CHEMIJA. 2012. Vol. 23. No. 3. P. 223–232

Biocatalytic properties of quinohemoprotein alcohol dehydrogenase IIG from *Pseudomonas putida* HK5

Liucija Marcinkevičienė[1],

Jonita Stankevičiūtė[1]*,

Irina Bachmatova[1],

Regina Vidžiūnaitė[2],

Ana Chaleckaja[2],

Rolandas Meškys[1]

[1] *Department of Molecular Microbiology and Biotechnology, Institute of Biochemistry, Vilnius University, Mokslininkų 12, Vilnius LT-08662, Lithuania*

[2] *Department of Enzyme Chemistry, Institute of Biochemistry, Vilnius University, Mokslininkų 12, Vilnius LT-08662, Lithuania*

Quinohemoprotein alcohol dehydrogenases (ADHs) are attractive catalysts because of their wide substrate specificity and non-diffusible cofactor. ADH IIG from *Pseudomonas putida* HK5 is capable to oxidize various primary or secondary aliphatic and cyclic amino alcohols stereoselectively. An optimal pH range for ADH IIG activity has been shifted to more acidic side (pH 4–6) when amino alcohols were used as substrates. ADH IIG covalently immobilized on silica gel by cross-linking with glutaraldehyde or 1-ethyl-3(3-dimethylaminopropyl)carbodiimide exhibits a higher storage stability and thermostability as well as an improved activity in the presence of organic solvents comparing with the free enzyme. The affinity of ADH IIG to substrates has not been significantly changed in the presence of organic solvents or after the immobilization of the enzyme.

Key words: alcohol dehydrogenase, pyrroloquinoline quinone, immobilization, organic solvents, enantioselective oxidation

INTRODUCTION

Enantiomerically pure target compounds have tremendous importance in the pharmaceutical, agricultural, and food industries, therefore, many efforts have been made to establish the methods for enantioselective synthesis [1]. Biocatalytic steps in organic synthesis have already proven to be an efficient, highly stereoselective and flexible alternative in the preparation of many industrially relevant compounds [2].

Alcohol dehydrogenases (ADHs) are the enzymes containing various cofactors (NAD(P)), pyrroloquinoline quinone (PQQ), heme *c*) which may be very useful in industrial enantioselective oxidation of various alcohols because of their wide substrate specificity [3–9]. It was previously reported that quinohemoprotein ADHs, isolated from *Comamonas testosteroni* [10, 11], *Acetobacter pasteurianus* [12] and *Ralstonia eutropha* [13], exhibited stereopreference, when enantiomerically pure alcohols were used as substrates.

Quino(hemo)protein ADHs are classified into three types [14]. Type I ADH is a soluble simple quinoprotein having PQQ as the only prostetic group, while type II and type III ADHs have heme *c* and PQQ as cofactors. Type II ADHs are soluble periplasmic enzymes, produced by different *Proteobacteria* such as *Ralstonia* [13], *Pseudomonas* [15–17] or *Comamonas* [10, 18]. Type III ADH is a membrane bound enzyme, located on the periplasmic surface of acetic acid bacteria [12, 19].

* Corresponding author. E-mail: jonita.stankeviciute@bchi.vu.lt

Quinohemoproteins catalyze the oxidation of a great variety of primary or secondary aliphatic and cyclic alcohols. These enzymes are capable to catalyze the oxidation of mono- and polyhydroxylic compounds. Many natural and artificial electron acceptors such as azurin, citochromes, phenazine methosulphate, dichlorophenol indophenole, ferricyanide and other are used for the biocatalysis with quinohemoproteins [14, 20–24]. Therefore, PQQ-dependent ADHs may find a wide application in industrial synthesis or design of biosensors and catalytic systems [14, 23, 25–28]. Furthermore, quinoprotein ADHs do not require external addition of expensive cofactors, unlike in the case of NAD(P)-dependent ADHs [29]. The benefits of these enzymes would increase if the conditions at which they can transform water insoluble substrates would be created. The organic solvents may increase the solubility of substrates or at least emulsify them for the better catalysis on the interface. The important problem is whether enzyme can work and be stable (retains its native structure and does not loose cofactors) in the presence of organic solvents or elevated temperatures [30].

An immobilization of the enzyme can improve its resistance against organic solvents and / or elevated temperatures. Moreover, immobilization can solve some important problems, such as long-term operational stability and re-use of the enzyme. There are many reports about the covalent immobilization of PQQ-dependent enzymes on the surface of electrodes. A membrane bound ADH (type III) was used for the design of the new biosensors based on modified screen printed electrodes [31] or a polypyrrole film [23, 25, 32]. However, there is scarce information about a covalent immobilization of PQQ-dependent ADHs applicable for a bioconversion of hydroxylic groups containing compounds.

Pseudomonas putida HK5, grown on different alcohols, produces three different alcohol dehydrogenases (ADH I, ADH IIB and ADH IIG) each of which contains the prostetic group PQQ [16]. ADH IIG is synthesized by *Pseudomonas putida* HK5 cells grown on 1,2-propanediol or glycerol. This enzyme is able to oxidize a broad range of primary and secondary aliphatic and cyclic alcohols including diols and glycerol. Moreover, ADH IIG can resolve the enantiomeric forms of linear secondary alcohols and 1,2-cyclohexanediols [16, 17, 33].

