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Stability, activity and substrate specificity of alcohol dehydrogenases in media containing organic solvents

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The stability, activity and substrate specificity of two alcohol dehydrogenases, membrane bound (mADH) from *Gluconobacter* sp. 33 and soluble (ADH IIG) from *Pseudomonas putida* HK5, in media containing organic solvents were investigated. ADH IIG was more stable in organic solvents than mADH. Organic solvents in the reaction mixture decreased the rate of substrate oxidation and increased the K_m. The substrate specificity of ADH IIG and mADH was different for water-insoluble substrates. ADH IIG used cyclo-, primary and secondary alcohols with long carbon chain as substrates, but mADH used effectively only 1-octanol when organic solvents were present in the reaction mixture.

Key words: alcohol dehydrogenase, pyrroloquinoline quinone (PQQ), organic solvents, enzyme inhibition, log P

INTRODUCTION

Enzymes have found wide applications as practical catalysts in chemical syntheses and industrial processes [1, 2]. The realization that enzymes can be made active in organic solvents containing little or no water [3] has given further boost to this utility [4]. Most synthetic uses of enzymes to date [1], including those in nonaqueous solvents [4], have involved hydrolases. However, the use of enzymes that catalyze the selective oxidations and reductions (especially asymmetric ones) of organic molecules should have the greatest impact in this field [5]. For example, the use of horseradish peroxidase in organic solvents (either alone or in combination with other enzymes) has been proposed for the production of phenolic resins [6, 7]. Another illustration of the benefits of nonaqueous enzymatic oxidoreductions involves horse liver alcohol dehydrogenase [8]. Like other dehydrogenases, this enzyme requires a coenzyme (NAD). However, NAD is costly and not very stable, which makes its use in large-scale asymmetric transformations doubtful, even assuming efficient coenzyme regeneration [5].

Alcohol dehydrogenases (ADH), containing pyrroloquinoline quinone (PQQ) as the prostetic group and produced by several aerobic bacteria [9–12], are currently of special interest in the fields of biosensors design and catalytic systems. ADHs can be classified according their prostetic group(s) and localization in the cell. Type I ADH is a simple quinoprotein having PQQ as the only prostetic group. It is produced by *Pseudomonas* spp. [10, 13]. Type II ADH is a monomeric periplasmic enzyme and has both PQQ and heme *c* as prostetic groups. It is produced by *Pseudomonas*, *Comamonas* and *Ralstonia* [10, 12, 13]. Type III ADH is a membrane-bound enzyme produced by *Gluconobacter* and *Acetobacter* spp. and acts on the periplasmic surface [13, 14]. The enzymes of this group consist of three different subunits, the first harbouring PQQ and heme *c*, the second containing three citochromes *c*, and the third subunit being of unknown function. Many catalytic systems based on electrochemical oxidation / reduction of products were designed for the detection of alcohols in the blood, vines and other biological media, based on the type II and III ADHs [15–18]. Different ADHs can oxidize a broad variety of primary and secondary, cyclic and aliphatic, mono- and polyalcohols and can be of great interest for chemical synthesis of various chemical compounds, including resolution of enantiomers [10, 14, 19]. Moreover, electrochemistry of redox proteins, especially direct electron transfer, is a broadly employed technology with important applications in biosensors, biofuel cells and chemical syntheses [20]. Escalating attention in this area is driven by remarkable progress in designing efficient interfaces for transferring electrons between electrode surfaces and quino(hemo)proteins including ADHs [21]. Keeping in mind that not all potential ADH substrates are soluble in water and a conversion of substrates with low solubility in water is hardly possible using the standard conditions, the use of organic solvents may increase the solubility substrates or at least emulsify them for the better catalysis on the interface.

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The purpose of the current investigation was a comparative study of the effect of organic solvent on the stability, activity and substrate specificity of two alcohol dehydrogenases: type II ADH from *Pseudomonas putida* HK5 (ADH IIG) and type III membrane-bound ADH from *Gluconobacter* sp. 33 (mADH).

