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E. coli JM109 pHBP461, a recombinant biocatalyst for the regioselective monohydroxylation of *ortho*-substituted phenols to their corresponding 3-substituted catechols

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Abstract

An expression system for the 2-hydroxybiphenyl-3-monooxygenase gene (*hbpA*) from *Pseudomonas azelaica* HBP1 was developed based on *E. coli* JM109 and the high copy vector pUCBM20. The system was used for the conversion of 2,2′-dihydroxybiphenyl to 2,2′,3-trihydroxybiphenyl on a preparative scale with a productivity of 0.06 g h^{-1} l^{-1}. This system also allows the biocatalytic production of various 3-aryl-, 3-alkyl-, and 3-halo-substituted catechols.

Keywords: Recombinant biocatalyst; Biodegradation; *Pseudomonas azelaica* HBP1; Hydroxylation; 2-Hydroxybiphenyl; 3-Substituted catechols; Phenols

1. Introduction

Microbial degradation pathways of hydrocarbons offer a tremendously high metabolic diversity and can be used as a source for the development of biocatalysts for organic synthesis. Generally, aerobic microbial degradation of aromatic hydrocarbons is initiated by the incorporation of one or two hydroxyl moieties into the aromatic nucleus yielding catechol derivatives as central pathway intermediates. These initial reaction steps are catalyzed by dioxygenases in concert with dehydrogenases when non-activated aromatic structures are attacked. In case of the degradation of phenol derivatives, monooxygenases are yielding catechol derivatives in a one-step reaction. These enzymes often exhibit a broad substrate spectrum, which in nature allows the funnelling of a variety of structurally similar hydrocarbons into a few central metabolic pathways. Because of their broad substrate spectrum and regioselective manner of hydroxylation, these enzymes provide a challenge for the development of new biocatalysts for organic synthesis.

Here, we describe a recombinant biocatalyst for the preparation of various 3-aryl-, 3-alkyl-,

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and 3-halo-substituted catechols employing 2-hydroxybiphenyl-3-monooxygenase (HbpA). HbpA is the first enzyme in the biodegradation pathway of 2-hydroxybiphenyl in *Pseudomonas azelaica* HBP1 [1,2]. Accordingly, 2-hydroxybiphenyl is hydroxylated to 2,3-dihydroxybiphenyl by action of HbpA. 2,3-Dihydroxybiphenyl in turn is further oxidized to its *meta*-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid and further metabolized via catechol and a second, so-called lower *meta*-cleavage pathway. Therefore, application of wildtype *P. azelaica* HBP1 for the synthesis of catechols is limited by the fast degradation of the products catalyzed by pathway enzymes exhibiting a broad substrate spectrum [3]. To overcome these limitations, we developed a recombinant biocatalyst, *E. coli* JM109 pHBP461, which regioselectively hydroxylates *ortho*-substituted phenols to their corresponding 3-substituted catechols. This biocatalyst only shows heterologous expression of *hbpA* and is free of product degrading enzymatic activities.

2. Materials and methods

2.1. Bacterial strains and plasmids

P. azelaica HBP1 [1] was grown with 2,2′-dihydroxybiphenyl as carbon source on minimal medium as previously described [3]. *E. coli* JM109 (*recA1*, *supE44*, *endA1*, *hsdR17*, *gyrA96*, *relA1*, *thi*, *Δ*(*lac-proAB*) F′ (*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZΔ*M15)) [4] was used for cloning and biotransformation experiments and was grown on L-broth [5] supplemented with 100 μg ml^{-1} ampicillin and 0.1 mM IPTG when appropriate. The plasmid pUCBM20 (Boehringer Mannheim, FRG) was used as basic cloning vector. Construction of pHBP100, carrying a 13.2 kb *Mlu*I-DNA fragment from the genome of *P. azelaica* HBP1 [6] will be published elsewhere. The plasmid pHBP461 (Fig. 1) was constructed by inserting a 3.2 kb *Sal*I/*Xho*1 DNA-fragment of pHBP100 in the *Sal*I polylinker-site of pUCBM20.

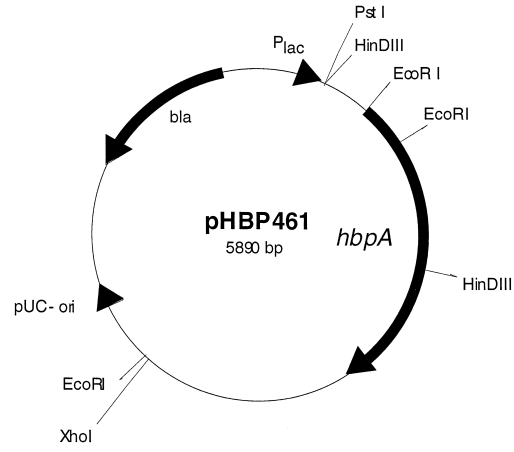

Fig. 1. Restriction map of plasmid pHBP461.