Optically active amino ketones and amino alcohols are promising intermediates for synthesis of various biologically active compounds such as neuraminidase inhibitor oseltamivir phosphate (Tamiflu™) [34] or anti-tuberculosis agent ethambutol [35]. Biocatalytic resolution of enantiomers of amino alcohols using NADP-dependent L-1-amino-2-propanol dehydrogenase from *Rhodococcus erythropolis* MAK154 has been applied for the synthesis of *d*-pseudoephedrine [36]. However, the application of PQQ-dependent ADHs for selective oxidation of amino alcohols has not been studied yet.

In this paper, a process of covalent immobilization of ADH IIG from *Pseudomonas putida* HK5 on a water insoluble silica gel is presented. The enantioselectivity of the enzyme as well as oxidation of amino alcohols is analysed.

EXPERIMENTAL

Materials

All chemicals used in the study were commercial products of guaranteed grade. The organic solvents were of analytical grade. Dimethyl sulfoxide (DMSO), acetonitrile (AC), 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (CDI) were obtained from Sigma; *N*,*N*-dimethylformamide (DMF) from Merck; 1,4-dioxane (DO) from Reachim. 3-Aminopropyltriethoxysilane (APTS), glutaraldehyde (GA) and silica gel spherical (particle size 0.035–0.045 mm, pore diameter 260–340 Å) and all substrates were purchased from Fluka. Alcohol dehydrogenase (ADH IIG) from *Pseudomonas putida* HK5 was isolated and purified as described earlier [17].

Modification of silica gel with APTS

20 ml of 1% APTS (pH 3.5) was mixed with 3 g of silica gel and heated in the boiling water bath with mild stirring. After one hour the pellet was collected, washed with acetone and distilled water. Then the silica gel was suspended into 10 ml of distilled water and stored at 4 °C. Aminated silica gel (SS) was further used for the immobilization of the enzyme.

Immobilization of ADH IIG by GA cross-linking

0.3 ml (90 mg) of SS was washed once with 2.5 volumes of 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$. 0.02% GA was added and the mixture was stirred at 30 °C for 30 min. The pellet was washed five times with 1 ml of the same buffer. 0.6 ml of ADH IIG (27 μg protein for 1 mg SS) was added to the wet pellet and the mixture was stirred at 30 °C for 30 min. The immobilization was stopped by the addition of 200 mM methylamine. After the immobilization, the pellet suspension (ADH IIG-GA-SS) was washed with 1 M NaCl in the same buffer several times. Thereafter, washing procedure was repeated with the buffer without NaCl. The pellet was suspended in the final volume of 0.3 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$.

Immobilization of ADH IIG by CDI cross-linking

0.06 ml of ADH IIG (15 μg of protein for 1 mg of SS) was mixed with 0.02 ml of 200 mM CDI solution, prepared in 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$. The mixture was stirred at 30 °C for 20 min. 0.3 ml (90 mg) of SS was washed once with 2.5 volumes 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$. The pellet was mixed with ADH IIG-CDI solution and was stirred at 30 °C for 2 h. After the immobilization, the pellet (ADH IIG-CDI-SS) was washed with 1 M NaCl in the same buffer several times. Thereafter, washing procedure was repeated with the buffer without NaCl. The pellet was suspended in the final volume of 0.3 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$. Immobilization efficiency was calculated from the equation: Efficiency (%) = (total units of immobilized enzyme / total units of enzyme used for immobilization) × 100.

Enzyme assay

Enzyme assays were performed at 30 °C with intensive stirring. The reaction mixture consisted of 2 mM $CaCl_2$, 1 mM $K_3[Fe(CN)_6]$, 20 mM substrate (glycerol or another alcohol), 5–10 µl of free or immobilized enzyme suspension in a total volume of 1.0 ml of 50 mM Tris-HCl buffer, pH 8.0. Immobilized enzyme was separated from the reaction mixture by short centrifugation after a defined period of time. The activity of ADH IIG was assayed spectrophotometrically by measuring a decrease in absorption at 420 nm resulting from reduction of potassium ferricyanide. One unit of the activity was defined as the amount of the enzyme that catalyses the oxidation of 1 µmol of substrate per min.

