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Tetrahedron: *Asymmetry* 15 (2004) 2955–2958
Tetrahedron: Asymmetry

Immobilization of benzaldehyde lyase and its application as a heterogeneous catalyst in the continuous synthesis of a chiral 2-hydroxy ketone

Nils Kurlemann and Andreas Liese*

Institute of Biochemistry, Westfälische Wilhelms-Universität Münster, 48149 Münster, Germany

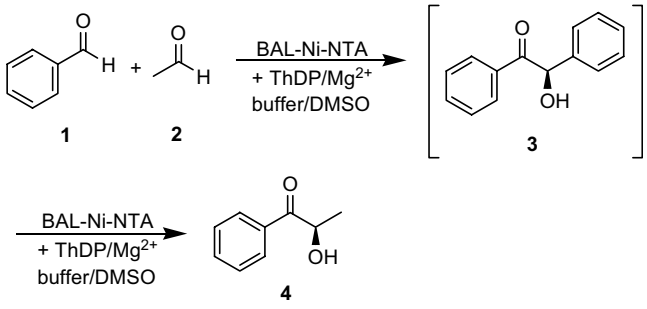
Received 12 May 2004; accepted 1 July 2004
Available online 11 September 2004

Abstract—Hexahistidine-tagged benzaldehyde lyase from *E. coli* SG13009/BAL_{HIS} was immobilized by means of metal ion affinity binding to a nickel(II)–nitrilotriacetic acid derivatized carrier and applied as a heterogeneous biocatalyst in the synthesis of (*R*)-2-hydroxy-1-phenyl-propanone. The applicability of the immobilization by metal ion affinity binding was proven in repetitive batch reactions and in a continuously operated plug flow reactor.

1. Introduction

Enantiopure 2-hydroxy ketones are important building blocks in the synthesis of biologically active compounds. Both (*R*)-2-hydroxy-l-phenyl-propanone **4** [(*R*)-HPP, Scheme 1] and its derivatives are applied as pharmaceutical intermediates.[1,2] Chemical routes towards hydroxy ketones include the stereoselective oxidation of enol ethers with OsO_4[3] and the regio- and enantioselective hydrogenation of 1,2-diketones.[4] The 2-hydroxy ketones are also synthesized by other biocatalytic methods, such as the oxidation of 1,2-diols into 2-hydroxy ketones by glycerol dehydrogenase[5] and the microbial reduction of 1,2-diketones.[6] An efficient approach is the stereoselective condensation of aldehydes catalyzed by the thiamine diphosphate (ThDP)-dependent enzymes benzaldehyde lyase and benzoyl formate decarboxylase yielding enantio-complementary 2-hydroxy ketones.[7,8] Benzaldehyde lyase (BAL, EC 4.3.2.38) from *Pseudomonas fluorescens* Biovar **1** is able to catalyze the enantioselective C–C-bond formation between benzaldehyde **1** and acetaldehyde **2**, leading to (*R*)-2-hydroxy-l-phenyl-propanone **4** with an ee greater than 99%.[9,10] Benzoin **3** is formed as an intermediate in low concentrations.

To improve its cost effectiveness a catalyst must be recovered after a discontinuous reaction. During continuous operation modes, the catalyst must be retained inside the reactor. This can be achieved by immobilizing soluble catalysts, that is, enzymes on a solid phase or by membrane retention.[11,12] In general, the immobilization of enzymes possesses many advantages such as stabilization, reusability of the enzymes and simplified isolation of the product.[13] One approach to controlled immobilization is to attach a tag—a peptide sequence with an affinity to a material—to the *N*- or *C*-terminus of a protein and take advantage of the specific interaction of the tag with a surface bound group.[14] Ideally the catalyst is reversibly bound to the solid phase. This enables the removal of the exhausted catalyst from the carrier without having to dismantle the reaction vessel, that is, a chromatography column. The solid carrier is



Scheme 1. Enantioselective synthesis of (*R*)-2-hydroxy-1-phenyl-propanone with histidine-tagged benzaldeyde lyase (BAL–Ni–NTA) immobilized on Ni–NTA–sepharose as a heterogeneous catalyst.