MATERIALS AND METHODS

Materials. All chemicals used in the study were commercial products of guaranteed grade. The organic solvents were of analytical grade and obtained: dimethyl sulfoxide (DMSO), and acetonitrile (AC) from Sigma; *N*,*N*-dimethylformamide (DMF) from Merck; 1,4-dioxane (1,4-DO) from Reachim. Alcohol dehydrogenase (ADH IIG) from *Pseudomonas putida* HK5 was isolated and purified by a previously published method [19]. Alcohol dehydrogenase (mADH) from *Gluconobacter* sp. 33 was isolated and purified from *Gluconobacter* sp. 33 biomass [14].

Enzyme assay. All enzyme assays were performed at 30 °C as follows. Activity of ADH from *Pseudomonas putida* HK5 was assayed spectrofotometrically measuring a decrease of potassium ferricyanide absorbtion at 417 nm in the reaction mixture containing 50 mM Tris/HCl, pH 8.0, 2 mM Ca^{2+}, 1 mM $K_3[Fe(CN)_6]$, 20 mM glycerol, 5–10 µl of enzyme solution in a total volume of 1 ml. Activity of ADH from *Gluconobacter* sp. 33 was assayed as described above, in the reaction mixture consisting of 50 mM $K_3C_6H_5O_7$, pH 5.0, 2 mM Ca^{2+}, 1 mM $K_3[Fe(CN)_6]$, 20 mM ethanol and 5–10 µl of enzyme solution. One unit of the enzyme activity was defined as the amount of the enzyme catalysing the oxidation of 1 µmol of substrate per minute under the conditions described above.

All the data presented in tables and figures were the mean from three experiments.

RESULTS

Stability of mADH and ADH IIG in organic solvents. Most of alcohols with a carbon chain longer than seven atoms are practically insoluble in water, but they can be dissolved in organic solvents. One of the main problems of enzymatic conversion of water-insoluble substrates is the stability of enzymes in organic solvents. The presence of organic solvent may both inactivate or activate the enzyme [20–22]. In this work, organic solvents miscible in water were used to dissolve insoluble substrates. The stability of soluble and membrane ADH was investigated (Tables 1 and 2). The organic solvents acted on the stability of soluble and membrane ADH in different manner. Membrane-bound mADH was 70–100% stable in all organic solvents (the final concentration up to 5%) but only for one hour. A prolonged incubation and higher organic solvent concentrations resulted in a partial or full inactivation of the enzyme. mADH was most stable in 1,4-dioxane (Table 1). Soluble ADH IIG was more than 85% stable in DMSO, AC and DMF (the final concentration up to 10%) for 24 hours. An exception was 1,4-dioxane which rapidly inactivated the ADH IIG (Table 2).

Table 1. **Stability of mADH from *Gluconobacter* sp. 33 in the presence of organic solvent**

Time,	DMSO			DMF			AC			1,4-DO		
h	1%	5%	10%	1%	5%	10%	1%	5%	10%	1%	5%	10%
0	100	83.9	62.3	100	91.3	75.1	100	100	100	100	100	100
1	91.1	76.9	45.3	100	69.5	27.4	100	100	88.6	100	100	100
3	82.5	48.6	19.4	77.0	35.9	20.9	93.9	87.0	57.4	96	95	95
7	67.4	33.0	15.3	63.8	25.1	11.1	80.5	73.6	31.6	89.5	88.6	76.6
24	46.0	19.9	9.2	35.9	13.7	4.2	79.9	43.4	18.7	79.1	67.1	45.8

The enzyme was incubated at room temperature in 50 mM Tris/HCl buffer, pH 8.0, containing 1 mM Ca^{2+} and organic solvent. The remaining activity was determined as described in Materials and Methods. 100% was initial activity without organic solvent.