Kinetic measurements and calculations

Kinetic measurements of substrate oxidation were performed spectrophotometrically using a computer-controlled "Nicolet evolution 300" spectrophotometer (Thermo electron corporation, USA) in 50 mM Tris-HCl buffer solution, pH 8.0, containing 2 mM $CaCl_2$. The kinetic curves were recorded by the decrease in absorption of potassium ferricyanide at 25 °C in the presence of ADH IIG from *Pseudomonas putida* HK5 and an appropriate alcohol. The reaction was started by adding the solution of the enzyme. The concentration of oxidized substrate was calculated according to the reduction of ferricyanide to ferrocyanide ($\varepsilon_{420} = 1.02\ mM^{-1} \cdot cm^{-1}$ [37]). Kinetic parameters for the alcohols and ferricyanide were determined with 1 mM ferricyanide and different concentrations of alcohols or with 10–30 mM alcohols and different concentrations of ferricyanide, respectively. The initial rates (V_0) of the oxidation of different substrates were calculated by fitting the kinetic curves with the linear function. For the linear dependence, the initial rate was calculated as a slope. To analyze the dependence of V_0 on the substrate concentration and determine the apparent kinetic parameters V_{max} and K_M of the reactions, the Michaelis-Menten equation was used. For data fitting, the program GraFit (Erithacus Software LTD) was used. Catalytic constants (k_{cat}) were calculated as a ratio of V_{max} and the total concentration of the enzyme. Reactivity constants of the enzyme and substrates (k_{ox} and k_{red}) were calculated as a ratio of catalytic constant (k_{cat}) and K_M.

The dependence of enzyme activity on pH was investigated using 50 mM Tris-HCl buffer solution, containing 2 mM $CaCl_2$ (pH range 5.5–9.5) and 50 mM phosphate-citrate buffer solution, containing 2 mM $CaCl_2$ (pH range 3.5–5.5). The pH of the buffer solution was changed within the range from pH 3.5 to pH 9.5.

Enzyme thermostability

Samples of ADH IIG, ADH IIG-GA-SS and ADH IIG-CDI-SS were incubated separately with 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ at different temperatures in the range from 25 °C to 65 °C for 10 min. The residual activity of the enzyme was measured as described in "Enzyme assay". For evaluation of the rate of thermoinactivation, samples of free and immobilized enzyme were incubated at 55 °C and the residual activity was measured after incubation (see "Enzyme assay" above). The half-lives of free and immobilized enzyme were calculated from the equation $X = X_0\ (1/2)^{t/g}$. This equation can also be written as $\log X = \log X_0 - [(\log 2)/g]t$ where X is remaining enzyme activity, X_0 is initial activity, t is time, and g is half-life.

Storage stability of free and immobilized ADH IIG in the presence of organic solvents

Storage stability in the presence of organic solvents was examined by incubation of 100–200 µl of the free and immobilized enzyme in tightly closed test tubes in 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and 45% DMSO, 25% AN, 25% DMF or 1% DO, respectively, at 25 °C for 24 h. The residual activity was determined after incubation as described in "Enzyme assay".

Activity of free and immobilized ADH IIG in the presence of organic solvents

5–10 µl of the free or immobilized enzyme suspension in a total volume of 1.0 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$, 1 mM $K_3[Fe(CN)_6]$, 20 mM glycerol and 0–25% of the corresponding organic solvent was prepared. The activity was determined as described in "Enzyme assay".

RESULTS AND DISCUSSION

Covalent immobilization of ADH IIG

The immobilization procedure was optimized as described in "Experimental" (data not shown) by changing APTS and protein concentration, duration of silica gel modification, buffers, concentrations of GA and CDI, duration and temperature of activation of SS. After optimization an immobilization efficiency of ADH IIG was achieved 29 ± 6% and 12.5 ± 1.5% with GA and CDI, respectively. Less efficient immobilization of ADH IIG using CDI as a cross-linker could be due to surface localization of the ADH IIG carboxyl or hydroxyl groups involved in covalent binding with SS. Moreover, some of the groups involved in covalent binding with the cross-linker could be important in catalytic activity or stability of the native structure. Therefore, such immobilization could be crucial for activity and stability of the enzyme. GA forms longer chains between the support and the enzyme. The covalent binding to the insoluble support, in contrary, may improve the stability and protect from inactivation at the higher temperatures and harmful environment, but not always it improves the activity of enzymes. The tight fixation of the enzyme on the support may interfere for the catalytic activity.

The immobilized form of ADH IIG enabled the separation of the particles from the reaction mixture by centrifugation or simple sedimentation. This property was applied for the bioconversion of glycerol and the operational stability of the

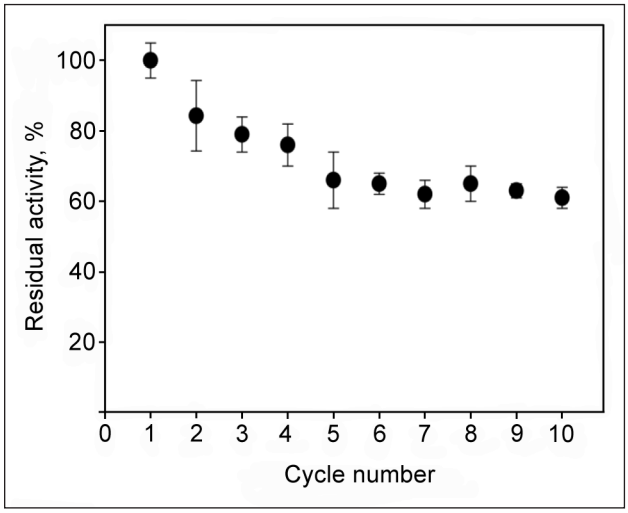


Fig. 1. Operational stability of ADH IIG-GA-SS. Each reaction was carried out at 30 °C. The duration of one cycle was 1 min. After each cycle the residual activity of ADH IIG was determined as described in Experimental. The enzyme activity of the first reaction cycle was assigned a value of 100%

