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# Preparative application of 2-hydroxybiphenyl 3-monooxygenase with enzymatic cofactor regeneration in organic-aqueous reaction media

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**Abstract**

2-Hydroxybiphenyl 3-monooxygenase (HbpA, E.C. 1.14.13.44) was partially purified on a gram scale from recombinant *E. coli* JM101 by expanded bed adsorption (EBA) chromatography. In order to develop an effective in vitro biotransformation system using isolated HbpA as catalyst, its stability and activity in the presence of different apolar and polar organic solvents was investigated. HbpA showed good stability in 80% (v/v) decanol and 10% (v/v) methanol. Since the reaction catalyzed by HbpA is cofactor dependent, enzymatic NADH cofactor regeneration by yeast alcohol dehydrogenase (YADH) was tested in the presence of 10% (v/v) methanol used both as a cosolvent and substrate for YADH. In an in vitro biotransformation on a 25 ml scale 5.4 mM of 2,2′-dihydroxybiphenyl was hydroxylated to 2,2′,3-trihydroxybiphenyl in 2.5 h and 2,2′,3,3′-tetrahydroxybiphenyl in 23 h at a mean volumetric productivity of 0.43 g/(l h) and 0.05 g/(l h), respectively. Additionally, HbpA and formate dehydrogenase (FDH) were applied in 80% (v/v) decanol to hydroxylate 2-hydroxybiphenyl with a productivity of 0.46 g/(l h) and a total turnover number of 503. In 10% (v/v) aqueous methanol the total turnover number of 30 and enzyme stability for at least 60 h were shown. This demonstrated the feasibility of using isolated HbpA for syntheses in the presence of polar as well as apolar solvents with enzymatic regeneration of reduced cofactors.


*Keywords:* Monooxygenase; Organic solvent tolerance; NADH regeneration; In vitro biotransformation; Expanded bed adsorption chromatography

## 1. Introduction

The interest in biocatalysts able to hydroxylate organic compounds has significantly increased in recent years [1]. Oxygenases are known to catalyze hydroxylations with high selectivity which is difficult to achieve by chemical means. As oxygenases require expensive cofactors, usually NAD(P)H, in stoichiometric amounts, these enzymes are mostly used in whole cell biotransformations [2]. However, independent optimization of enzyme production and biotransformation reactions can better be achieved by in vitro approaches. Enzymes have to be at least partially purified from crude extracts in preparative quantities to avoid enzyme degradation and to remove remaining cell debris. In addition, economical syntheses necessitate efficient cofactor regeneration systems [3,4].

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In this paper we report the production of 2-hydroxybiphenyl 3-monooxygenase (HbpA, E.C. 1.14.13.44) and its application in a cell-free biotransformation. The soluble and NADH-dependent monooxygenase HbpA catalyzes the specific *ortho*-hydroxylation of different 2-substituted phenols [5–7]. The resulting catechols are high value substances and of interest for the synthesis of pharmaceutical compounds, like, e.g. hydroxyl or peroxide radical scavengers [8–10]. Recently, HbpA was partially enriched by anionic exchange chromatography using the expanded bed adsorption (EBA) technique [11,12].

We have optimized the protocol for enzyme purification and tested the stability of HbpA in the presence of different polar and apolar solvents. The solvents were added to increase the solubility of substrates and formed monophasic or biphasic organic-aqueous reaction systems. For enzymatic cofactor regeneration, we used yeast alcohol dehydrogenase (YADH) in a monophasic system and examined formate dehydrogenase (FDH) stability in apolar solvents [12].

HbpA was used in the presence of YADH in a monophasic biotransformation system (Fig. 1) for the hydroxylation of 2,2′-dihydroxybiphenyl at different concentrations of $NAD^+$ and NADH, using methanol serving both as a cosolvent and substrate for YADH. The application of the FDH system in biphasic organic-aqueous reaction media has been recently described by Schmid et al. [12]. In the present work, we compared the stability of both FDH and HbpA in different apolar organic cosolvents. In a biotransformation system containing HbpA and FDH, formate was added stepwise to increase the total turnover number of the cofactor. Finally, we discuss our results with respect to other preparative in vitro applications of oxygenases.

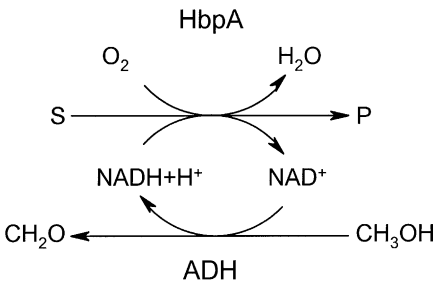



Fig. 1. Reaction principle of biotransformations with cofactor regeneration using ADH in presence of methanol as cosolvent and substrate (S: substrate, P: product, HbpA: 2-hydroxybiphenyl 3-monooxygenase, ADH: alcohol dehydrogenase).

