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Chiral Aromatic Alcohols Production by Thermophilic NADH-dependent Carbonyl Reductase

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Dehydrogenases/reductases (ADHs) are nowadays among the most requested enzymes used for the preparation of optically active compounds, for the inherent advantages over chemocatalysts in terms of their high chemo-, enantio-, and regioselectivity as well as to be environmentally friendly. Our aim was to identify and characterize novel ADHs for the selective bioreduction of sterically demanding ketones or alcohols, preferably NAD(H)-dependent and endowed with good operational stability. The gene encoding a novel dehydrogenases/reductase that belongs to the short-chain dehydrogenases/reductases (SDRs) superfamily has been identified in the genoma of *Thermus thermophilus* HB27. The *ttadh* gene has been heterologously overexpressed in *E. coli* and the protein, named TtADH, has been purified to homogeneity and characterized. The enzyme was found to be a thermophilic and thermostable NAD(H)-dependent SDR, active on both aromatic alcohols as well as a discrete spectrum of carbonyl compounds, including substituted benzaldehydes, aromatic ketones, and α-ketoesters. TtADH shows a good tolerance towards common organic solvents such as methanol, acetonitrile, ethyl acetate, and 2-propanol used at 2–10% concentration and at a temperature range from 25° to 60°C. The preparative applicability of TtADH was investigated by way of an efficient *in situ* NADH-recycling system involving 2-propanol and a second NAD(H)-dependent ADH. TtADH enzyme catalyses the reduction of acetophenone, 2,2,2-trifluoroacetophenone, methyl and ethyl benzoylformate with high yields and excellent enantiomeric excess.

1. Introduction

Biocatalysis is increasingly being used in synthetic routes for the preparation of enantiomerically pure compounds due to their importance in the pharmaceutical, agricultural, fragrance and flavour industries (Kroutil et al., 2004, and refs. therein). This is reflected in the fact that the sales of single-enantiomer small-molecule drugs has reached about US $10 billion in 2002 and the percent of chiral compounds as pharmaceutical intermediates is expected to grow to 70% by 2010. Moreover, the FDA has become increasingly reluctant to permit the introduction of additional racemic drugs, as these therapies are by definition saddled with 50% of chemical ballast (Rouhi, 2003).

Among many kinds of biocatalysts, dehydrogenases/reductases from various microorganisms have been used to prepare optically pure alcohols from carbonyl compounds. Furthermore, NAD(H) dependence and preference for bulky side-chained ketones/alcohols are two features that make a dehydrogenase/reductase more attractive from an application perspective; however, there is still a striking lack of sturdy ADHs with these characteristics that are even stable and active in organic solvents and in a broad range of temperature (Kroutil et al., 2004). Therefore, our aim was to search for novel activities, following the criterion to identify one or more gene(s) in the genome sequence of thermophilic microorganisms present in the database with the highest amino acid sequence identity with respect to that of *Lactobacillus brevis* ADH, a very well-known enantioselective NADPH-dependent SDR, active mainly on various aromatic ketones (Schlieben et al., 2005).

2. Materials and Methods

2.1 Chemicals. $NAD(P)^+$ and NAD(P)H were obtained from AppliChem (Darmstadt, Germany). Alcohols, aldehydes, ketones and ketoesters were obtained from Sigma-Aldrich. Other chemicals were A grade substances from Applichem. Recombinant *Bacillus stearothermophilus* ADH (BsADH) was prepared and assayed as previously described (Fiorentino et al., 1998).

2.2 Purification and characterization. The cloning and expression of the *ttadh* gene, and the purification of recombinant enzyme were performed according to Pennacchio et al., (2008).

2.3 Enzyme assay. TtADH was assayed spectrophotometrically at 65°C by measuring the change in absorbance of NADH at 340 nm using a Cary 1E spectrophotometer equipped with a Peltier effect-controlled temperature cuvette holder. The standard assay for the reduction reaction was performed by adding 5–25 μg of the enzyme to 1 ml of preheated assay mixture containing 20 mM methyl benzoylformate and 0.3 mM NADH in 50 mM potassium phosphate, pH 6.0. The standard assay for the oxidation reaction was performed using a mixture containing 20 mM (*S*)-(−)-1-phenylethanol and 3 mM NAD^+ in 100 mM glycine-NaOH, pH 10.5. 1 unit of TtADH and BsADH represented 1 μmol of NADH produced or utilized per min at 65° and 60°C, respectively on the basis of an absorption coefficient of 6.22 mM^{-1} for NADH at 340 nm.