immobilized ADH IIG was evaluated in the repeated batch process. Each reaction was carried out for 1 min as described in "Enzyme assay". Thereafter, a new portion of reaction mixture was added to the separated particles. The dependence of residual activity of ADH IIG-GA-SS on the number of the repeated cycles is presented in Fig. 1. The ADH IIG-GA-SS showed high operational stability. The activity of immobilized ADH IIG was as high as 61 ± 3% of the initial catalytic activity after 10 runs using the same sample of the enzyme. The decrease in ADH IIG activity during the first five cycles might be influenced by several effects such as a loss of the enzyme, which was adsorbed on the surface of silica gel, an inactivation of the enzyme and / or a physical loss of the particles after each cycle of bioconversion.

Analysis of substrate specificity of ADH IIG

The possibility of oxidation of aliphatic and cyclic amino alcohols and vicinal diols by ADH IIG from *Pseudomonas putida* HK5 was investigated. Comparing the results shown in Table 1, it was noticed that introduction of an amino group on the cyclohexanol *para* position was crucial for the catalysis by ADH IIG. In the case of 1,2-propanediol, it was shown that 3-chloro substituted 1,2-propanediol was better substrate for ADH IIG than (±)-3-amino-1,2-propanediol. In addition, it was observed that the position of the amino group in propanediol was important and the rate of oxidation differed more than three-fold between 2-amino-1,3-propanediol and (±)-3-amino-1,2-propanediol. On the other hand, a new and promising ADH IIG ability of amino alcohol oxidation has been shown for the first time. Summarizing the results obtained with all tested amino alcohols, it was obvious that ADH IIG preferred 2-amino-1,3-propanediol as a substrate. In this case, 2-amino-3-hydroxypropionaldehyde should be formed as the product of oxidation by ADH IIG. Similar compounds are very unstable, but they can undergo self-condensation due to their structure. Self-condensation of two molecules of α-aminocarbonyl compounds is a well known process and describes a classical synthesis scheme of pyrazines. Therefore, there is a good possibility of ADH IIG application for the synthesis of different pyrazines at appropriate conditions [38].

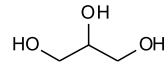
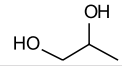
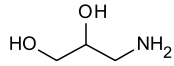
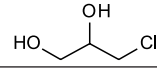
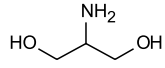
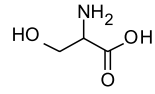
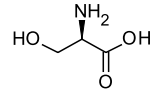
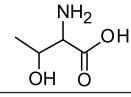
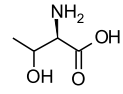
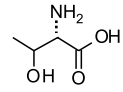
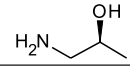
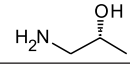
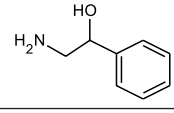
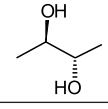
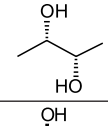
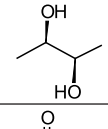
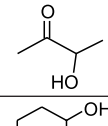
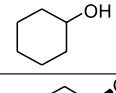
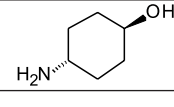
In parallel experiments enantioselectivity of ADH IIG was determined. The enantioselectivity of the enzyme was studied using 2,3-butanediol as a test substrate. As outlined in Table 1, ADH IIG oxidized (2S,3S)-(+)-2,3-butanediol preferably, whereas relative activity of the enzyme decreased more than five-fold using *meso*-2,3-butanediol as a substrate. Moreover, (2R,3R)-(−)-2,3-butanediol was poor substrate for ADH IIG and relative activity of the enzyme differed more than twenty-fold when (2S,3S)-(+)- and (2R,3R)-(−)- butanediols were used. It should be mentioned that no ADH IIG activity was found in the reaction mixture with acetoin as a substrate. Previously studies of quinohemoprotein ADHs isolated from *Acetobacter*, *Gluconobacter* and *Comamonas* species elucidated an appreciable enantioselectivity in the oxidation of (R)- or (S)-secondary alcohols [20]. During the oxidation of chiral 2,3-butanediol a preference of ADH IIG from *Pseudomonas putida* HK5 for (S)-(+) alcohol was revealed. Because of the evident enantio preference of the enzyme, ADH IIG can become an attractive tool for a stereospecific oxidation.

Kinetic investigations

For characterization of ADH IIG from *Pseudomonas putida* HK5, some kinetic parameters were defined. Oxidation of different alcohols catalysed by ADH IIG was investigated according to the decrease in absorption of ferricyanide (mediator) as the second substrate. The dependence of an initial rate on the concentration of various alcohols and ferricyanide was measured. The latter dependence largely fitted the Michaelis-Menten equation. The values of K_M and V_{max} for ADH IIG, catalysing oxidation of the alcohol in the presence of 1 mM ferricyanide, were determined, and then the enzyme reduction constants (k_{red}) for different alcohols were calculated (Table 2). Moreover, the values of K_M and V_{max} for the enzyme, catalyzing ferricyanide reduction at fixed concentrations of alcohols, were determined and enzyme oxidation constants (k_{ox}) were calculated (Table 2).