## 2. Experimental

### 2.1. Materials

Yeast alcohol dehydrogenase, $NAD^+$ and NADH were obtained from Sigma (St. Louis, MO, USA). FDH of *Candida boidinii* was purchased from Fluka (Buchs, Switzerland) and 2,2′-dihydroxybiphenyl was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were obtained from Fluka, Sigma or Merck. Organic solvents, salts and buffer components were of the highest quality available.

### 2.2. Enzyme preparation

Cultivation of *E. coli* JM101 (pHBP461) [13] on a 30 l scale was done as described previously [8]. Preparation of HbpA was based on a modified protocol of Schmid et al. [12]. Two hundred grams of *E. coli* cell paste was suspended in 800 ml of 20 mM Tris–HCl, pH 7.5, containing 0.5 mM protease inhibitor phenylmethylsulfonylfluoride. The cells were disrupted in a continuous mode in a cooled 0.6 l bead mill with a flow rate of 2.5 l/h (Dyno-Mill, type KDL, Willy A. Bachofen, Basel, Switzerland) filled up to 87 (vol.%) with glass beads of 0.1–0.25 mM diameter and operated at 3000 rpm. Ten Units/ml of Benzonase (Merck, Darmstadt, Germany) and 1.25 mM $MgSO_4$ were added to reduce viscosity and the mixture was incubated on ice for 1 h. The crude extract was diluted with buffer to 3 l and stored on ice until loading on the column.

For preparative scale enrichment of HbpA a Streamline 50 column (Amersham Biosciences, Uppsala, Sweden) filled with 510 ml Streamline DEAE anion exchanger was used. The column was equilibrated at room temperature with 50 mM NaCl in 20 mM Tris (pH 7.5) with an upward flow rate of 200 cm/h forming a stable expanded bed with a 1.9-fold expansion. During sample loading the degree of expansion increased to 2.2. One-third of the sample volume was recycled and loaded on the column again to ensure proper binding. After washing, the column elution in packed bed mode was started by raising the NaCl

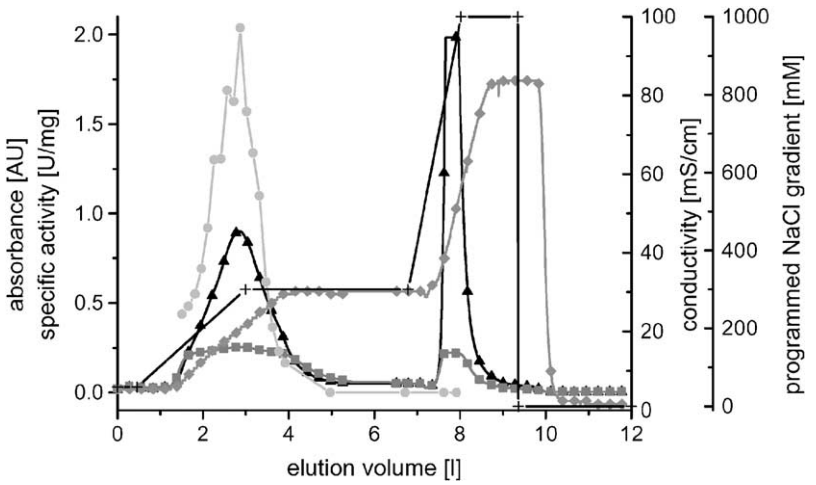



Fig. 2. EBA chromatogram of *E. coli* JM101 (pHBP461) crude extract for enrichment of HbpA with Streamline$^{TM}$ DEAE. Legends: (●) specific HbpA activity; (■) absorbance at 450 nm; (▲) absorbance at 280 nm; (◆) recorded conductivity; (+) programmed NaCl gradient.

concentration from 50 to 300 mM over 5 column volumes and holding this concentration for 8 column volumes at a downward flow rate of 200 cm/h (Fig. 2). Conductivity and absorbance at 280 and 450 nm were recorded. Fractions of 150 ml volume were collected on ice. Seven fractions with the highest activities were pooled and stored at −20 °C.

### 2.3. Activity assays, protein determination and SDS PAGE

HbpA activity was measured by recording NADH oxidation at 340 nm (ATI Unicam UV/Vis spectrometer UV4, Unicam vision software) in 20 mM phosphate buffer (pH 7.2), containing 0.2 mM NADH and approximately 10–50 μg protein at 30 °C. Non-specific oxidation of NADH was measured for 1 min to establish the baseline. Substrate specific oxidation of NADH was measured for 1 min after adding 0.1 mM 2-hydroxybiphenyl (100 mM methanol stock solution). The activity was calculated from the difference in activity before and after substrate addition using the NADH extinction coefficient of $\varepsilon_{340} = 6.22/(\text{mM cm})$. FDH activity was determined spectrophotometrically as described previously [12].

Protein concentrations were determined according to the Bio-Rad protein microassay (Bio-Rad, Munich, Germany). Enrichment of HbpA was checked by SDS PAGE (12% acrylamide) and gels were stained with Coomassie Brilliant Blue G-250.

