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Efficient resolution of prostereogenic arylaliphatic ketones using a recombinant alcohol dehydrogenase from *Pseudomonas fluorescens*

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Abstract—A broad range of arylaliphatic ketones is efficiently reduced to the corresponding optically active (*R*)-alcohols by a recombinant alcohol dehydrogenase from *Pseudomonas fluorescens* (PFADH) produced by overexpression in *Escherichia coli*. PFADH shows high activity and stereoselectivity in the reduction of acetophenone and various derivatives (45–99% e.e.), as well as in the reduction of 3-oxobutyric acid methyl ester (>99% e.e.). The highest activity was observed between 10 and 20°C. The cofactor NADH can be efficiently recycled by the addition of 10–20% (v/v) of *iso*-propanol.

1. Introduction

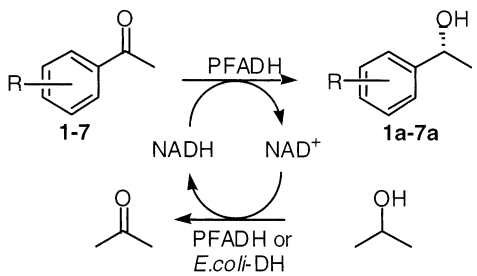
Dehydrogenases are enzymes belonging to the class of oxidoreductases (E.C. 1.x). Within this class, alcohol dehydrogenases (E.C.1.1.1.1, also called keto-reductases) represent an important group of biocatalysts because they can be used efficiently in the synthesis of optically pure compounds by reduction of prostereogenic ketones to the corresponding optically active alcohols. From a practical point of view, only those dehydrogenases which use NADH as cofactor are of importance, because for biocatalysts depending on NADPH, much less efficient cofactor recycling systems are available.

A range of alcohol dehydrogenases useful for organic synthesis has been described in the literature.[1–6] Amongst these, the most frequently used are from yeast, horse liver and *Thermoanaerobium brockii*, which also differ considerably in their substrate specificity and stereopreference.

Recently, we identified a gene encoding an alcohol dehydrogenase (PFADH) within the genomic library of *Pseudomonas fluorescens* DSM50106 and succeeded in the functional expression of the corresponding protein in the host *Escherichia coli*.[7] Herein, we describe the application of PFADH in the synthesis of optically active alcohols from the corresponding prostereogenic ketones (Scheme 1).

2. Results and discussion

The recombinant alcohol dehydrogenase from *Pseudomonas fluorescens* can be efficiently produced in the host organism *Escherichia coli* by induction with L-rhamnose.[7] The enzyme was harvested 4–5 h after



Scheme 1. Principle of the PFADH-catalyzed reduction of arylaliphatic ketones **1**–**7** in combination with cofactor-recycling catalyzed by PFADH or a dehydrogenase present in the crude cell extract from *E. coli*.

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induction by cell disruption using sonification followed by centrifugation. The resulting crude extract can be directly used for reductions or lyophilized for storage and later use.

Initially, we performed reductions with this crude extract using acetophenone **1** as a model compound in the presence of NADH, these reactions yielded (*R*)-α-phenylethanol in high yield and enantiomeric purity (Scheme 1, Table 1). Accordingly, PFADH delivers the hydride ion from the *Si* face and thus shows anti-Prelog stereopreference.[8] Addition of *iso*-propanol revealed efficient regeneration of NAD^+ with concomitant formation of acetone. Preliminary results using an *E. coli* strain lacking the PFADH gene suggests that this regeneration is probably catalyzed by a constitutive dehydrogenase produced by *E. coli*, e.g. AdhE.[9,10] However, control experiments confirmed that only the recombinant enzyme accepted the ketones **1**–**8** used in this study. Thus, from a practical point of view, the crude extract of the recombinant PFADH resembles an extremely versatile catalyst as no addition of a second dehydrogenase—for example, the frequently used formate dehydrogenase from *Candida boidinii*[11]—is required for recycling of NADH.