Table 2. **Stability of ADH IIG from *Pseudomonas putida* HK5 in the presence of organic solvent**

Time,	DMSO			DMF			AC			1,4-DO		
h	5%	10%	25%	5%	10%	25%	5%	10%	25%	1%	5%	10%
0	100	100	100	100	100	74.2	100	100	71.6	100	85	81
1	100	100	100	100	100	60.2	100	100	8.0	11.3	0	0
3	100	100	100	100	100	52.9	100	100	0.6	1.1	0	0
7	100	100	96.8	97.8	87.0	38.2	100	100	0	0	0	0
24	100	94	88	95	85.1	10	95.2	90.4	0	0	0	0

The enzyme was incubated at room temperature in 50 mM Tris/HCl buffer, pH 8.0, containing 1 mM Ca^{2+} and organic solvent. The remaining activity was determined as described in Materials and Methods. 100% was initial activity without organic solvent.

Ability of mADH and ADH IIG to oxidize the substrate in the media containing various organic solvents. The second important problem of using ADH IIG and mADH for chemical synthesis is its ability to catalyse conversion of substrates in the presence of organic solvents in the reaction mixture. The relative activity of these enzymes in the presence of 0–50% of organic solvent is shown in Figure. The results indicated that the rate of substrate oxidation strongly decreased with increasing the concentration of organic solvent. The best results were obtained with 0.5–5% di-

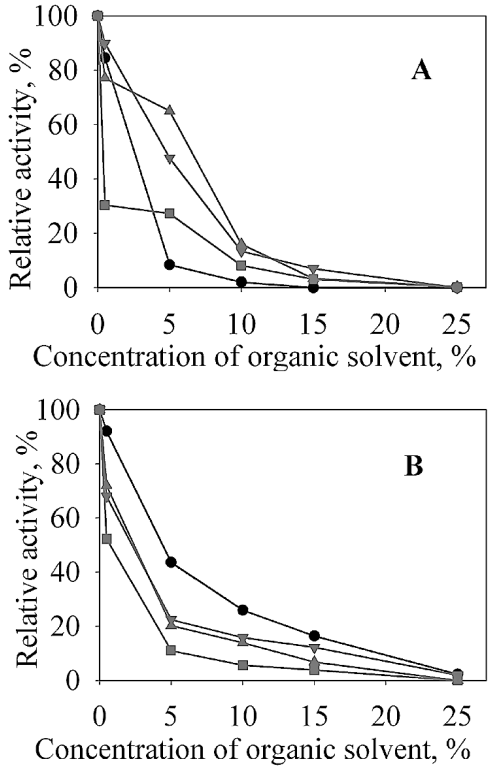

Figure. Dependence of ADH activity on the nature of organic solvent and its concentration in the reaction mixture. A – mADH from *Gluconobacter sp.* 33. B – ADH IIG from *Pseudomonas putida* HK5. Activity was determined as described in Materials and Methods. 100% was activity without organic solvent. DMSO (●), DMF (■), AN (▲), DO (▼)

methylsulfoxide which decreased the activity of ADH IIG by 8–56%, and 0.5–5% 1,4-dioxane decreased the activity of mADH by 10–52%.

Substrate specificity of mADH and ADH IIG. The substrate specificity of mADH and ADH IIG for water-soluble substrates has been investigated in previous papers [14, 19]. Five substrates insoluble in water (2-decanol, cyclohexanol, cyclooctanol, 1-octanol, benzyl alcohol) were tested in this work. Four different organic solvents in the concentration range of 0.5–25% were used for preparing the reaction mixtures. In the case of 1-octanol and 2-decanol, an emulsion was observed when the concentration of organic solvent was 0.5%. Benzyl alcohol formed emulsions in all mixtures but not in the presence of 25% of organic solvent. The results showed that substrate specificity of mADH was different from that of ADH IIG. ADH IIG used cyclo- primary and secondary alcohols with long carbon chain as substrates (Table 3). The mADH used effectively (76–89%) only 1-octanol, the only primary alcohol used in the study, when 0.5% of organic solvents were present in the reaction mixture. The relative activity of ADH IIG was dependent on the solvent and substrate. The nature of the solvent did not show an influence on mADH activity. It should be noted that 0.5% emulsions were unstable and partial lamination was observed, therefore, higher concentrations of organic solvents (DMSO and 1,4-DO) were tested. The results are presented in Tables 4 and 5. An increase of DMSO concentration in the reaction mixture decreased the relative activity of both ADHs; even 5% of DMSO decreased ADH IIG activity by 37–57% versus 0.5% (Table 4). In contrast, mADH was very sensitive to DMSO, but not to 1,4-DO (Table 5).

