDA-epoxy-magnetic support (Fig. 3A). These results suggest that previous physical adsorption of the enzyme is necessary to obtain covalent immobilization of proteins in epoxy supports (Scheme 1) [19,21,23,24]. In order to determine the amount of immobilized BAL, the Co^{2+}-IDA-epoxy-magnetic support was incubated with crude extract of *E. coli* BL21(DE3)pLysS containing BAL_{HIS} in the 100 mM phosphate buffer at pH 8.0. Periodically, 0.1 mL aliquot of supernatant was withdrawn for assay to determine the amount of immobilized enzyme. The amount of immobilized enzyme was calculated by the difference between the amount of enzyme in the supernatant before and after the immobilization procedure. The enzyme content was determined by the

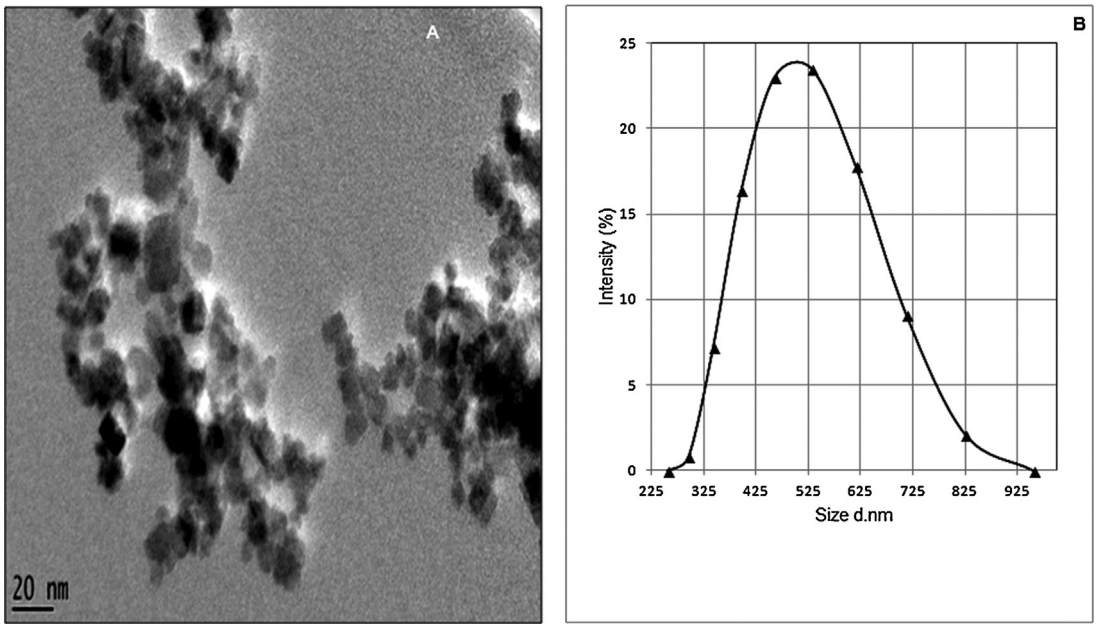


Fig. 2. (A) TEM graphs of SCMPs. (B) Particle size distribution for magnetic nanoparticles.

Bradford method using bovine serum albumin as a standard protein [31]. As observed in Fig. 3A, the enzyme loading onto the Co^{2+}-IDA-epoxy-magnetic support increased as the enzyme loading time increased up to 60 min, and remained constant in the subsequent periods. The maximum amount of immobilized enzyme was found as 5.3 mg per gram of support. Since the enzyme-support system was highly responsive to the magnetic field (Fig. 3B), samples were withdrawn after the slurry of the reaction was clarified by the application of the magnet.

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 (see Fig. 4). In Fig. 4 was showed that our Co^{2+}-IDA-epoxy-magnetic support were efficient for the selective binding of HIS-tagged BAL. Over expressed BAL was observed as correspondent band in the induced cell lane (56 kDa). As can be seen from Fig. 4, the single line was observed in lanes 2–4 which is belonged to the BAL although a series of many bands of different sizes was observed for a crude sample in lane 1.