### 2.4. Stability tests of HbpA and FDH in organic solvents

The stability of HbpA and FDH was examined in the presence of different organic solvents with different log *P* values (−1.0–8.8) [14]. The tested solvents are listed in Table 1. HbpA was incubated for 60 min in polar solvents (*N*,*N*-dimethylformamide and methanol) at different solvent concentrations (0–30% (v/v)). Enzyme activities in these monophasic organic-aqueous systems were determined spectrophotometrically.

The effect of 80% (v/v) of apolar solvents on the activity of HbpA and FDH was measured by incubating the enzymes in the biphasic systems for up to 8 h under stirring at 30 °C. After separation of the organic and aqueous phases by centrifugation (15,800 g for 10 s), the enzyme activities were measured spectrophotometrically.

### 2.5. Biotransformation with cofactor regeneration

#### 2.5.1. Optimization of cofactor concentrations

HbpA (0.5 mg powder, 0.025 U/mg powder), YADH (0.5 mg, 250 U/mg) and different concentrations of $NAD^+$ or NADH (0.14–0.75 mM) were dissolved

Table 1
Properties of different solvents

| Solvent | Costs (US$/l)[a] | Boiling point (°C) | Density (g/cm$^3$) | Vapor pressure (mbar) | log *P* [14] |
|---|---|---|---|---|---|
| *N*,*N*-dimethylformamide[b] | 11.6 | 153 | 0.944 | 26.0 | −1.00 |
| Methanol[c] | 5.5 | 65 | 0.786 | 169.3[d] | −0.76 |
| Octanol[c] | 74.9 | 194 | 0.822 | 0.1[d] | 2.90 |
| Hexane[c] | 15.6 | 69 | 0.655 | 201.7[d] | 3.50 |
| Decanol[c] | 14.9 | 231 | 0.830 | 0.1 | 4.00 |
| Heptane[c] | 13.3 | 98 | 0.679 | 60.9[d] | 4.00 |
| Octane[b] | 75.3 | 126 | 0.703 | 14.5 | 4.50 |
| Hexadecane[b] | 118.3 | 287 | 0.773 | n.d.[e] | 8.80 |

[a] Prices of www.acros.be, based on >99% quality and 0.5–10 l quantity (2001–2002).
[b] Chemfinder: http://chemfinder.cambridgesoft.com.
[c] Solvent database: http://solvdb.ncms.org.
[d] 25 °C.
[e] n.d.: no data.

in 1 ml of 20 mM potassium phosphate buffer, pH 7.5, containing 5.4 mM 2,2′-dihydroxybiphenyl. The reaction was run at room temperature. Periodically, aliquots of the reaction mixture were taken and diluted with acetonitrile to obtain substrate and product concentrations appropriate for analysis by HPLC. The samples (10–20 μl) were analyzed on a Shimadzu VP-series HPLC system with a photodiode array detector (SPD-M10A) using an Adsorbosphere XL C18 column (Alltech, 4.6 mM × 150 mM) and a linear water/acetonitrile gradient (30–100% acetonitrile over 8 min at 1 ml/min flow rate). To confirm the identity of reaction products, samples were analyzed by flow injection mass spectrometry on a PE-Sciex API100 electrospray mass spectrometer with turbo-ion spray head by collecting mass spectra in the negative mode.

#### *2.5.2. Preparative scale biotransformation with cofactor regeneration by ADH*

HbpA (50 mg powder, 0.025 U/mg powder), YADH (50 mg, 250 U/mg) and NADH (20 mg or 28 μmol) were dissolved in 25 ml of 20 mM potassium phosphate buffer, pH 7.5, containing 5.4 mM 2,2′-dihydroxybiphenyl. The reaction was run at room temperature. Periodically, aliquots of the reaction mixture were taken, diluted with acetonitrile and analyzed by HPLC as described in Section 2.4.

#### *2.5.3. Preparative scale biotransformation with cofactor regeneration by FDH*

HbpA and FDH were used as catalysts in a two liquid phase biotransformation in 80% (v/v) on a 200 ml scale to hydroxylate 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl [12]. The initial formate concentration in the aqueous phase was 50 mM. Sodium formate was added (10 mM, 27.2 mg) 11 times every 45 min.