* Corresponding author. Tel.: +49 0251 83 33042; fax: +49 0251 83 32122; e-mail: aliese@uni-muenster.de

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doi:10.1016/j.tetasy.2004.07.039

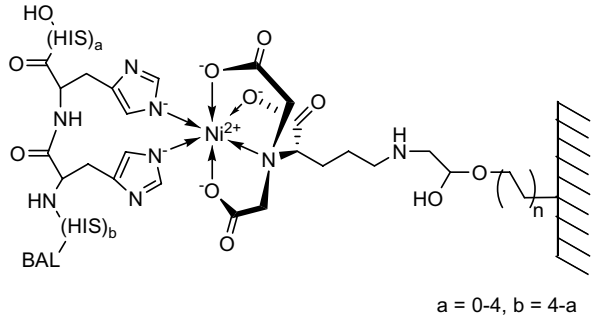
kept inside the reactor while replacing the catalyst, which considerably reduces set-up times. This should be advantageous in fields requiring high-priced components such as in the case of micro structured reactor systems: a reversible linking of enzymes can be achieved by complex formation between metal ions, that is, Ni^{2+}, Cu^{2+} or Ti^{2+} and the functional groups of certain amino acids like histidine and arginine.[15] The hexahistidine-tag (His_6-tag) interacts with the vacant coordination positions of nickel or copper chelates. Two neighbouring histidine amino acids attach strongly and reversibly to nickel(II)-ions,[16] which are bound to nitrilotriacetic acid (NTA)-derivatized agarose resin.

The His_6-tag was designed for protein purification through chromatography using Ni–NTA agarose beads as a packed bed. The tag is widely used in this field but also offers the possibility of employing the carrier bound enzymes as immobilized catalysts. In the field of carbohydrate chemistry, a few applications involving multiple enzymes immobilized on Ni–NTA agarose beads acting as heterogeneous biocatalysts have been reported.[17,18]

Benzaldehyde lyase with a hexahistidine tag was bound to Ni–NTA–Superflow (highly cross-linked agarose, Qiagen, Germany). The synthesis of (*R*)-2-hydroxy-l-phenyl-propanone **4** starting from benzaldehyde **1** and acetaldehyde **2** was carried out with immobilized benzaldehyde lyase in repetitive batch reactions and in a continuously operated plug flow reactor. To the best of our knowledge, this is the first example of an Ni–NTA immobilized enzyme being applied in the enantioselective C–C bond formation.

2. Results and discussion

After purification,[19] the recombinant His_6-tagged enzyme benzaldehyde lyase was immobilized on Ni–NTA–Superflow[20] (Scheme 2).



Scheme 2. Structure of the chelate complex formed by Ni–NTA and two histidine molecules from hexahistidine-tagged benzaldehyde lyase.

Both the purification and immobilization processes are based on the strong affinity of the imidazole group of histidine molecules to nickel(II)–nitrilotriacetic acid. The formation of the chelate complex is reversible. The histidine linker can be replaced by pure imidazole, which possesses a stronger affinity to the Ni^{2+} binding site. The immobilization of the enzyme on the Ni–NTA was almost quantitative. In the supernatant, a residual fraction of 1% protein was detected.

For continuous operation of a reactor containing an immobilized catalyst, the binding must be strong enough to avoid wash out of the catalyst. To ensure this, no other compound with heteroatoms in the reaction mixture should bind to the Ni–NTA carrier. A binding of neither HPP nor benzaldehyde to the Ni–NTA resin could be detected. On incubation with 5 mM thiamine diphosphate (a 10-fold excess compared to the reaction conditions), a protein concentration of 2% was observed in the supernatant. This points to an unspecific binding of ThDP to Ni–NTA resulting in a desorption of a small amount of enzyme.