As shown in Table 1, amino alcohols could be oxidized by ADH IIG. Based on the obtained $K(alc)_M$ and calculated k_{red} values as well as on relative activity of free ADH IIG (Table 1), it can be stated that introduction of the 3-chloro functional group on 1,2-propanediol had minor influence on the affinity of the enzyme. On the other hand, the introduction of 3-amino substitute considerably decreased the affinity of the enzyme ($K(alc)_M$ value increased about six-fold) and decreased k_{red} value from 0.1 ± 0.02 to 0.004 ± 0.0007 $\mu M^{-1} \cdot s^{-1}$. It is important to note that the position of the amino group on propanediol was also

Table 1. **Substrate relative activity of free and immobilized ADH IIG. Activity was determined as described in Experimental**

Substrate		Relative activity of free ADH IIG, %	Relative activity of ADH IIG-GA-SS, %
Glycerol		100	100
1,2-Propanediol		119	na*
(±)-3-Amino-1,2-propanediol		23.8	34
(±)-3-Chloro-1,2-propanediol		88.8	100
2-Amino-1,3-propanediol		74.4	83
rac-2-Amino-3-hydroxypropionic acid		0	na
(*R*)-2-Amino-3-hydroxypropionic acid		0	na
2-Amino-3-hydroxybutyric acid		0	na
(*2R,3S*)-2-Amino-3-hydroxybutyric acid		0	na
(*2S,3R*)-2-Amino-3-hydroxybutyric acid		0	na
2-Propanol		5.7	na
(*S*)-(+)-1-Amino-2-propanol		0.8	0.2
(*R*)-(–)-1-Amino-2-propanol		0	0
(±)-2-Amino-1-phenylethanol		3.0	5.2
meso-2,3-Butanediol		19.6	na
(*2S,3S*)-(+)-2,3-Butanediol		106.8	115
(*2R,3R*)-(–)-2,3-Butanediol		5.2	na
Acetoin		0	na
Cyclohexanol		47.1	na
trans-4-Aminocyclohexanol		1.3	3.4

* – not analysed.

Table 2. **Kinetic parameters for oxidation of alcohols and reduction of ferricyanide catalyzed by free ADH IIG. Parameters were determined as described in Experimental**

Alcohol	$K(alc)_M$, μM	k_{red}, $\mu M^{-1} \cdot s^{-1}$	[Alcohol], mM	$K(f)_M$, μM	k_{ox}, $\mu M^{-1} \cdot s^{-1}$
Glycerol	2 540 ± 340	0.013 ± 0.005	25	1 090 ± 90	0.026 ± 0.002
1,2-Propanediol	111 ± 24	0.02	30	580 ± 46	0.035 ± 0.002
(±)-3-Amino-1,2-propanediol	662 ± 110	0.004 ± 0.0007	20	213 ± 33	0.016 ± 0.002
(±)-3-Chloro-1,2-propanediol	159 ± 4	0.076 ± 0.018	10	620 ± 110	0.047 ± 0.005
2-Amino-1,3-propanediol	14 170 ± 2 400	0.0021 ± 0.0003	20	1 167 ± 115	0.036 ± 0.002
2-Propanol	13 200 ± 1 900	$(0.075 \pm 0.01) \cdot 10^{-3}$	20	132 ± 38	0.014 ± 0.026
S-(+)-1-Amino-2-propanol	860 ± 310	$(0.4 \pm 0.1) \cdot 10^{-3}$	–	na*	na
(±)-2-Amino-1-phenylethanol	32 100 ± 2 400	0.002 ± 0.0005	20	1 540 ± 230	0.018 ± 0.005
(2S,3S)-(+)-2,3-Butanediol	751 ± 108	0.025 ± 0.005	20	352 ± 60	0.093 ± 0.016
Cyclohexanol	1 550**	$(4.66 \cdot 10^{-3})$**	–	na	na
trans-4-Amino-cyclohexanol	3 470 ± 400	$(0.20 \pm 0.05) \cdot 10^{-3}$	20	45 ± 7	0.045 ± 0.005

* – not analysed, ** – previously published data [17].

important. $K(alc)_M$ value was more than twenty-fold higher for 2-amino-1,3-propanediol than for (±)-3-amino-1,2-propanediol, whereas k_{red} value decreased twice, respectively. ADH IIG oxidized (2S,3S)-(+)-2,3-butanediol too, but affinity of this substrate to the enzyme was lower than with 1,2-propanediol. Introduction of an amino group to primary or secondary aliphatic and cyclic alcohols strongly reduced the relative activity of the enzyme and decreased substrate affinity to the enzyme as well as the reduction constants. $K(f)_M$ and k_{ox} values, obtained from the dependence of the initial rate on the concentration of ferricyanide, also depended on the nature of the alcohol.