Dependence of K_m on organic solvent. The K_m values were determined for ADH IIG and mADH

Table 3. **The substrate specificity of ADH IIG from *Pseudomonas putida* HK5 in the presence of 0.5% of organic solvent**

Substrate	Relative activity of ADH IIG, %			
	DMSO	DMF	AC	1,4-DO
2-decanol	47	30.5	18.5	50.1
cyclohexanol	47.1	23.4	22.0	41.4
cyclooctanol	41	29.8	28.8	34.3
1-octanol	100	84.4	100	100
benzyl alcohol	82	58.4	62.6	70.7

The substrates were dissolved in organic solvent. Activity was determined in 50 mM Tris/HCl buffer, pH 8.0, containing 0.5% of organic solvent, 2 mM Ca^{2+}, 1 mM $K_3[Fe(CN)_6]$ and 5 mM substrate at 30°C. 100% was ADH IIG activity with 5 mM glycerol as a substrate without organic solvent.

Table 4. **Dependence of substrate oxidation rate by ADH IIG from *Pseudomonas putida* HK5 on the concentration of DMSO in the reaction mixture**

Substrate	DMSO, %				
	0.5	5	10	15	25
2-decanol	100	56.4	36	25.7	8
cyclohexanol	100	62.4	30	22.4	1.4
cyclooctanol	100	46	34.7	22.7	2
1-octanol	100	59.4	32.9	18	4.8
benzyl alcohol	100	62.7	32.5	23.9	2.5
glycerol	100	43.6	25.9	16.4	2.3

The substrates were dissolved in DMSO. Activity was determined in 50 mM Tris/HCl buffer, pH 8.0, containing organic solvent, 2 mM Ca^{2+}, 1 mM $K_3[Fe(CN)_6]$ and 5 mM substrate at 30°C. 100% was ADH IIG activity with 5 mM glycerol as a substrate without DMSO.

Table 5. **Dependence of substrate oxidation rate by mADH from *Gluconobacter* sp. 33 on the concentration of organic solvent in the reaction mixture**

Substrate	DMSO, %					1,4-DO, %				
	0.5	5	10	15	25	0.5	5	10	15	25
ethanol	100	6.81	0.26	0	0	100	41	10.6	8.8	0
1-octanol	100	10.2	1.74	0	0	100	34.7	19.4	11.8	4.9

The substrates were dissolved in organic solvent. Activity was determined in 50 mM potassium citrate buffer, pH 5.0, containing organic solvent, 2 mM Ca^{2+}, 1 mM $K_3[Fe(CN)_6]$ and 5 mM substrate at 30°C. 100% was mADH activity with 5 mM ethanol as a substrate without organic solvent.

Table 6. **Dependence of K_m of ADH IIG and mADH on organic solvents in the reaction mixture**

Organic solvent, 5%	K_m, mM	
	ADH IIG	mADH
none	2.28	0.846
DMSO	3.268	n.a.
DMF	23.35	427.3
AC	n.a	566.4
1,4-DO	n.a	505

n. a – not analysed. The activity was determined as described in Materials and Methods. Substrates were glycerol and ethanol for ADH IIG and mADH, respectively.