To compare the ligase activities of immobilized BAL and homogeneously dissolved BAL, the biotransformation of acetaldehyde and benzaldehyde to (*R*)-HPP was performed with dissolved BAL and Ni–NTA bound BAL in batch reactors (Fig. 1).

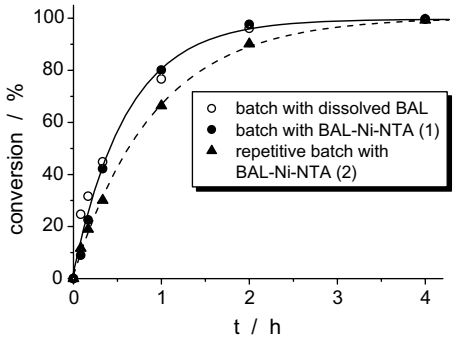


Figure 1. Repetitive batch synthesis of (*R*)-2-hydroxy-l-phenyl-propanone with immobilized benzaldehyde lyase (BAL–Ni–NTA) in KPG-stirred tank reactors (10 mg BAL [(**1**) and (**2**): 10 mg BAL on 1 mL Ni–NTA–Superflow], 50 mM triethanolamine buffer, pH = 8, 0.5 mM ThDP, 0.5 mM $MgSO_4$, 30 vol% DMSO, 10 mM benzaldehyde, 60 mM acetaldehyde).

The levels of conversion reached in the first batch reaction involving immobilized BAL (**1**) are comparable to those of the experiment applying dissolved enzyme. This demonstrates that the immobilization process does not alter the enzyme activity noticeably. The decrease in conversion observed during the second batch reaction with immobilized BAL (**2**) is probably due to material losses, which occurred during filtration and recovery of the catalyst rather than to any enzyme deactivation.

As a model for the plug flow reactor with heterogeneously immobilized biocatalyst, a packed bed of 7 mL in a flash chromatography column was established with BAL–Ni–NTA agarose and perfused with substrate-containing solution. The experiment was continuously operated for 95 h (Fig. 2). The given residence time τ is the quotient of the reactor volume and the flow rate.

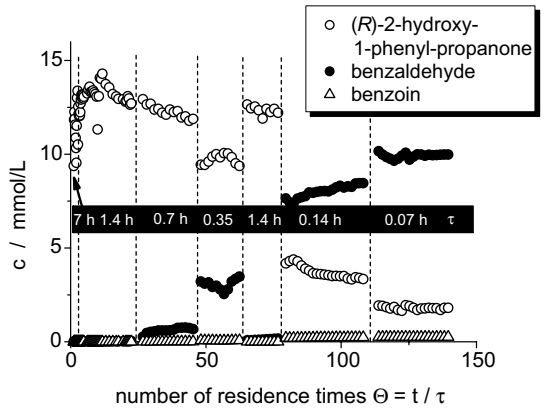


Figure 2. Continuous synthesis of (*R*)-2-hydroxy-l-phenyl-propanone in a plug flow reactor, the residence time is indicated in the graphic (50 mg BAL on 5 mL Ni–NTA–Superflow, 50 mM triethanolamine buffer, pH = 8, 0.5 mM ThDP, 0.5 mM $MgSO_4$, 30 vol% DMSO, 13 mM benzaldehyde, 60 mM acetaldehyde).

Different residence times were applied by changing the flow rate, yielding different levels of space–time yield (*sty*) and conversion (Fig. 3).