The calculated k_{red} constants for different alcohols covered the range of $0.075 \cdot 10^{-3}$–0.1 $\mu M^{-1}s^{-1}$, whereas k_{ox} constants were in the range of 0.014–0.093 $\mu M^{-1}s^{-1}$ (Table 2).

Influence of pH on the activity of ADH IIG

The effect of pH on ADH IIG activity with different alcohols is presented in Fig. 2. The highest activity was observed in the pH interval between 6.0 and 8.0 when ADH IIG oxidized

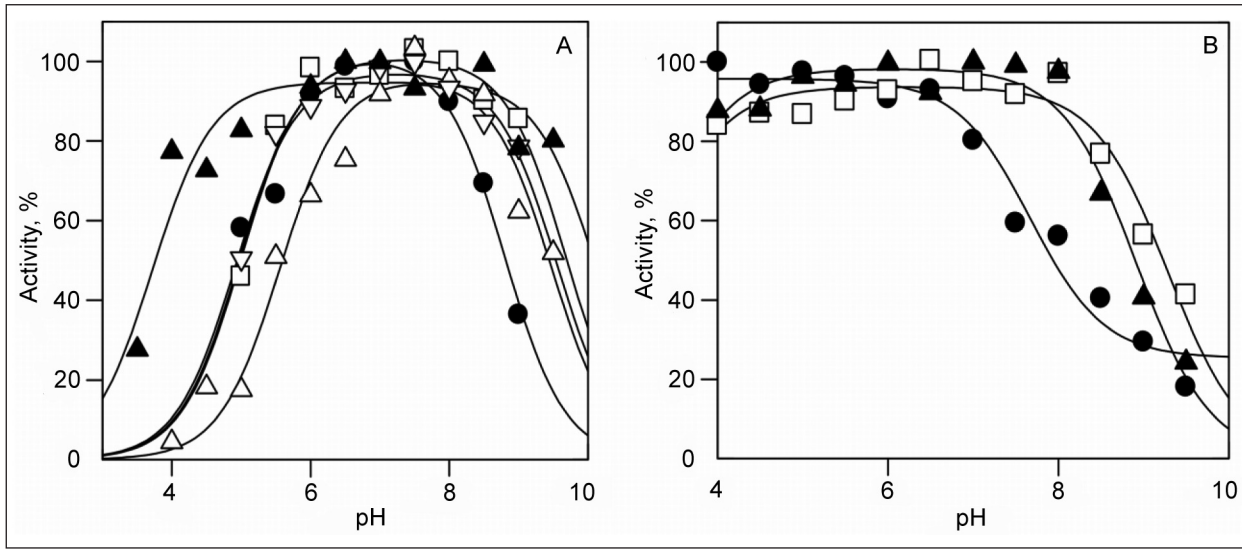


Fig. 2. The dependence of ADH IIG activity on pH. The concentrations of substrates used in the reaction were as follows: 1 mM ferricyanide; A. Closed circle – 10 mM 1,2-propanediol, open square – 10 mM 3-chloro-1,2-propanediol, closed triangle – 20 mM 2-propanol, open down pointing triangle – 30 mM glycerol, open triangle – 20 mM 2,3-butanediol; B. Closed circle – 10 mM 2-amino-1-phenylethanol, open square – 20 mM 3-amino-1,2-propanediol, closed triangle – 20 mM 2-amino-1,3-propanediol. 100% is equal to the maximal ADH IIG activity

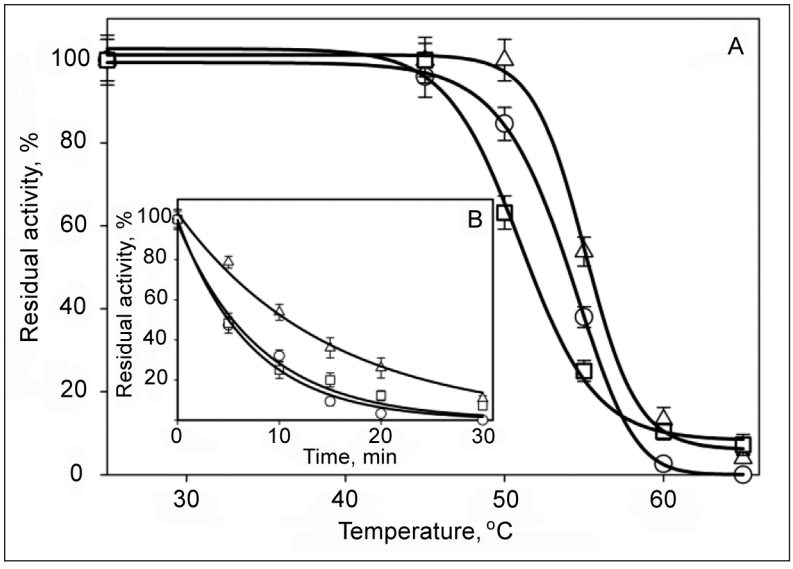


Fig. 3. A. The thermostability of free and immobilized ADH IIG. Open circle – free ADH IIG, open square – ADH IIG-CDI-SS, open triangle – ADH IIG-GA-SS. Samples of the free and immobilized enzyme were incubated for 10 min at 25–65 °C. The residual activity was measured as described in Experimental. B. Thermoinactivation of free and immobilized ADH IIG. Open circle – free ADH IIG, open square – ADH IIG-CDI-SS, open triangle – ADH IIG-GA-SS. Samples of free and immobilized enzymes were incubated for 0–30 min at 55 °C. At different time points the residual activity was measured as described in Experimental. 100% corresponds to the initial activity of the enzyme