Next, we subjected the aryl-substituted acetophenone derivatives **2**–**7** to PFADH-catalyzed reductions. From Table 1 it is obvious that the enzyme also accepts these compounds, but conversions and the e.e.s of the alcohol products generated were markedly influenced by the nature of the ring substituent. Whereas methyl **2** and methoxy substituents **5** in the 4-position led to low product e.e.s of 42–45%, high selectivities, with e.e.s of 91 to >99%, were achieved in the reactions of 2- and 3-methoxy acetophenones and the 4-fluoro derivative **6**. 3-Nitro-, 4-nitro- and 4-aminoacetophenones were not accepted as substrates (data not shown). 3-Oxobutyric acid methyl ester **8** was converted with high activity and excellent stereoselectivity (e.e. >99%).

Table 1. Results for the desymmetrizations of substrates **1**–**8** using recombinant PFADH. All reactions were performed using the NADH-recycling system depicted in Scheme 1 at 20°C (see also experimental section).

Substrate/R[a]	Conversion[b] (%)	Enantiomeric excess (%e.e.)[b]	Time (h)
1/H	95	92	21
2/4-Me	82	42	19
3/2-MeO	31	>99	21
4/3-MeO	89	92	19
5/4-MeO	38	45	20
6/4-F	91	91	21
7/4-Cl	29	79	19
8[c]	83	>99	19

[a] R for **1**–**7** as depicted in Scheme 1.
[b] As determined by GC analysis.
[c] 3-Oxobutyric acid methyl ester.

2.1. Influence of temperature

Reactions carried out at different temperatures revealed that PFADH surprisingly shows highest activities in the reduction of **1** at 10–20°C, albeit *Pseudomonas fluorescens* is known as a mesophilic organism. Even at 5°C the activity was higher than above 30°C (Fig. 1). Moreover, the highest optical purity (>99% e.e.) was found at 10°C. Similar observations were made in the reactions of **3**, **4** and **6** (data not shown).

2.2. Influence of solvents

To investigate the influence of different organic solvents, *iso*-propanol, acetone or ethanol were added to the reaction mixture in concentrations of up to 10% (v/v) (Fig. 2, left). The highest conversions and e.e.s were achieved using *iso*-propanol, which is unsurprising as *iso*-propanol also serves as a substrate for NADH regeneration. Fig. 2 (left) shows that PFADH is also active in the presence of acetone and therefore stability in the presence of this NADH-regeneration product from *iso*-propanol is ensured. Reactions at 20°C in the presence of varying amounts of *iso*-propanol (Fig. 2, right) revealed that 20% (v/v) is a good compromise between high conversion and acceptable product e.e., which can be further enhanced to >99% e.e. by lowering the reaction temperature. Addition of >35% *iso*-propanol led to inactivation of the alcohol dehydrogenase.

So far, a range of bacterial alcohol dehydrogenase accepting arylaliphatic ketone substrates such as ace-

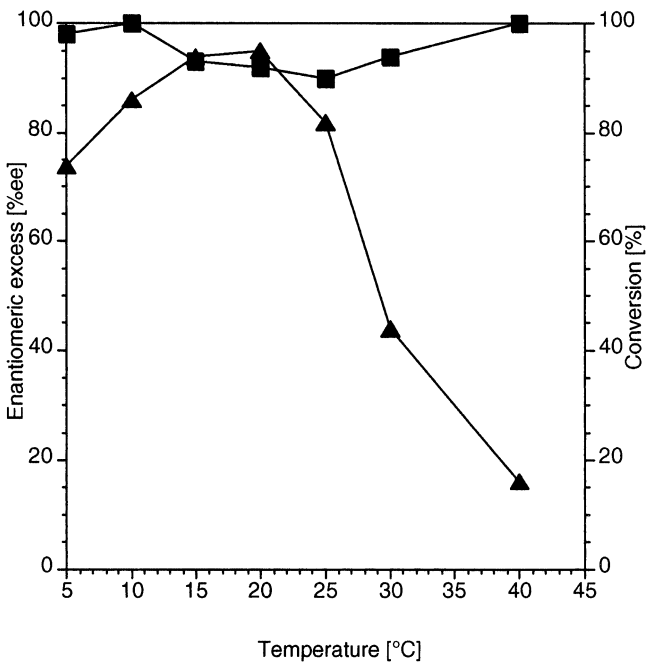

Figure 1. Influence of temperature on conversion and enantiomeric excess in the PFADH-catalyzed reduction of acetophenone **1** to (*R*)-α-phenylethanol **1a** (■, e.e.; ▲, conversion).