In order to analyze the covalent immobilization time for the BAL, the enzyme was incubated with Co^{2+}-IDA-epoxy-magnetic support in incubation medium. Periodically, BAL immobilized on Co^{2+}-IDA-epoxy-magnetic support was withdrawn, washed several times with 50 mM phosphate buffer (pH 7.0) and incubated 100 mM imidazol and 100 mM NaCl in 5.0 mM sodium phosphate buffer

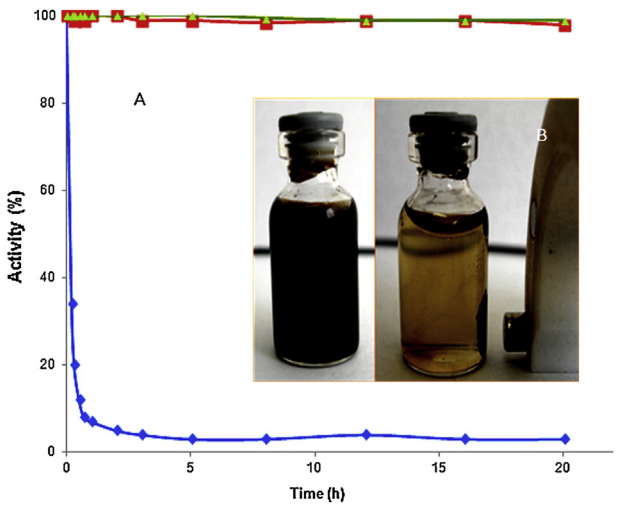


Fig. 3. (A) The activity of the BAL that remains in the supernatant during incubation with different supports is shown under different conditions. (♦) Immobilization on Co^{2+}-IDA-epoxy-magnetic support in 50 mM of sodium phosphate at pH 7.5; (■) immobilization on Co^{2+}-IDA-epoxy-magnetic support 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. (B) Response of the BAL-Co^{2+}-IDA-epoxy-magnetic support biocatalyst to the magnetic field.

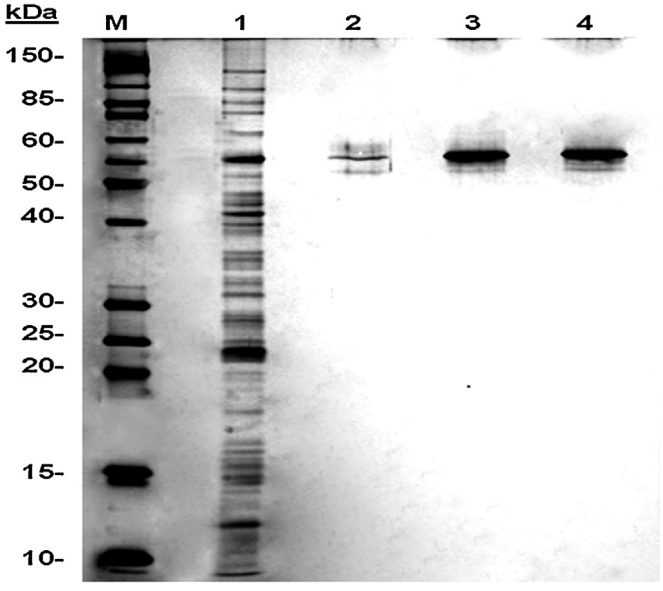


Fig. 4. SDS-PAGE analysis of the purified 6Xhis tagged BAL. M, SDS-PAGE molecular mass markers; lane 1, crude extract of *E. coli* BL21(DE3)pLysS containing BAL_{HIS}; lanes 2–4, protein eluted from 100, 700, 1500 μL crude extract loaded resin (50 μL sample from eluents).

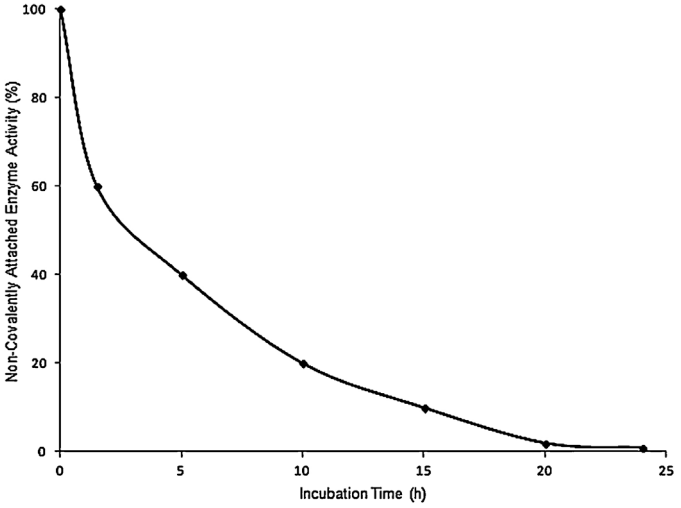


Fig. 5. Effect of incubation time on the covalent immobilization. Specific time intervals immobilized BAL on Co^{2+}-IDA-epoxy-magnetic support were withdrawn and incubated 100 mM imidazole and 100 mM NaCl at 30 min. The BAL activity of the supernatant was analyzed.