2.4 Enantioselective biosynthesis. The enantioselectivity of TtADH was determined by reduction of aryl ketones and α-ketoesters using an NADH-regeneration system consisting of BsADH and 2-propanol. The reaction mixture contained 1 mM NAD^+, 20 mM carbonyl compound (3 mg), 11 U of BsADH , 260 mM 2-propanol, and 7 U TtADH in 1 ml of 100 mM MES, pH 6.0, 5 mM 2-mercaptoethanol and 100 mM KCl. The reactions were carried out at 50° and 60°C at different times in a temperature controlled water bath. Upon termination, the reaction mixture was extracted twice with ethyl acetate. The enantiomeric excess of the product (ee) and conversion were determined by GLC (Agilent 6850) equipped with a dimethylpentyl, β-cyclodextrin 25 m, 0.25 mm ID,

MEGA column (Legnano, Italy). Conditions for 1-phenyl-ethanol and α-(trifluoromethyl)benzyl alcohol were: oven temperature from 90°C (initial time 10 min) to 110°C (final time 5 min), with a heating rate of 2.5°C/min. Conditions for ethyl and methyl mandelate were: oven temperature from 100°C (initial time 5 min) to 130°C (final time 5 min), with a heating rate of 2.5°C/min. The conversion yield was determined on the basis of the peak areas of carbonyl substrate and alcohol products on GC chromatograms.

3. Results and Discussion

3.1 Enzyme properties. Gel filtration chromatography, SDS-PAGE and kinetic studies assessed that TtADH is a homotetrameric SDR (MW =108 kDa), markedly thermophilic (optimal temperature ≈ 80°C) and thermostable ($T_{1/2,\ 30\ min}$ ≈ 90°C). The optimal pH for the reduction and oxidation reaction catalysed by TtADH resulted around 6 and 10, respectively (Pennacchio et al., 2008).

3.2 Coenzyme and substrate specificity. The enzyme showed no activity with NADP(H) and full activity with NAD(H). Moreover, the following compounds resulted substrates of TtADH (reported in decreasing order of relative %). Alcohols: (*S*)-(−)-1-phenylethanol (100), 4-methoxybenzyl alcohol (99), (±)-1-phenyl-1-propanol (59), 1-(4-fluorophenyl)ethanol (45), 1-(4-chlorophenyl)ethanol (26), *trans*-cinnamyl alcohol (25), 2-methoxybenzyl alcohol (25), 1-phenyl-2-propanol (16), (*R*)-1-phenyl-2-propen-1-ol (14) and 3-methoxybenzyl alcohol. Carbonyl compounds: 1-phenyl-1,2-propanedione (146), 2,2,2-trifluoroacetophenone (100), 2,2-dichloroacetophenone (32), benzaldehyde (14), 2-, 3-, and 4-methoxybenzaldehyde (13, 14, 13). α-Ketoesters: ethyl benzoylformate (100) and methyl benzoylformate (57). Kinetic constants analysis indicated that the enzyme is *S* stereospecific and that the physiological direction of the catalytic reaction is reduction rather than oxidation (data not shown).

3.3 Stability in organic solvents. The effect of common organic solvents such as methanol, 2-propanol, acetonitrile, dioxane and ethyl acetate was studied by assaying TtADH activity in their presence at two different times and temperatures. Table 1 shows that the enzyme activity increases significantly in aqueous buffer, as well as in the presence of all the solvents tested. Moreover, the activity measured after 65 h incubation at 25°C in TtADH samples containing 10% methanol, 2-propanol, acetonitrile, dioxane, ethyl acetate, 1-propanol, and *n*-hexane resulted increased by 172, 110, 167, 160, 115, 173 and 182%, respectively, whereas that of the control remained unchanged. Furthermore, standard assays performed in the presence of 0.01 to 0.5% 2-propanol resulted in no change in activity, suggesting that the activity enhancement is not due to an immediate effect of solvent on the protein structure. A considerable body of literature exists which describes the activation of thermophilic enzymes by loosening of their rigid structure in the presence of protein perturbants (Fontana et al., 1998). To account for the observed enhancements of TtADH activity, the organic solvent is proposed to induce a

Table 1. Effect of organic solvents on *T. thermophilus* ADH.

Solvent	Activity[a] (%)[b] 50°C		Activity[a] (%)[b] 60°C	
	5 h	24 h	5 h	24 h
None	131	142	129	134
5% Methanol	138	137	185	185
2% 2-Propanol	165	151	193	168
10% 2-Propanol	174	180	211	174
5% Acetonitrile	131	158	167	177
5% 1,4-Dioxane	152	180	177	161
5% Ethyl acetate	151	183	176	145

[a] Activity assays were performed as described in M. M. using ethyl benzoylformate as the substrate. [b] The percent of activity was calculated with respect to the value measured before the addition of solvent.

conformational change in the protein molecule to a more relaxed and flexible conformation into one which is optimal for activity.

3.4 Enantioselective biosynthesis. The enantioselectivity of TtADH was tested using acetophenone, 2,2,2-trifluoroacetophenone, methyl and ethyl benzoylformate as substrates, and an efficient NADH-regeneration system (Figure 1) consisting of Zn-containing, homotetrameric ADH from the moderate thermophilic bacterium *Bacillus stearothermophilus LLD-R strain* (BsADH) (Fiorentino et al., 1998). This NAD(H)-dependent ADH is mainly active on aliphatic and aromatic primary and secondary alcohols and aldehydes, but not on aliphatic and aromatic ketones, nor on the carbonyl substrates of TtADH and corresponding alcohols (data not shown). Since 2-propanol is not a substrate of TtADH, it may be a suitable substrate for BsADH in NADH recycling, as well as being used as a co-solvent. Experimental conditions including buffer, pH,