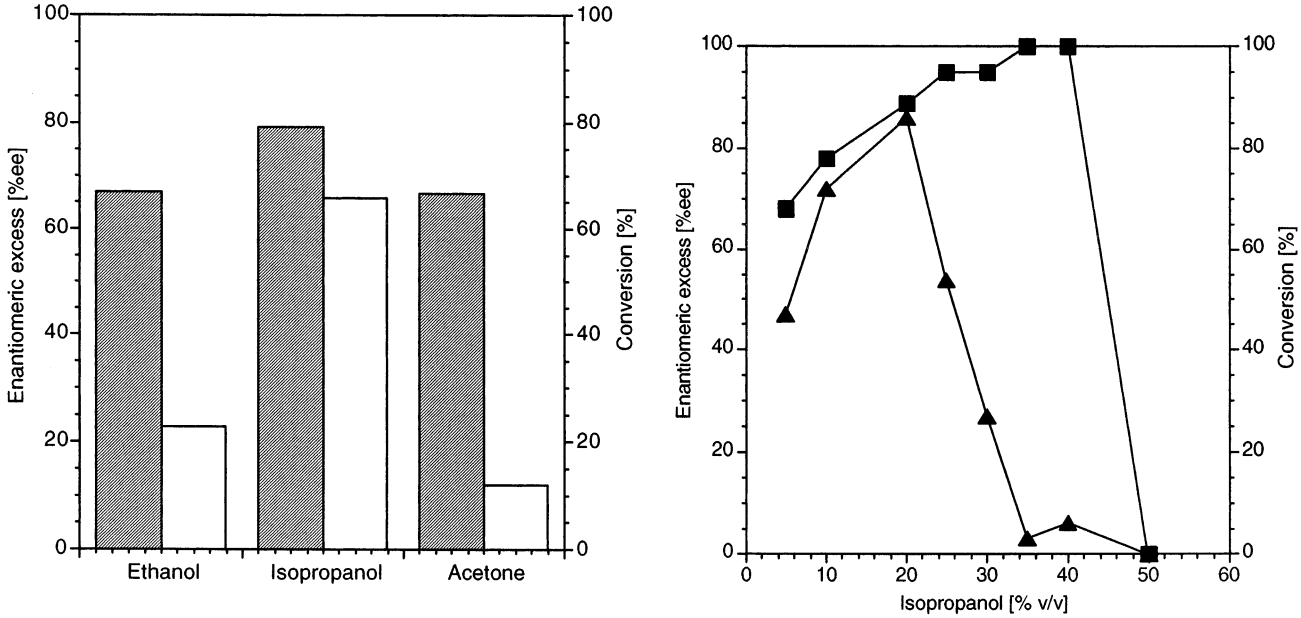

Figure 2. Influence of cosolvents (10% (v/v), left graph) and of varying concentrations of *iso*-propanol (right graph) on conversion and enantiomeric excess in the PFADH-catalyzed reduction of acetophenone **1** to (*R*)-α-phenylethanol **1a** (hatched bars/■, e.e.; white bars/▲, conversion).

tophenone have been described. An enzyme present in the crude acetone powder obtained from *Geotrichum candidum* cultivations[12] was found to be useful for the conversion of acetophenone and derivatives to the corresponding (*S*)-alcohols with similar yields and optical purities as the new PFADH described here. Similar observations were made for a carbonyl reductase from *Candida parapsilosis*[11] and an ADH from *Rhodococcus erythropolis*.[13] In contrast, reductions with ADH from *Lactobacillus kefir* lead to the formation of (*R*)-α-phenylethanol.[14,15] Only a few ADHs from *Pseudomonas* sp. have been described to date. An enzyme from strain ATCC49688[16] exhibits only limited substrate tolerance, whereas an enzyme from *Pseudomonas* sp. PED[17] accepts a wide range of substrates similar to PFADH and also shows activity in the presence of *iso*-propanol. However, these ADHs are not available in recombinant form, whereas a recombinant alcohol dehydrogenase from *Ps. putida* expressed in *E. coli* seems to be restricted to the conversion of allylic alcohols.[18] Moreover, the efficient cofactor recycling using the crude PFADH extract from *E. coli* cultivation appears to be a very versatile procedure for the efficient production of homochiral alcohols.