2.2. Preparation of crude extracts and determination of enzyme activity

P. azelaica cells were incubated in minimal medium with 2,2′-dihydroxybiphenyl until the late exponential growth-phase. *E. coli* cells were incubated for 16 h in L-broth, harvested by centrifugation and resuspended into phosphate buffer (50 mM Na_2HPO_4–KH_2PO_4 pH 7.2). Cells were disrupted with a French press (Aminco, Silver Spring, USA) and cell debris was removed by centrifugation (35 min, 100 000 $\times$ g, 4°C). Protein was determined by the Bradford procedure (Bio-Rad, Munich, FRG). Spectrophotometric (in crude extracts) and polarographic (with whole cells) determination of 2-hydroxybiphenyl-3-monooxygenase-activity was performed as described previously [1] using phosphate buffer (50 mM Na_2HPO_4–KH_2PO_4 pH 7.2).

2.3. DNA manipulation, SDS polyacrylamide gel electrophoresis and HPLC

All DNA techniques were carried out according to established procedures as described elsewhere [7]. SDS-PAGE was performed with a gel chamber system from Biometra (Göttingen, FRG). The protein bands on the gels were stained with Coomassie brilliant blue G250.

High-pressure liquid chromatography (HPLC) was carried out as described elsewhere [8].

2.4. Preparative bioconversion

Prior to every bioconversion experiment, *E. coli* JM109 was de novo transformed with plasmid pHBP461. One resulting colony was incubated in 500 ml L-broth (100 μg ml^{-1} carbenicillin) and grown at 30°C to an optical density (546 nm) of 0.6. Cells were harvested by centrifugation, washed in L-broth, transferred in 10-l L-broth (100 μg ml^{-1} ampicillin, 0.1 mM IPTG, 5 mM sodium succinate) in a bioreactor and incubated at 30°C until the late exponential growth phase ($OD_{546\ nm}$ 7; 5.5 g cell wet weight l^{-1}). The bioconversion was performed in the growth medium and started by the addition of 0.85 g 2.2-dihydroxybiphenyl, provided as stock solution in methanol (1 M). After 10 min and after 30 min an additional amount of 0.85 g 2,2′-dihydroxybiphenyl was added. The reaction medium was supplemented after 90 min with 5 mM of sodium succinate supplied as 1 M stock solution. After 170 min the reaction was stopped by a shift to pH 2.0 (addition of 61 ml 70% perchloracid). This also decreased product autooxidation and increased adsorption to Serdolit AD-4. Biomass was separated from the culture broth by centrifugation and filtration. Product in the clear supernatant was extracted by adsorption to Serdolit AD-4 (400 g; Serva, FRG) and eluted with acidic methanol. Product purification was performed by preparative HPLC.

3. Results

3.1. Expression system for hbpA with 2-hydroxybiphenyl-3-monooxygenase activity

Recently, we cloned a 13.2 kb *Mlu*I-DNA fragment from the genome of *P. azelaica* HBP1. This DNA fragment carried a cluster of genes coding for the first three enzymatic steps of 2-hydroxybiphenyl degradation [6]. The gene coding for 2-hydroxy-biphenyl-3-monooxygenase was subcloned on a 3.2 kb *Sal*I/*Xho*I DNA-fragment in pUCBM20. This new construct was named pHBP461 (Fig. 1). Cells expressing 2-hydroxybiphenyl-3-monooxygenase activity were identified by spectrophotometric determination of enzyme activity in cell-free extracts. Cell-free extracts were obtained from cultures resulting from the incubation of single colonies for 16 h in 40 ml LB-medium (100 μg ml^{-1} ampicillin, 0.1 mM IPTG). A specific activity of 0.42 U mg^{-1} protein was determined in cell-free extracts of *E. coli* JM109 pHBP461. Proteins of crude extracts of *E. coli* JM109, *E. coli* JM109 pUCBM20 *and E. coli* JM109 pHBP461 were separated by SDS gel electrophoresis. A polypeptide with a molecular weight of 60 kDa was detectable in crude extracts of *E. coli* JM109 pHBP461 (Fig. 2). This corresponds to the molecular weight of a subunit of 2-hydroxybiphenyl-3-monooxygenase purified from *E. coli* JM109 pHBP461 and *P. azelaica* HBP1 [2].