(substrates were glycerol and ethanol, respectively) in water/solvent mixtures. Results are presented in Table 6. It was found that the Km values for both enzymes were higher in the presence of organic solvent.

DISCUSSION

The use of aqueous mixtures of organic co-solvents for conducting enzymatic reactions has become highly promising in biotechnology processes. By total or partial replacement of water as the reaction medium with an organic solvent, hydrophobic substrates can be more efficiently converted, hydrophobic products can be produced with higher yields, and the thermo dynamic equilibrium of hydrolytic reactions can be shifted to the synthetic way [22, 23]. The use of organic solvents has broadened the range of possible substrates and increased the possibility to use water-immiscible compounds in enzymatic conversion. A comparative analysis of mADH and ADH IIG has revealed their difference as regards stability, substrate specificity and ability to oxidize different substrates in media containing organic solvents. The solvents tested included acetonitrile, dioxan, dimethylformamide and dimethylsulfoxide. The effect of different water-miscible organic solvents on the activity and stability of free ADH was evaluated. For all of them, as the co-solvent content increased, ADHs activities decreased. The organic solvents used in this investigation differed in their polarity index, log *P,* dielectric constant, and denaturing capacity: DMSO (7.2; -1.3; 47; 60.3), DMF (6.4; -1.0; 37; 63.3) acetonitrile (5.8; -0.33; 38; 64.3), dioxane (4.8; -1.1; 2.2; 92.2) [24]. The soluble enzyme ADH IIG from *Pseudomonas putida* HK5 was most stable in the most hydrophilic organic solvent (DMSO), contrary to mADH which was isolated from the membrane fraction of *Gluconobacter* sp. 33 and which was more stable in the solutions containing more hydrophobic organic solvents. The inhibitory effect of different organic solvents was detected for alcohol dehydrogenases from *Thermoanaerobacter brockii, Lactobacillus kefir* and yeast [25, 26]. On the contrary, alcohol dehydrogenase from *Aeopyrum pernix* was activated by organic solvents with a large log P [27]. It was shown that the catalytic efficiency (k_{cat}/K_m) of a carboxylesterase from the extremely thermoacidophilic archaeon *Sulfolobus solfataricus* P1 decreased with increasing DMSO concentrations. Interestingly, the effect of DMSO on the Michaelis constant (K_m) was most noticeable at lower temperatures [24].

In spite of enzymes, stability in the presence of 5–10% of organic solvents, the rates of catalysis were reduced. The mADH was more sensitive, maybe because of its more complicated protein structure [14]. The ADH IIG is a monomeric protein [10] and was more resistant to organic solvents. A stabilisation of enzymes by various immobilisation procedures is a known state-of-the-art. The application of these techniques for PQQ-dependent ADHs is in progress.

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L. Marcinkevičienė, I. Bachmatova, R. Meškys

ALKOHOLIŲ DEHIDROGENAZIŲ STABILUMAS, AKTYVUMAS IR SUBSTRATINIS SPECIFIŠKUMAS TERPĖSE SU ORGANINIAIS TIRPIKLIAIS

S a n t r a u k a

Ištirtas organinių tirpiklių poveikis dviejų alkoholių dehidrogenazių (ADH) – membraninės ADH iš *Gluconobacter* sp. 33 ir tirpios ADH IIG iš *Pseudomonas putida* HK5 – stabilumui, aktyvumui ir substratiniam specifiškumui. Nustatyta, kad ADH IIG yra stabilesnė daugumai tirtų tirpiklių, lyginant su mADH, tačiau organiniai tirpikliai reakcijos mišinyje mažino substratų oksidacijos greitį ir didino fermentų K_m. ADH IIG ir mADH pasižymėjo skirtingu substratiniu specifiškumu vandenyje netirpių substratų atžvilgiu. ADH IIG substratai buvo pirminiai, antriniai ir cikloalkoholiai, tuo tarpu mADH substratai – tik pirminiai alkoholiai.