## 3. Results and discussion

### *3.1. Enzyme preparation*

Efficient and simple methods are necessary to provide enzymes on a gram scale for synthetic applications. This was achieved for 2-hydroxybiphenyl 3-monooxygenase (HbpA) using EBA chromatography. One chromatographic step clarified the crude *E. coli* JM101 (pHBP461) extract and reduced the volume to one third. The elution profile of HbpA from anionic exchange Streamline$^{TM}$ DEAE material is shown in Fig. 2. Fractions containing HbpA were yellow in color and specifically absorbed at 450 nm, due to the cofactor FAD present in the enzyme. Activity was detected in the broad first peak, eluted at a calculated NaCl concentration of 140 mM. From 200 g cell wet weight *E. coli*, 5.7 g protein with a specific activity of 1.7 U/mg and 59% of initial activity (Table 2) were recovered. Assuming the specific activity of pure HbpA of 3.5 U/mg [5], HbpA was approximately 50% pure. The enrichment of HbpA from the crude extract by EBA chromatography was confirmed by SDS PAGE (Fig. 3). The unbound HbpA protein eluted in the void volume (“flow through”),

Table 2
Enrichment of 2-hydroxybiphenyl 3-monooxygenase overexpressed in *E. coli* via EBA chromatography

| Fraction[a] | Total volume (ml) | Total protein (mg) | Total activity (U) | Specific activity (U/mg) | Recovery (%) | Purification factor (–) |
|---|---|---|---|---|---|---|
| Crude extract | 3000 | 16830 | 16230 | 0.96 | 100 | 1.00 |
| Anion exchange[b] | 1028 | 5747 | 9622 | 1.67 | 59 | 1.74 |
| Flow through[c] | 4881 | 4557 | 498 | 0.11 | 3.0 | – |

[a] Conditions as described under experimental.
[b] Seven most active fractions were pooled.
[c] Unbound proteins during loading/washing of the column.

presumably as inactive inclusion bodies, because its activity corresponded to only 3% of the total activity. Dialysis was used to remove high chloride concentrations which inhibit HbpA [5]. The enzyme fraction could be used directly for in vitro biotransformations or stored at −18 °C. These results demonstrate that EBA is an effective one step method for the large scale enrichment of HbpA for cell free biotransformations.

### 3.2. Apolar solvents

Apolar or polar organic solvents can be added to reaction mixtures to increase solubilities of hydrophobic substrates and products. However, organic solvents often reduce enzyme activities [15]. The stability of HbpA and FDH in the presence of 80% (v/v) apolar solvents with different log *P* values was examined in order to find a suitable apolar solvent for the HbpA/FDH bienzyme system in an organic-aqueous two-phase reaction medium (Fig. 4). The only solvent in which HbpA and FDH showed good activity after incubation for 8 h was decanol. The residual activity was approximately 100% of the initial activity for HbpA and 45% for FDH. In general, HbpA is less tolerant than FDH towards different apolar solvents. This is in accordance with the findings of Schmid et al. [12] who compared activities of HbpA and FDH in 50% (v/v) organic solvents. Although decanol is a relatively expensive solvent, its physical properties like vapor pressure, high boiling point and suitable partition coefficients for hydrophobic substrates make it valuable for applications in two liquid phase biotransformations.

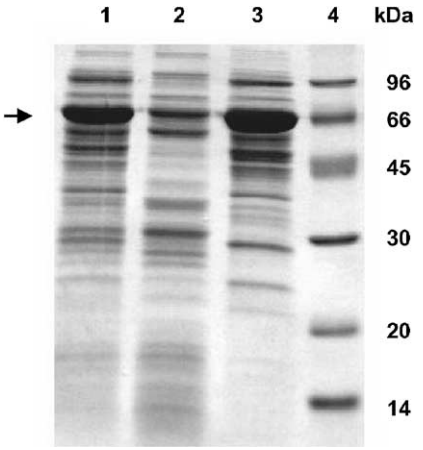



Fig. 3. SDS PAGE of enriched HbpA by anion exchange EBA chromatography on Streamline™ DEAE. HbpA band marked. Lane 1 crude extract (12 μg), lane 2 unbound flow through while loading the column (10 μg), lane 3 seven most active fractions pooled (11 μg), lane 4 protein marker.

### 3.3. Polar solvents

Water-miscible polar solvents, such as *N*,*N*-dimethylformamide (DMF) or methanol, are an alternative to the use of apolar solvents. The stability of HbpA was tested at different concentrations of these polar solvents over time (Fig. 5). While both solvents caused loss of the enzyme activity, methanol produced a milder inactivation effect compared to DMF at comparable concentrations of the cosolvents. The addition of both cosolvents resulted in a fast initial drop in enzyme activity, which then remained relatively stable during further incubation. Half of the initial enzyme activity was lost after addition of approximately 10% (v/v) DMF or 15% (v/v) methanol, and almost complete inactivation was observed at approximately 20%