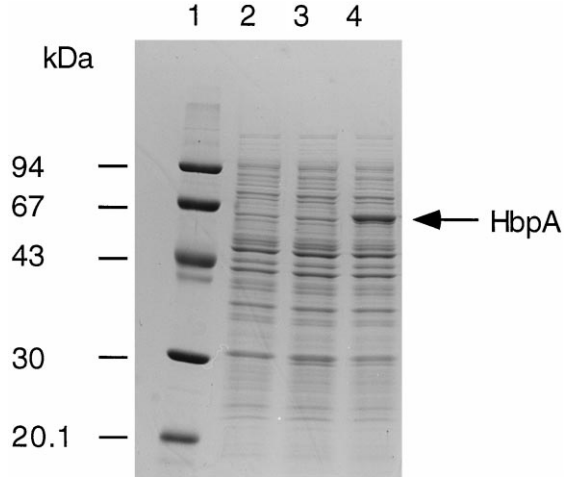

Fig. 2. SDS-PAGE of proteins (12 μg) from crude extracts of *E. coli* JM109 (lane 2), *E. coli* JM109 pUCBM20 (lane 3), and *E. coli* JM109 pHBP461. Lane 1, molecular weight marker.

3.2. Substrate spectrum of recombinant 2-hydroxybiphenyl-3-monooxygenase

The substrate spectrum of *E. coli* JM109 pHBP461 for *ortho*-substituted phenols was de-

Table 1
Specific oxygen uptake rates of *E. coli* JM109 pHBP461 cells

Substrate	Product	Relative activity[a] (%)
2,2′-Dihydroxybiphenyl	2,2′,3-Trihydroxybiphenyl[c]	100[b]
2-Hydroxybiphenyl	2,3-Dihydroxybiphenyl[c]	49
2,2′,3-Trihydroxybiphenyl	2,2′,3,3′-Tetrahydroxybiphenyl[c]	34
2-Propylphenol	n.d.[d]	36
2-Methylphenol	3-Methylcatechol[c]	24
2-Bromphenol	n.d.[d]	33
2-Chlorphenol	3-Chlorcatechol[c]	20
2-Fluorphenol	3-Fluorcatechol[c]	10
2-Mercaptophenol	n.d.[d]	74
1-Naphthol	n.d.[d]	11
8-Hydroxychinolin	n.d.[d]	12

[a]Relative activities were determined polarographically with whole cells of *E. coli* JM109 pHBP461 as described in Section 2.
[b]The activity determined with *E. coli* JM109 pHBP461 was 110% of the activity of wildtype *P. azelaica* HBP1 grown on 2,2′-dihydroxybiphenyl determined under the same conditions.
[c]The product was characterized with HPLC by comparison with authentic material.
[d]Formation of a more polar product was observed by HPLC. Reaction mixtures turned brown after incubation at room temperature for an hour.

termined polarographically as described in Section 2. The relative activities are shown in Table 1. The highest activity was determined for 2,2′-dihydroxybiphenyl. The activity of recombinant 2-hydroxybiphenyl-3-monooxygenase with 2,2′-dihydroxybiphenyl was 110% of the activity in the wildtype *P. azelaica* HBP1 grown on 2,2′-dihydroxybiphenyl, determined under the same conditions. Interestingly, 2-fluorophenol, 2-chlorophenol, and 2-bromophenol were also substrates of HbpA. Formation of products was confirmed by HPLC analysis (Table 1). No activity could be measured with phenol, 2-nitrophenol, 2-aminophenol, 2-methoxyphenol, 2-aminobiphenyl, salicylate, 2-hydroxyphenylacetate, 2-hydroxydiphenylmethan, dibenzofuran, 3,3′-dihydroxy-2,2′-bipyridyl, and all isomeric dimethylphenols, di- and trichlorophenols.

3.3. Biotransformation of 2,2′-dihydroxybiphenyl to 2,2′,3-trihydroxybiphenyl

2,2′,3-Trihydroxybiphenyl was produced by incubating 2,2′-dihydroxybiphenyl with *E. coli* JM109 pHBP461 on a 10-l scale as described in Section 2. Substrate was added in three steps to prevent intoxication of the cells with the toxic phenol derivative. After 90 min sodium succinate was added to allow regeneration of cofac-