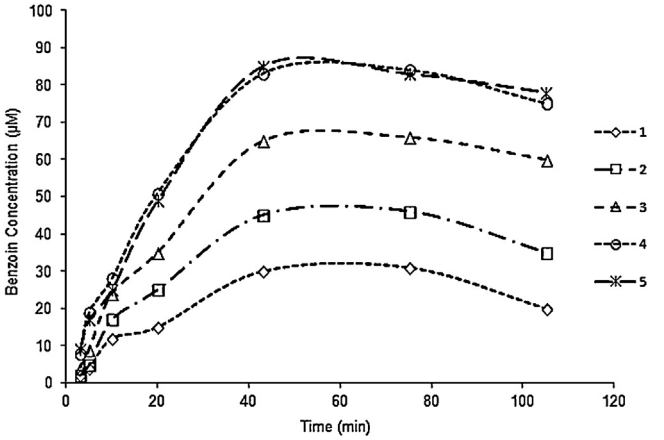


Fig. 6. Time course of the benzoin formation reaction with free enzyme (5.3 mg, 18 U) and immobilized BAL (650 mg of immobilized BAL on to magnetic support) at different benzaldehyde concentrations (50 mM phosphate buffer; 2,5 mM Mg^{2+}, 0.1 mM ThDP, 30 °C, 20% DMSO). (1) 20 mM; (2) 40 mM, (3) 60 mM, (4) 80 mM and (nr. 1, nr. 2, nr. 3 and nr. 4 with immobilized enzyme) and (5) 80 mM (with free enzyme).

(pH 7.0) at 30 min which is enough to desorb all non-covalently attached BAL. The BAL activity of the supernatant was analyzed as described in Section 2. The proper covalent immobilization time for the enzyme-resin system was determined as 20 h (see Fig. 5). No protein desorption was observed when the amount of protein was quantified at the end of the incubation time. This result can be verified well with the high reusability of the BAL enzyme as shown in Fig. 5.

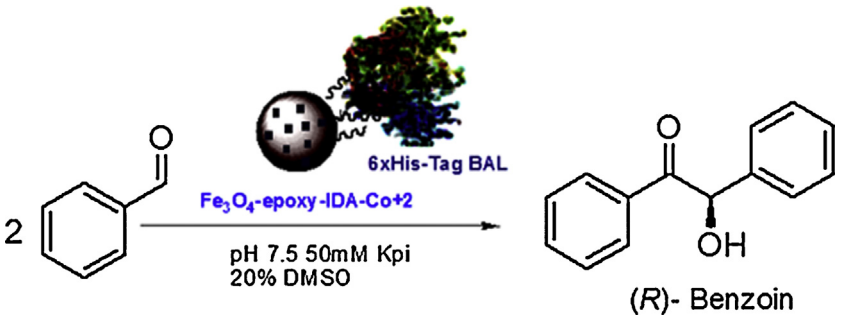
3.3. Benzoin condensation reaction

In the present study, we performed the benzoin condensation reaction with free and immobilized enzymes. The reactions were carried out until the desired conversions were achieved (the reaction was monitored by HPLC). The benzoin condensation reaction of covalently immobilized BAL was conducted in a batch synthesis (Scheme 2). A benzoin condensation reaction was performed with different benzaldehyde concentrations (Fig. 6) that were determined according to the literature [37–40].

Benzaldehyde amounts were chosen as 20 mM, 40 mM, 60 mM, 80 mM and enzyme activities determined as 3.0 U, 5.1 U, 7.4 U and 9.7 U for the 650 mg immobilized BAL. When 100 mM and 200 mM benzaldehyde amount were applied, we have observed that enzyme activities were decreased to 3 U and 0.7 U due to the enzyme inactivation and inhibition caused by the reaction product. The reaction conducted with 80 mM benzaldehyde resulted in a 87% yield and ≥96% *ee* after 43 min as a result of the higher catalytic activity of this reaction (9.7 U), in which a decline in the benzoin concentration occurred at subsequent time points due to product inhibition. In all the cases, the reactions were followed up to 110 min. The reaction was repeated with free enzyme upon obtaining the same results. The reactions performed with free enzyme were started with 18 U enzyme purified by Ni affinity chromatography and freshly applied for the reaction (0.15 mM TPP, 2.5 mM $MgSO_4$, 50 mM pH:7.5 phosphate buffer, 80 mM benzaldehyde). The free enzyme specific activity was determined as 3.4 U according to benzoin condensation reaction in 50 mM potassium phosphate buffer at pH 7.5 (0.15 mM TPP, 2.5 mM $MgSO_4$, 20% DMSO, 80 mM benzaldehyde).