glycerol, 1,2-propanediol and 3-chloro-1,2-propanediol. The activity of ADH IIG decreased at more acidic or alkaline pH. The maximal activity of the enzyme was observed in the wider range of pH when 3-amino-1,2-propanediol and 2-amino-1,3-propanediol (pH 4.5–8.0) or 2-propanol (pH 5.0–9.0) were oxidized. The maximal activity was observed in more alkaline pH interval (7.0–8.5) when ADH IIG oxidized (2S,3S)-(+)-2,3-butanediol, whereas 2-amino-1-phenylethanol was oxidized better at more acidic pH. The maximal oxidation rate of 2-amino-1-phenylethanol was observed in the pH interval 4.0–6.5. A decrease in enzyme activity took place at higher pH values.

Thermostability of free and immobilized ADH IIG

Initially the study was performed to investigate whether the covalent immobilization of ADH IIG on SS by GA or CDI cross-linking has any influence on the termostability of the enzyme.

In the present case, the operating temperature of free and immobilized enzyme was raised within the range from 25 °C to 65 °C. It was found that the free ADH IIG was stable up to 50 °C (Fig. 3A). The thermostability of the ADH IIG-GA-SS was shifted upward by 5 °C compared with that of the free enzyme. Moreover, the free ADH IIG was almost completely inactivated at 60 °C, while the enzyme immobilized by GA still maintained more than 13% of activity. In contrary, ADH IIG-CDI-SS was more temperature sensitive in the range of 46–58 °C, but maintained more than 7.2% of activity at 60–65 °C. It was obvious that covalent immobilization of ADH IIG on SS protected the enzyme from complete thermal inactivation at 60 °C. The different manner of inactivation of the immobilized ADH IIG by the CDI and GA cross-linking could depend on different localization of covalent bonds and the distance between enzyme and SS.

The comparison of the inactivation rate of the free and immobilized ADH IIG indicated that covalent immobilization on SS via GA caused higher stability (Fig. 3B). The free ADH IIG had the half-life of 5 min, whereas the half-life of the ADH IIG-GA-SS was approximately twice longer. The thermostability of free and ADH IIG-CDI-SS was almost the same; however, the immobilization via CDI slightly protected the enzyme from the thermal inactivation during a prolonged incubation.

Storage stability of free and immobilized ADH IIG in the presence of organic solvents

The storage stability of both the free and immobilized ADH IIG in the presence of different organic solvents was compared (Fig. 4). The rate of inactivation of the free ADH IIG differed depending on the nature of organic solvents used and correlated with partition coefficients (log P) of the solvents [39]. The most inactivating effect was observed for the most hydrophobic solvents. The covalent immobilization of ADH IIG stabilized the enzyme against inactivation by organic solvents significantly except the case with DMSO, when the rate of inactivation was similar for both, the native and the immobilized ADH IIG.

Activity of free and immobilized ADH IIG in the presence of organic solvents

The effect of organic solvents on the activity of ADH IIG was also tested. Activity of the free and immobilized ADH IIG was analysed in the presence of 0–25% of organic solvents. As shown in Fig. 5, the addition of organic solvent resulted in sharp decrease of the activity of the free ADH IIG. Even 5% of the organic solvent in the reaction mixture reduced the activity from 2 to 10 times. On the other hand, the better retention of the enzyme activity was observed with the immobilized ADH IIG in the presence of 5% of the organic solvent when compared with the free ADH IIG.

The higher retention of the immobilized ADH IIG storage stability and activity in the presence of the solvents can be attributed to the rigidity of the enzyme. Significant stabilizing effect on the enzyme may be due to multiple covalent