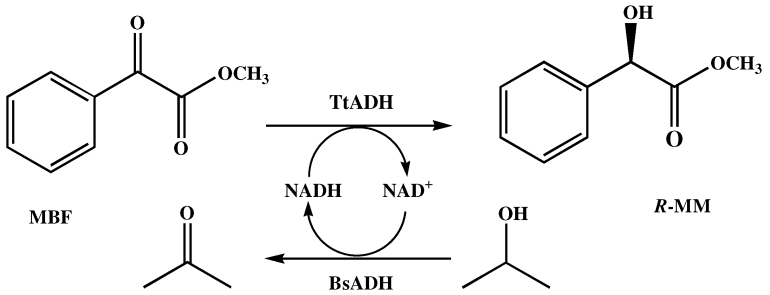

Figure 1. Coenzyme recycling in the production of (*R*)-methyl mandelate with *T. thermophilus* ADH (TtADH) utilizing *B. stearothermophilus* ADH (BsADH) and 2-propanol. MBF, methyl benzoylformate; *R*-MM, methyl (*R*)-(–)-mandelate.

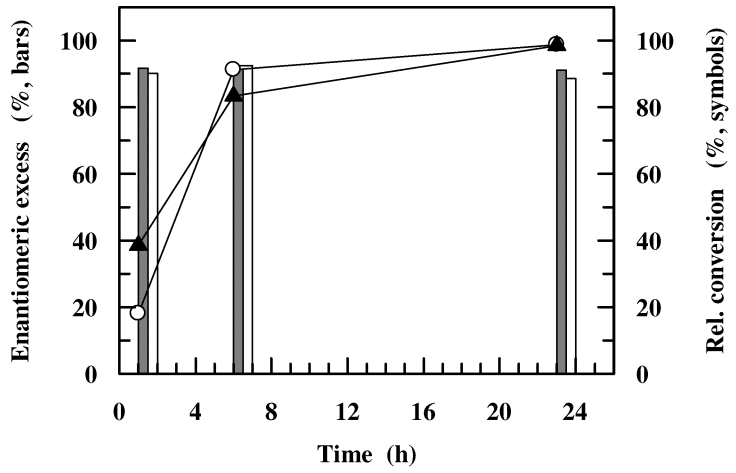

Figure 2. Conversion and enantiomeric excess of methyl benzoylformate by *T. thermophilus* ADH at different reaction times. Biotransformations were carried out at 50°C (white bars and circles) and 60°C (grey bars and triangles) as described in M. M. The reactions were stopped by addition of ethyl acetate at the times indicated. The dried extracts were analysed by chiral GC and the relative conversion calculated as the area of alcohol products divided by the total area.

temperature and reaction time were chosen to optimise productivity. The k_{cat}/pH profiles indicated that MES buffer, pH 6.0 and 60°C were the optimal pH and temperature conditions for catalysis by the two enzymes. Conversion experiments were carried out at 50° and 60°C, and the reactions allowed to proceed for 1, 6 and 24 h. Figure 2 shows that methyl benzoylformate was reduced by TtADH to methyl (*R*)-(−)-mandelate with 18, 91, and 99% conversion at 50°C and 38, 83, and 98% conversion at 60°C following 1, 6, and 24 h, respectively. The higher ee value (92%) was achieved after 6 h reaction at 50°C, whereas it was 91% at 60°C after 6 h, and 89 and 91%, at 50 and 60°C, respectively after 24 h reaction. However, the reaction with larger amounts of substrate and enzyme (100 mg, 92 and 62 U of BsADH and TtADH, respectively, in 30 ml) resulted in 93.5% ee and 90% conversion after 24 h reaction at 50°C.

The reaction with ethyl benzoylformate performed for 6 h at 50°C yielded ethyl (*R*)-(−)-mandelate with a conversion of 90% and 95% ee. Acetophenone was reduced to (*S*)-(−)-1-phenylethanol following a 6 h reaction at 50°C, with a conversion of 70% and 99% ee. The conversion of 2,2,2-trifluoroacetophenone was 20 and 40% in 1 h at 50° and 60°C respectively, approximating 100% in 6 h at both the temperatures. TtADH preferably reduced this arylketone to (*R*)-α-(trifluoromethyl)benzyl alcohol with an ee of 90 and 93% at 60° and 50°C respectively, after 3 h of reaction. However, reaction times extended to 24 h did not improve the yield or ee of the biotransformation (data not shown).

On the whole, enantioselectivity data indicate that the hydride ion of NADH is transferred to the *re* face of the carbonyl of acetophenone, 2,2,2-trifluoroacetophenone,

as well as that of methyl and ethyl benzoylformate, suggesting that TtADH exhibits Prelog specificity (Prelog, 1964). Noteworthy, the optically active alcohols produced are used as chiral building blocks in organic synthesis. *O*-protected methyl (*R*)-(−)-mandelate is used as an intermediate for the synthesis of pharmaceuticals (Kobayashi et al., 1990), and optically pure trifluoromethyl-substituted benzyl alcohols are important precursors for specialty chemicals used in electro-optical devices such as LCDs (Fujisawa et al., 1993).

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