The determined enzyme activities were higher even in the case of 40 mM benzaldehyde use compared to the literature values due to the increased stability of covalently immobilized enzyme on Co^{2+}-IDA-epoxy-magnetic support through multipoint covalent attachment [37].

Besides for the improved enzyme activities, immobilized enzyme systems offer the reusability of the enzyme (Fig. 7) that is the result of the improved stability of the enzyme. This stability can be achieved through multipoint covalent interaction between the enzyme and the support. Although enzymes can be recovered when immobilization was affinity supported, enzyme-support system gives limited reusability and after the 4th trial, a decrease in the enzyme activities was reported [37–40]. However, when BAL enzyme was immobilized onto Co^{2+}-IDA-epoxy-magnetic support through covalent interactions, a decrease in the activity was not determined even after the 5th trial that brings about increased conversions and selectivities during carboligation reactions. As a



Scheme 2. Covalently immobilized BAL catalyzed benzoin condensation reaction.

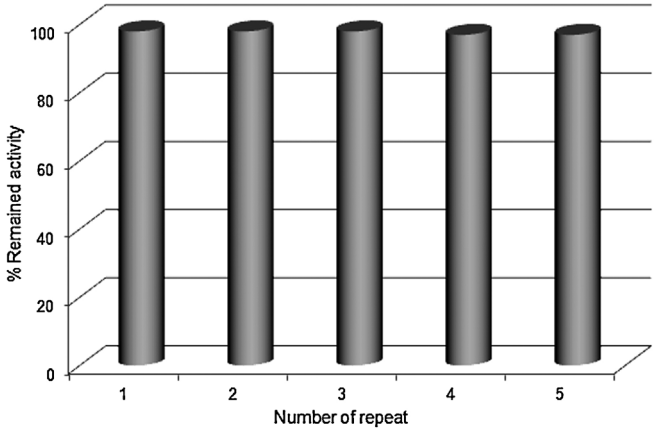


Fig. 7. Activity of BAL after a repeated set of experiments with covalently immobilized BAL.

result, the recovery of the enzyme compensated with the increased reusability through covalent interactions.

The benzoin condensation reaction was repeated with free and immobilized enzymes in another laboratory upon obtaining the same results.

4. Conclusion

In this study we have taken advantage of the two-step mechanism of immobilization of histidine-tagged recombinant benzaldehyde lyase (BAL, EC 4.1.2.38) in epoxy-chelate magnetic 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, immobilization, and stabilization of BAL to take place in one step.

This strategy produced the enhanced enzyme stability resulted in an increase in the enzyme activity to the highest value, which has not been reported previously for this class of enzymes. This system was used for the representative BAL catalyzed enantioselective homo- and cross coupling of aldehydes to form acyloin reactions via C—C-bond formation and bond cleavage in high chemical and optical yield ($\geq$96% *ee*, 87% yield). This reaction can be used for the synthesis of key synthons for pharmaceutically important compounds. The covalent immobilization offers high enzyme activity and stability (at least 5 repeats without losing its activity). These results are a good demonstration of the fact that covalent attachment of the enzyme is achieved.

Acknowledgements

This project is funded by the financial support from Middle East Technical University, Dicle University Research Fund (DUBAP, Project No. 13-ZEF-28, 10-ZEF-129, 10-ZEF-28 and 10-ZEF-128, 12-ZEF-25) and the Scientific and Technological Research Council of Turkey (TUBITAK, 109T497), COST Action CM0701.