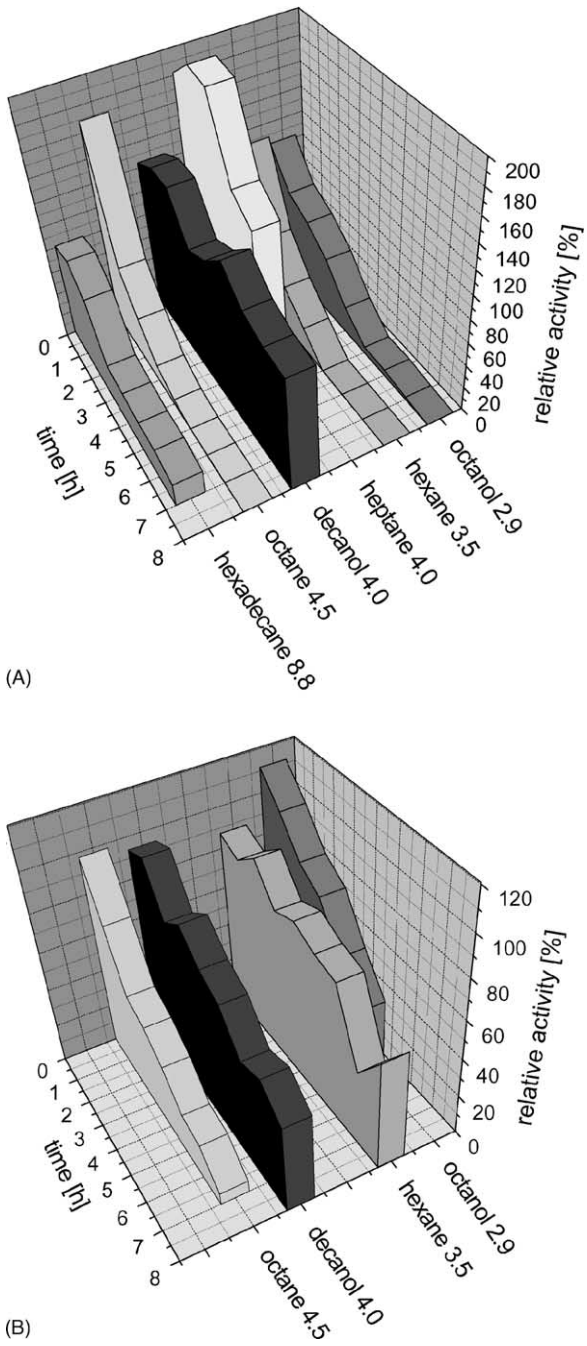



Fig. 4. Stability of 2-hydroxybiphenyl 3-monooxygenase (A) and formate dehydrogenase (B) in the presence of 80% (v/v) apolar solvents with different log *P* values (2.9–8.8) over time.

(v/v) DMF and 30% (v/v) methanol. Based on these observations, methanol was selected as a cosolvent for all further experiments. In addition to providing better enzyme stability, methanol has further advantage of being able to serve as a substrate of YADH for cofactor regeneration.

### 3.4. In vitro biotransformation

In further experiments, the water–methanol monophasic system was tested as a reaction medium for the cell-free biotransformation according to Fig. 1. The substrate 2,2′-dihydroxybiphenyl was hydroxylated by HbpA in the presence of 10% (v/v) methanol and YADH used for cofactor regeneration. In order to optimize reaction conditions for higher conversions of the phenolic substrate, the effect of the cofactor concentration and cofactor form ($NAD^+$ versus NADH) on the different reaction parameters was studied in detail (Table 3). It was found that regardless of the cofactor form applied in the reaction (NADH or $NAD^+$) substrate conversions close to 100% were achieved. Lowering the cofactor concentration from 0.75 to 0.14 mM resulted in the increase of the total turnover number (TTN) from 7 to 34. However, at the same time space-time yields decreased by approximately 30%, despite the fact that cofactor concentrations were still significantly higher than the $K_M$ value of HbpA of 26.8 μM for NADH [5]. When the biotransformation was started with the reduced cofactor NADH, approximately 1.5-fold lower productivities were obtained than by starting the reaction with $NAD^+$. Equimolar combinations of NADH/$NAD^+$ resulted in productivities intermediate between those observed for NADH or $NAD^+$ alone (data not shown). The reasons for lower productivity observed for NADH compared to $NAD^+$ remain unclear and require further investigation.

The results of the optimization study were then applied to conduct a preparative scale biotransformation of 2,2′-dihydroxybiphenyl to 2,2′,3,3′-tetrahydroxybiphenyl in 10% MeOH (Fig. 6). A full conversion of the substrate was achieved after 3 h predominantly to the monohydroxylated product, 2,2′,3-dihydroxybiphenyl. The further conversion of 2,2′,3-dihydroxybiphenyl to 2,2′,3,3′-tetrahydroxybiphenyl was approximately eight times slower than the hydroxylation of 2,2′-dihydroxybiphenyl. The mean productivities