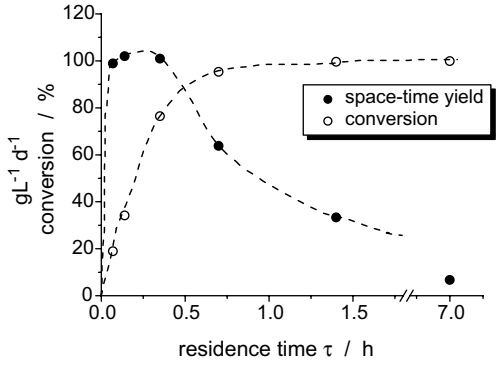


Figure 3. Space–time yield and conversion depending on the average residence time in the plug flow reactor. The data points correspond to the mean values obtained at different residence times and displayed in Figure 2. Dashed lines (---) are only drawn as visual aids.

Lower residence times lead to lower conversion and selectivity due to higher concentrations of both benzaldehyde and benzoin. A high level of selectivity for (*R*)-HPP is important because the maximum solubility of benzoin is 1.2 mM under the applied reaction conditions (30 vol% DMSO). Higher concentrations implicating a precipitation of benzoin would cause an obstruction of the enzyme molecules situated in the deeper sections of the polymer structures by blocking the channels of the porous agarose beads.

The highest space–time yields of 102 and 101 $g L^{-1} d^{-1}$ were reached at $\tau = 0.14$ and 0.35 h, respectively. At $\tau = 1.4$ h the conversion was above 99.5%. To achieve the optimal reaction conditions, both space–time yield and conversion must be taken into account.

Overall the activity of the enzyme decreases, leading to an overall reduction in conversion and yield. In a second continuous experiment involving the same reaction conditions, the residual protein in the packed bed reactor was quantified after 185 residence times. The BAL was eluted from the column with 250 mM imidazol solution (add 250 mM imidazol to 50 mM potassium phosphate buffer, pH = 7, 0.1 mM ThDP, 2 mM $MgSO_4$). The protein concentration corresponded to 78% of the total amount of enzyme suggesting a retention factor R_f >99.8% per residence time. The progressive decrease in enzyme activity is probably both due to thermal deactivation of the BAL and a slow process of washing out.

3. Conclusion

The recombinant hexahistidine-tagged enzyme benzaldehyde lyase was attached to Ni–NTA–Superflow by metal ion affinity binding, which proved to be a facile method for enzyme immobilization and thereby generation of a heterogeneous biocatalyst. The immobilized enzyme was applied in repetitive batch reactions to catalyze an enantioselective C–C-bond formation by synthesizing (*R*)-2-hydroxy-l-phenyl-propanone from benzaldehyde and acetaldehyde. The immobilized benzaldehyde lyase was employed as a heterogeneous biocatalyst in a packed bed in a continuously operated plug flow reactor for over 140 h and yielded (*R*)-2-hydroxy-l-phenyl-propanone in up to 102 $g L^{-1} d^{-1}$.

Acknowledgements

We thank Dipl.-Chem. S. Lütz from the Research Center Julich for generously providing the *E. coli* SG13009/BAL_{His} cells.

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20. The purification of benzaldehyde lyase was carried out as described previously.[19] The immobilization of BAL was carried out in a round-bottomed flask by suspending 1 mL (5 mL) Ni–NTA–Superflow (highly cross-linked agarose, Qiagen, Hilden, Germany) in 3 mL (15 mL) triethanolamine (TEA) buffer (50 mM, pH = 8) containing ThDP (0.5 mM), $MgSO_4$ (0.5 mM) and 30 vol% DMSO. After the addition of 10 mg (50 mg) lyophilized BAL, the suspension was stirred for 1 h with a KPG stirrer (a magnetic stirrer would disrupt the resin). After filtration, the immobilized BAL was washed several times with buffer solution and directly added to the batch or plug flow reactor. The determination of selectivity and conversion rates were performed via HPLC monitoring using a 25×0.4 cm RP-8 column (Merck, Darmstadt, Germany): 40% acetonitrile in aq 0.2% TEA-buffer, pH = 3, 1 mL/min, $\tau_{(HPP)} = 5.2$ min, $\tau_{(benzaldehyde)} = 8.0$ min, $\tau_{(benzoin)} = 11.2$ min.