References

[1] B. Gonzalez, R. Vicuna, J. Bacteriol. 171 (1989) 2401–2405.
[2] A.S. Demir, M. Pohl, E. Jansen, M. Müler, J. Chem. Soc. Perkin Trans. 1 (2001) 633–635.
[3] T.G. Mosbacher, M. Muelle, G.E. Schulz, FEBS J. 272 (2005) 6067–6076.
[4] Maraite, T. Schmidt, M.B. Ansörge-Schumacher, A.M. Brzozowski, G. Grogan, Acta Crystallogr. Sect. F: Struct. Biol. Cryst. Commun. 63 (2007) 546–548.
[5] P. Ayhan, I. Simsek, B. Cifci, A.S. Demir, Org. Biomol. Chem. 9 (2011) 2602–2605.
[6] A.S. Demir, P. Ayhan, S.B. Sopaci, Clean-Soil Air Water 35 (2007) 406–412.
[7] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, Enzyme Microb. Technol. 40 (2007) 1451–1463.
[8] R.A. Sheldon, Biochem. Soc. Trans. 35 (2007) 1583–1587.
[9] C.G.C.M. Netto, H.E. Toma, L.H. Andrade, J. Mol. Catal. B: Enzym. 85–86 (2013) 71–92.
[10] H. Gu, K. Xu, C. Xu, B. Xu, Chem. Commun. 9 (2006) 941–949.
[11] A.K. Johnson, A.M. Zawadzka, L.A. Deobald, R.L. Crawford, A.J. Paszczynski, J. Nanopart. Res. 10 (2008) 1009–1025.
[12] F.B. Anspach, J. Chromatogr. 672 (1994) 35–49.
[13] E.S. Hemdan, Y.J. Zhao, E. Sulkowski, Porath, J. Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 1811–1815.
[14] H. Hubert, J. Porath, J. Chromatogr. 198 (1980) 247–255.
[15] J. Porath, Protein Expres. Purif. 3 (1992) 263–281.
[16] M.Y. Wang, W.E. Bentley, V. Vakharia, Biotechnol. Bioeng. 43 (1994) 349–356.
[17] R. Woker, B. Champluvier, M.R. Kula, J. Chromatogr. 584 (1992) 85–92.
[18] B. Brena, L.G. Ryden, J. Porath, Biotechnol. Appl. Biochem. 19 (1994) 217–231.
[19] M.J. Hernaiz, D.H.G. Crout, Enzyme Microb. Technol. 27 (2000) 26–32.
[20] E. Katchalski-Katzir, D. Kraemer, J. Mol. Catal. B: Enzym. 10 (2000) 157–176.
[21] J.F. Kennedy, E.H.M. Melo, K. Jumel, Chem. Eng. Prog. 45 (1990) 81–89.
[22] K. Smalla, J. Turkova, J. Coupek, P. Herman, Biotechnol. Appl. Biochem. 10 (1988) 21–31.
[23] J.B. Wheatley, D.E. Schmidt, J. Chromatogr. A 849 (1999) 1–12.
[24] J.B. Wheatley, D.E. Schmidt, J. Chromatogr. 644 (1993) 11–16.
[25] C. Mateo, G. Fernandez-Lorente, R. Fernandez-Lafuente, E. Cortes, J.M. Guisan, J.L. Garcia, Biotechnol. Bioeng. 76 (2001) 269–276.
[26] C. Mateo, G. Fernandez-Lorente, O. Abian, R. Fernandez-Lafuente, J.M. Guisan, Biomacromolecules 1 (2000) 739–745.
[27] E. Janzen, M. Müller, D. Kolter-Jung, M.M. Kneen, M.J. McLeish, M. Pohl, Bioorg. Chem. 34 (2006) 345–361.
[28] A. Cosp, C. Dresen, M. Pohl, L. Walter, C. Röhr, M. Müller, Adv. Synth. Catal. 350 (2008) 759–771.
[29] C. Mateo, O. Abian, R. Fernandez-Lafuente, J.M. Guisan, Enzyme Microb. Technol. 26 (2000) 509–515.
[30] Z. Ma, Y. Guan, H. Liu, J. Magn. Magn. Mater. 301 (2006) 469–477.
[31] S. Sidney, in: M.M. Bradford (Ed.), Quantitative Organic Analysis, 3rd ed., Wiley, New York, 1967, p. 242.
[32] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[33] G. Schottner, Chem. Mater. 13 (2001) 3422–3435.
[34] Q. Zhang, R.F. Huang, L.H. Guo, Chin. Sci. Bull. 54 (2009) 2620–2626.
[35] M. Wanunu, S. Livne, A. Vaskevich, I. Rubinstein, Langmuir 22 (2006) 2130–2135.
[36] W.F. Chen, P.L. Kuo, Macromolecules 40 (2007) 1987–1994.
[37] W. Lijing, X. Xiangyu, D.G. Evans, X.D. Dianqing, J. Solid State Chem. 183 (2010) 1114–1119.
[38] B. Sopaci, I. Simsek, B. Tural, M. Volkan, A.S. Demir, Org. Biomol. Chem. 7 (2009) 1658–1664.
[39] N. Kurlemann, A. Liese, Tetrahedron: Asymmetry 15 (2004) 2955–2958.
[40] G. Drager, C. Kiss, U. Kunz, A. Kirschning, Org. Biomol. Chem. 5 (2007) 3657–3664.