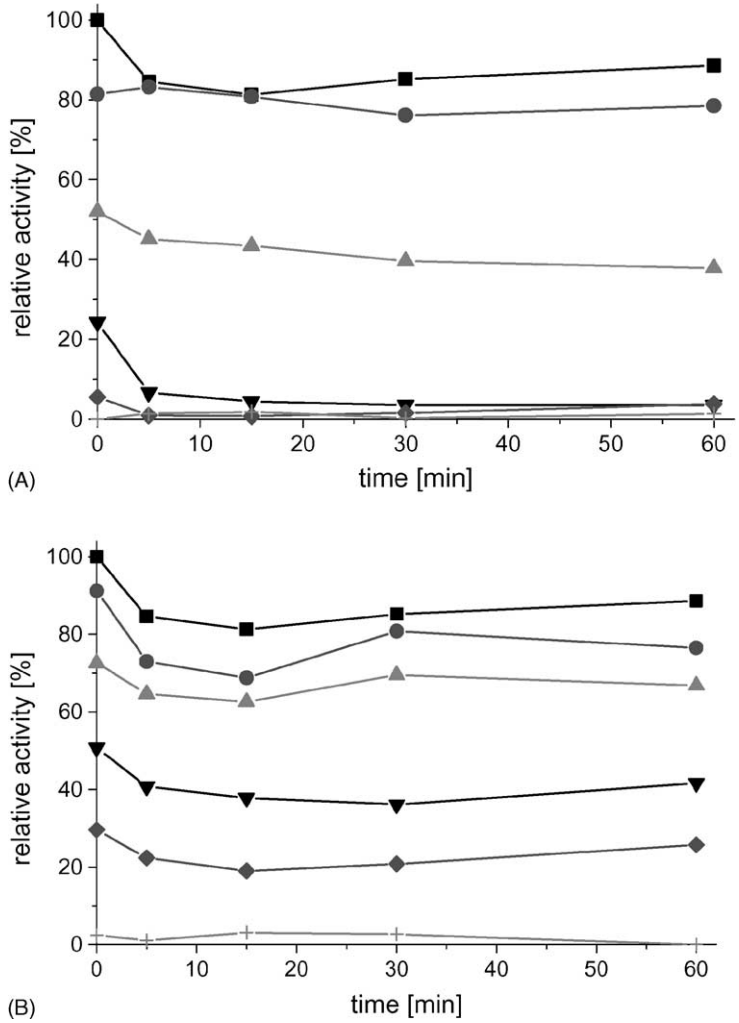



Fig. 5. Activity and stability of HbpA in presence of various concentrations of *N*,*N*-dimethylformamide (A, log *P*: −1.00) and methanol (B, log B: −0.76), 100% activity = 0.63 U/mg. Legends: (■) 0% (v/v); (●) 5% (v/v); (▲) 10% (v/v); (▼) 15% (v/v); (◆) 20%; (+) 30% (v/v).

Table 3
In vitro hydroxylation of 2,2′-dihydroxybiphenyl in the presence of 10% (v/v) methanol[a]

| Batch | Cofactor concentration (mM) | Space-time-yield[b] (mg/l h) | Specific activity (U/g) | Converted substrate[c] (mM) | Conversion[c] (%) | TTN[d] |
|---|---|---|---|---|---|---|
| NADH | 0.70 | 20.2 | 3.41 | 5.4 | 100 | 7 |
| $NAD^+$ | 0.75 | 31.2 | 5.28 | 5.3 | 98.5 | 7 |
| NADH | 0.35 | 19.7 | 3.34 | 5.2 | 97.1 | 14 |
| $NAD^+$ | 0.38 | 29.3 | 4.95 | 5.4 | 100 | 14 |
| NADH | 0.14 | 13.7 | 2.31 | 4.1 | 76.8 | 28 |
| $NAD^+$ | 0.15 | 20.1 | 3.39 | 5.0 | 93.9 | 34 |

[a] Yeast ADH was used for the regeneration of NADH.
[b] Linear phase until 30 h.
[c] After 65 h.
[d] Total turnover number (TTN) is defined as mol product formed/mol cofactor present.

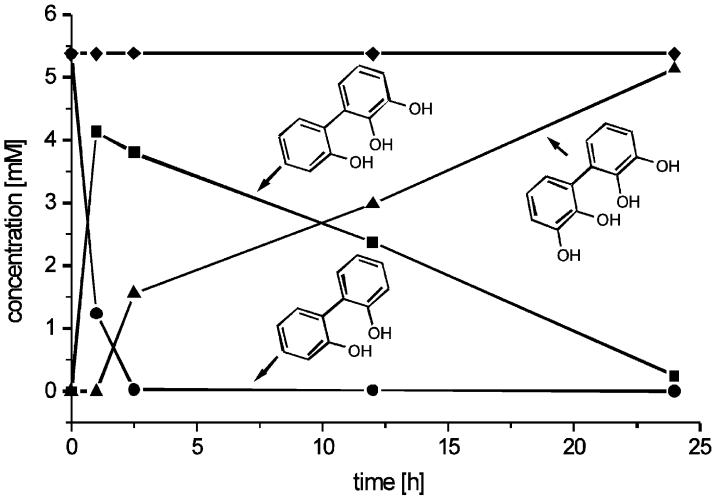



Fig. 6. Hydroxylation of 2,2′-dihydroxybiphenyl catalyzed by HbpA in the presence of NADH-regenerating system. Concentrations were estimated from corresponding peak areas in HPLC traces. Legends: (●) substrate 2,2′-dihydroxybiphenyl; (■) monohydroxylated product 2,2′,3-trihydroxybiphenyl; (▲) dihydroxylated product 2,2′,3,3′-tetrahydroxybiphenyl; (◆) sum balance.

were 0.43 g/(l h) (maximal value 0.83 g/(l h)) and 0.05 g/(l h) (maximal value 0.23 g/(l h)) for 2,2′,3-dihydroxybiphenyl and 2,2′,3,3′-tetrahydroxybiphenyl, respectively. The activity was stable over 24 h, which is significantly longer than in an analogous reaction setup without cofactor regeneration [16].