3. Experimental

3.1. Production of recombinant PFADH

Cloning and creation of the expression system are described elsewhere.[7] For expression, *E. coli* DH5α harboring the gene PFADH under control of the rhamnose inducible rhaBAD promoter[19] on the plasmid pJOE4016 were grown at 37°C in 500 mL LB media supplemented with ampicillin (100 μg/mL) until the early exponential phase (OD_{600} = 0.5–0.6). Dehydrogenase production was induced upon addition of L-rhamnose (final conc. 0.2% v/v) and cultivation continued for 4–5 h. Cells were collected by centrifugation (Heraeus Labofuge 400R, 4000×*g*, 10 min, 4°C) and washed twice with sodium phosphate buffer (50 mM, pH 7.5, 4°C). Cells were disrupted by sonification on ice for 12 min at 50% pulse and 50% power (Bandelin HD 2070, MS73). Cell debris was removed by centrifugation (4000×*g*, 10 min, 4°C) and the supernatant was directly used for reductions or lyophilized and stored at 4°C.

3.2. Substrate specificity of PFADH

The standard reaction mixture (250 μL) for the reduction of ketones **1**–**8** consisted of 6.4 μmol substrate dissolved in *iso*-propanol, 1.25 mg/mL protein (crude extract) in Tris–HCl (0.1 M, pH 8.0) and 20% (v/v) *iso*-propanol as substrate for the NADH-recycling dehydrogenase. No extra NADH was added. All reactions were performed at room temperature unless stated otherwise. All values were determined in triplicate.

At the end of the reaction, the mixture was extracted with two volumes of chloroform and the organic phase was dried over anhydrous Na_2SO_4. The reaction components were then analyzed by gas chromatography (Shimadzu GC 14A, Tokyo, Japan, equipped with a flame ionization detector, Integrator C5RA) using a chiral column (heptakis-(2,6-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin, 25 m×0.25 mm ID, Macherey–Nagel, Düren, Germany) using the following temperature programs: 120°C isothermal, retention times: acetophenone **1**, 1.55 min, (*R*)-α-phenylethanol **1a**, 2.77 min, (*S*)-**1a**, 2.99 min; 120°C for 2 min, heating at 10°C/min to 150°C, retention times: 2-methoxy-acetophenone **3**, 3.68 min, 2-methoxy-α-(*R*)-phenylethanol 4.90 min, 3-methoxy-acetophenone **4**, 3.98 min, (*R*)-3-methoxy-α-

phenylethanol **4a**, 5.61 min, (*S*)-**4a**, 5.86 min; 4-fluoro-acetophenone **6**, 1.58 min, (*R*)-fluoro-α-phenylethanol **6a**, 3.03 min, (*S*)-**6a**, 3.28 min; 90°C for 1 min, heating at 5°C/min to 120°C, retention times: 4-chloro-acetophenone **7**, 3.62 min, (*R*)-4-chloro-α-phenylethanol **7a**, 5.52 min, (*S*)-**7a**, 5.90 min; 4-methyl-acetophenone **2**, 2.63 min, (*R*)-4-methyl-α-phenylethanol **2a**, 3.40 min, (*S*)-**2a**, 3.76 min; 4-methoxy-acetophenone **5**, 4.94 min, (*R*)-4-methoxy-α-phenylethanol **5a**, 5.52 min, (*S*)-**5a**, 5.74 min; 70°C isothermal: 3-oxobutyric acid methylester **8**, 2.20 min, (*R*)-3-hydroxybutyric acid methylester **8a**, 3.84 min, (*S*)-**8a**, 3.99 min. Absolute configurations were assigned for **1a** and **8a** using commercially available (*R*)-alcohols and for **2a**–**7a** by assuming the same elution order as determined for **1a**.

3.3. Preparative scale reduction of acetophenone

Acetophenone **1** (200 mg, 50 mM) was dissolved in *iso*-propanol (6.58 mL) and Tris–HCl (0.1 M, pH 8.0, 26.52 mL). The reaction was started by addition of lyophilized crude cell extract (320 mg). Due to the moderate stability of the enzyme, further addition of crude PFADH (final amount: 2 g) was necessary until 73.5% conversion was achieved. After work-up of the reaction mixture as described above, (*R*)-α-phenylethanol was isolated in 63% yield (e.e. = 92%).

Acknowledgements

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