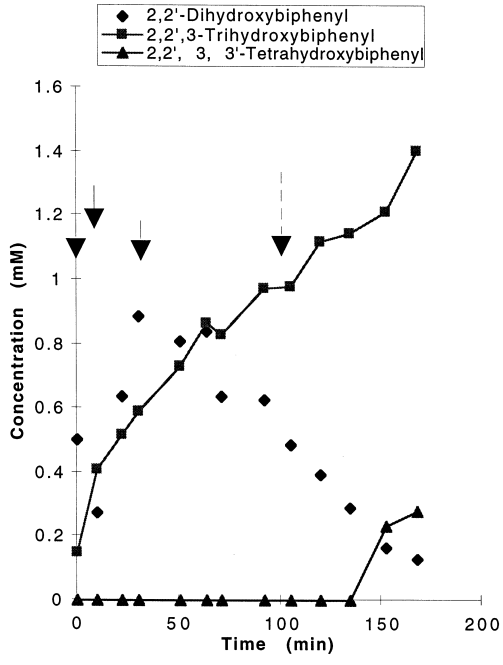

Fig. 3. Kinetics of the conversion of 2,2′-dihydroxybiphenyl by *E. coli* JM109 pHBP461. Arrows (full line), addition of 0.85 g 2,2′-dihydroxybiphenyl (85 mg l^{-1}) after 0 min, 10.5 min and 30 min reaction time. Arrow (dashed line), addition of 5 mM succinate.

Table 2
Quantification of the conversion[a] of 2,2′-dihydroxybiphenyl to 2,2′,3-trihydroxybiphenyl by *E. coli* JM109 pHBP461

Step	Amount (g)	Compound	Relative amount (%)
Substrate	2.6	2,2-Dihydroxybiphenyl	100
Product	1.8[b]	2,2′,3-Trihydroxybiphenyl	70
Byproduct	0.25[b]	2,2′,3,3′-Tetrahydroxybiphenyl	9
Substrate left in the medium (not converted)	0.13[b]	2,2′-Dihydroxybiphenyl	5
Substrate adhered to cell mass or polymerized product	0.42	2,2′-Dihydroxybiphenyl or corresponding amount of product	16

[a]Conversion time: 3 h; productivity 0.06 g l^{-1} h^{-1} (10-l scale).
[b]As determined by high pressure liquid chromatography.

tors. The reaction was followed by HPLC analysis. Depletion of substrate and formation of product is shown in Fig. 3. The byproduct, 2,2′,3,3′-tetrahydroxybiphenyl, generated by the monohydroxylation of 2,2′,3-trihydroxybiphenyl was formed when the concentration of 2,2′-dihydroxybiphenyl fell below 0.2 mM. The concentration of the main product, 2,2′,3-trihydroxybiphenyl, linearly increased over 170 min. The productivity was calculated as 0.06 g product l^{-1} h^{-1}. The product 2,2′,3-trihydroxybiphenyl was purified by solid-phase extraction and preparative HPLC as described in Section 2. The overall process is quantified in Table 2. During the time of the reaction, a significant amount of substrate (16%) was lost, probably due to adsorption effects or polymerization of the product. The product yield was 70%. The yield of the following purification procedure was 63%. The product was characterized by ^{1}H-NMR, COSY-NMR, ^{13}C-NMR, and GC–MS analysis and shown to be 2,2′,3-trihydroxybiphenyl (data not presented).

4. Discussion

E. coli JM109 pHBP461 proved to be a versatile biocatalyst for the production of 2,2′,3-trihydroxybiphenyl from 2,2′-dihydroxybiphenyl. As the substrate spectrum of recombinant 2-hydroxybiphenyl-3-monooxygenase (HbpA) is quiet broad (Table 1), also other 3-substituted catechols are accessible with this biocatalyst starting with the corresponding *ortho*-substituted derivatives of phenol. A preliminary characterization of the substrate spectrum of HbpA [1] had shown, that the *ortho*-substitution of phenol was essential for hydroxylation; *meta*- or *para*-substituted phenols were not hydroxylated by HbpA; nor were phenols carrying charged substituents in *ortho* position. In addition, we showed that *ortho* halo-substituted phenols are also hydroxylated by HbpA to their corresponding catechols. Alkylphenols like 2-*sec*-butylphenol are hydroxylated at very high rates [2], while phenols carrying very bulky substituents like 2-hydroxydiphenylmethane are not substrates (Table 1). Therefore, activity of HbpA is rather independent of electronic or inductive effects but influenced by the charge and the size of the respective substituent. Based on these results, a process including in situ recovery of the products has been developed and is described in the accompanying report [9]. Cloning and expression of *hbpA* in *E. coli* JM109 prevented the product from being degraded by normal pathway enzymes. Also, induction of *hbpA* gene expression was no longer dependent on 2-hydroxybiphenyl as in the wild-type. Instead, induction could be achieved by addition of IPTG. Nevertheless, IPTG turned out not to be essential for *hbpA* gene-expression

[9] as the leaky *lac*-promoter was used for construction of pHBP461. In this steady-state process, remaining HbpA-activity was still higher than the rate of in situ recovery of the products and therefore not rate-limiting for the overall process.

The use of recombinant techniques should provide fast access also to the vast variety of other enzymes of the natural metabolic pool being important in this respect.

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