In another experiment HbpA and FDH were used in 80% (v/v) decanol to hydroxylate 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl. In contrast to an experiment described previously [12], sodium formate was added sequentially to avoid high formate concentrations. High concentrations of anions were reported to inhibit HbpA [5]. The total space-time-yield of 0.46 g/(l h) was the same as reported in [12], but the conversion increased from 15 to 23% resulting in an increased TTN of 503 (Table 4).

The productivity of the application of HbpA in polar and apolar solvents is in the range of 0.4 g/(l h), but the TTN differs (ca. 30 for polar solvents and 500 for apolar solvents). The potential to increase the TTN in the application of HbpA in polar solvents is limited, as higher substrate concentrations would result in substrate inhibition of HbpA [5] and lower cofactor concentrations result in lower conversion rates and productivities. Therefore, the application of HbpA in apolar solvents appears to be more advantageous.

Comparison of these results with the data reported for synthetic applications of other isolated oxygenases (Table 4) shows that the concept of using HbpA in organic-aqueous media with concomitant cofactor regeneration is a promising strategy. Even without full optimization, productivities and TTN numbers are in the same range as for other various oxygenases. The use of an isolated oxygenase is still rare, partly because an effective system for cofactor regeneration is required but also because of the limited availability of these enzymes. In addition to HbpA, the use of isolated Baeyer–Villiger monooxygenases and P450 monooxygenases was reported using enzymatic or electrochemical systems for cofactor regeneration [12,17–24]. The highest TTN for the regeneration of reduced cofactor is 500. The systems are applied on scales from small (few ml) to preparative (30–200 ml). Space–time yields up to 0.77 g/(l h) have been reported (Table 4). These examples show that although the enzyme isolation procedure needs further improvement, in vitro biotransformations using oxygenases represent a feasible and convenient synthetic strategy, primarily due to easier handling compared to whole cell systems. This strategy will hopefully find its niche, for example, for the production of fine chemicals and derivatization of biologically active lead compounds.

Table 4
In vitro applications of oxygenases

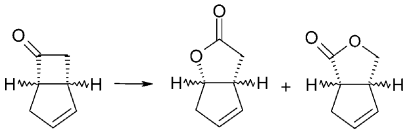
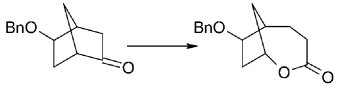
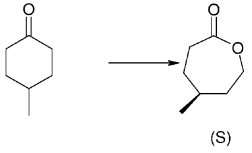
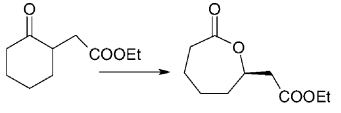
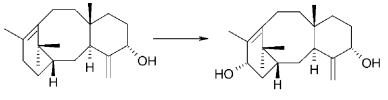
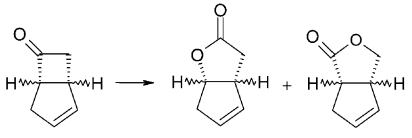
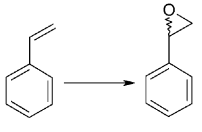
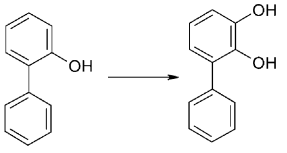
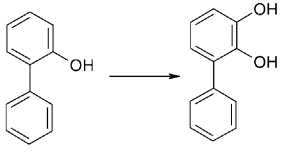
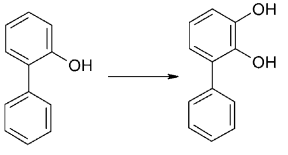
| Enzyme | Substrate/product | Cofactor/cofactor regeneration system | Volumetric oxygenase activity (U/ml) | Average productivity (g/l h) | Conversion (%) | TTN[a] |
|---|---|---|---|---|---|---|
| Monooxygenase *Pseudomonas putida* NCIMB 10007 grown on camphor [17][b] |  | NADH/formate dehydrogenase *Candida boidinii* | 5 | 0.12 (1 h) | 100 | 6 |
| Monooxygenase 1 from *Pseudomonas putida* NCIMB 10007 grown on camphor [18][c] |  | NADH/formate dehydrogenase *Candida boidinii* | – | – | 39 | – |
| Cyclohexanone monooxygenase [19][d] |  (S) | NADPH/formate dehydrogenase mutant *Pseudomonas sp.* 101 | 0.16 initial<br>0.066 average | 0.51 (10 h) | 100 | 160 |
| Cyclohexanone monooxygenase [20][e] |  (R) | NADPH/formate dehydrogenase mutant *Pseudomonas sp.* 101 | 0.064 average | 0.77 (2.5 h) | 48 | 24 |
| Taxane 13α-hydroxylase cytochrome P450 monooxygenase [21][f] |  | NADPH/glucose 6-phosphate dehydrogenase | – | – | – | Maximum 5 |
| Cyclohexanone monooxygenase [22][g] immobilized on Eupergit C |  | NADPH/alcohol dehydrogenase *Thermoanaerobium brockii* coimmobilized on Eupergit C | 2 U/ml initial | >0.24 | 100 | 92 |
| Cytochrome P450cam [23][h] |  | Putidaredoxin/electrode driven | 0.005 initial<br>0.002 average | 0.014 (0.45 h) | 30 μM | – |

Table 4 (*Continued*)

| Enzyme | Substrate/product | Cofactor/cofactor regeneration system | Volumetric oxygenase activity (U/ml) | Average productivity (g/l h) | Conversion (%) | TTN[a] |
|---|---|---|---|---|---|---|
| 2-Hydroxybiphenyl 3-monooxygenase [24][i] |  | NADH/electrochemical [Cp*Rh(bpy)Cl]Cl 2,2′-bipyridine | 1.9 initial | 0.20 (2 h) | 100 | Maximum 10 |
| 2-Hydroxybiphenyl 3-monooxygenase [12][j] |  | NADH/formate dehydrogenase *Candida boidinii* | 0.56 initial[l]<br>0.15 average[l] | 0.45 (7.5 h) | 15 | 403 |
| 2-Hydroxybiphenyl 3-monooxygenase[k] |  | NADH/formate dehydrogenase *Candida boidinii* | 0.56 initial[l]<br>0.15 average[l] | 0.46 (8 h) | 23 | 506 |

[a] TTN (total turnover number) is defined as mol product formed/mol cofactor present.

[b] Ca. 1 ml scale, 30 °C, 150 rpm, 1 mM substrate, 15 mM sodium formate, 0.16 mM NADH, monooxygenase:formate dehydrogenase 1.6:1 (U:U).

[c] 30 °C, 200 rpm, 50 mM Tris–HCl pH 8.1, 4–10 mM substrate, 7–12 mg crude monooxygenase, 0.2 U/ml formate dehydrogenase.

[d] 30 ml scale, 30 °C, pH 8.0, 40 mM substrate, 200 mM sodium formate, $NADP^+$ concentration 0.25 mM, formate dehydrogenase in two-fold activity excess, bubble free aeration.

[e] 30 ml scale, 30 °C, pH 8.0, 20 mM substrate, 200 mM sodium formate, $NADP^+$ concentration 0.4 mM, formate dehydrogenase in three-fold activity excess, bubble free aeration.

[f] 1 ml scale, 32 °C, 250 mM Tris–HCl pH 7.5, 200 μM NADPH, 2.5 μM FMN, 2.5 μM FAD, 2 mM glucose-6-phosphate, 2 units glucose-6-phosphate dehydrogenase, ca. 600 μg microsomal protein, 250 μM substrate (e.g. [20-$^3$H]taxadien-5α-ol, etc.) over night.

[g] 2 ml scale, 50 mM Tris-HCl pH 8.8, 46 mM substrate, 0.5 mM $NADP^+$, 5% (v/v) 2-propanol (0.65 M), cyclohexanone monooxygenase:alcohol dehydrogenase 1:1 (U:U).

[h] 1 ml scale, 22 °C, pH 7.4, 100 mM Tris–HCl, 0.2 M KCl, 0.02 M $MgCl_2$, Pt to avoid $H_2O_2$, antimony doped tin oxide electrode.

[i] 100 ml scale, 30 °C, pH 7.5, 2 mM substrate, 0.1 mM Rh-complex, 0.2 mM $NAD^+$.

[j] 200 ml scale, 30 °C, pH 7.5, 80% (v/v) decanol, 20% (v/v) potassium phosphate pH 7.5, 110 mM substrate in organic phase, 160 mM sodium formate and 0.2 mM NADH in aqueous phase, formate dehydrogenase in 1.5-fold activity excess.

[k] 200 ml scale, 30 °C, pH 7.5, 80% (v/v) decanol, 20% (v/v) potassium phosphate pH 7.5, 110 mM substrate in organic phase, initial 50 mM sodium formate, stepwise increased 10 mM every 45 min, 0.2 mM NADH in aqueous phase, formate dehydrogenase in 1.5-fold activity excess.

[l] Calculated for aqueous phase.

## 4. Conclusion

The results obtained in this work show that the EBA chromatography is an efficient one step method to partially purify HbpA on a multigram scale. HbpA showed good operational stability in decanol and methanol, allowing to use the partially purified enzyme to hydroxylate phenolic substrates in methanol/YADH and decanol/FDH systems with high productivities.

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