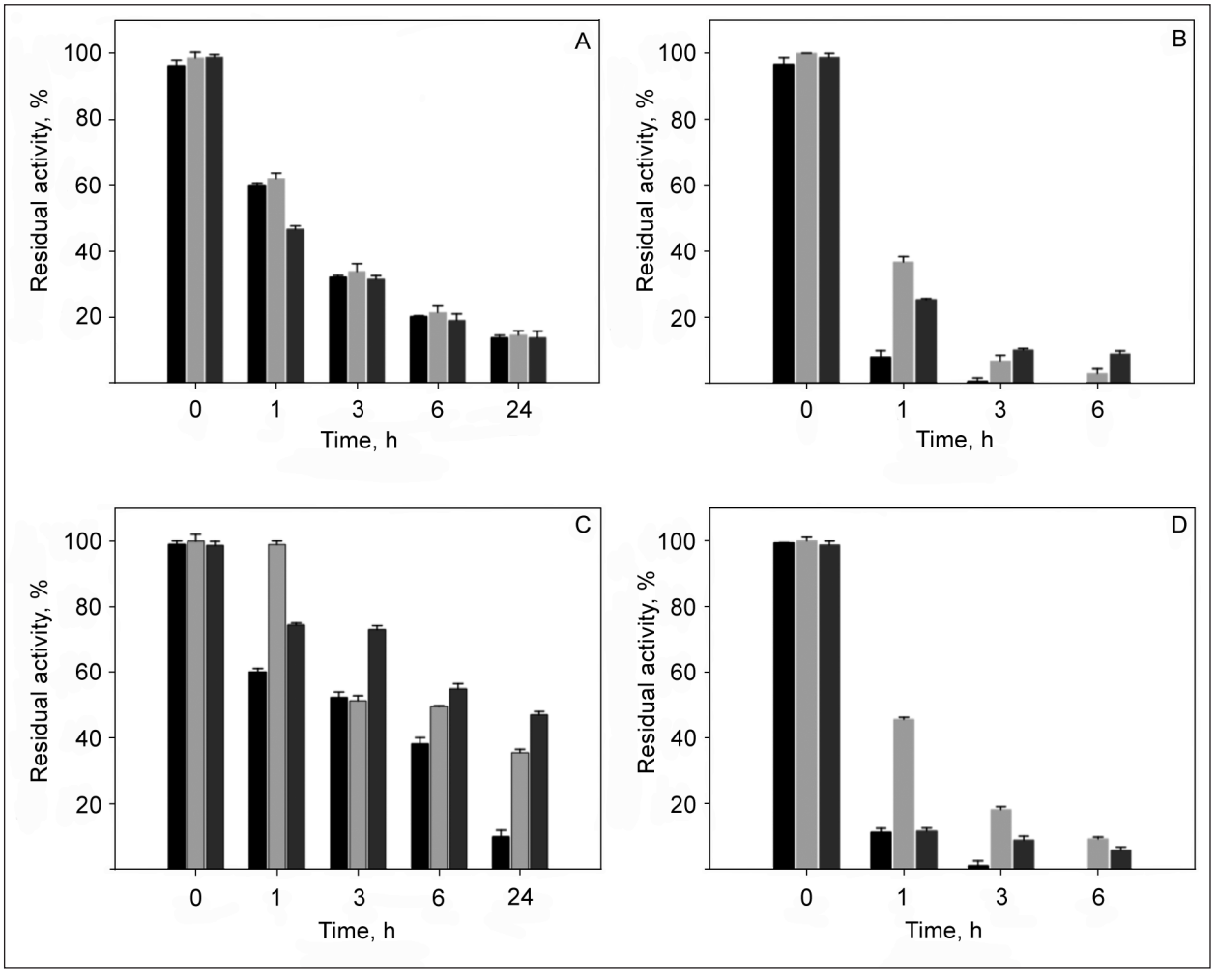


Fig. 4. Storage stability of free and immobilized ADH IIG in the presence of organic solvents. A – 45% DMSO, B – 25% AN, C – 25% DMF, D – 1% DO. Black columns – free ADH IIG, grey columns – ADH IIG-GA-SS, dark grey columns – ADH IIG-CDI-SS. Samples of the immobilized and free enzyme were incubated in the 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM $CaCl_2$ and appropriate concentration of organic solvent at 25 °C for 0–24 h. The residual activity was measured as described in Experimental. 100% is equal to the activity of the enzyme before incubation

linkages. Therefore, the structure of the enzyme is more rigid and it is expected to be more resistant to the conformational alterations induced by the solvents [40]. On the other hand, the solvents have different effect on the physical properties of the reaction mixture, thus the activity of the enzyme can be affected [41]. Moreover, PQQ is not covalently bound to the protein and can be lost in the presence of organic solvents. ADH IIG does not release the PQQ from the active site during dialysis (data not shown), but the organic solvents can induce the extraction of PQQ from the enzyme as in the case of a membrane bound ADH [19], hence impairing the activity of the enzyme.

Kinetic parameters of immobilized ADH

In general, enzyme immobilization procedures tend to reduce the rate of the enzyme inactivation and to alter the substrate specificity of the enzyme [40]. Here the oxidation of different amino alcohols and vicinal diols was examined using the covalently immobilized ADH IIG. In almost all investigated cases, the relative activity of ADH IIG-GA-SS slightly increased in comparison with the free ADH IIG (Table 1). Such differences could be due to the conformational changes of the native enzyme, since covalent bonds formed as a result of immobilization.

Many enzymes, including ADHs, were inactive or unstable in the presence of organic solvents. The addition of organic solvents to the reaction mixture could change kinetic properties of free and immobilized enzymes [40, 41]. Oxidation of glycerol catalysed by the free and immobilized ADH IIG was investigated in the presence and absence of DMSO. 5% of DMSO in the reaction mixture slightly increased the K_M value and decreased the rate of the reaction of the free ADH IIG. The K_M values of the enzyme working in the absence and presence of DMSO were 2.28 mM and 2.84 mM, respectively. The K_M values of ADH IIG-GA-SS or ADH IIG-CDI-SS were very similar and equal to 1.75 mM and 1.83 mM, respec-

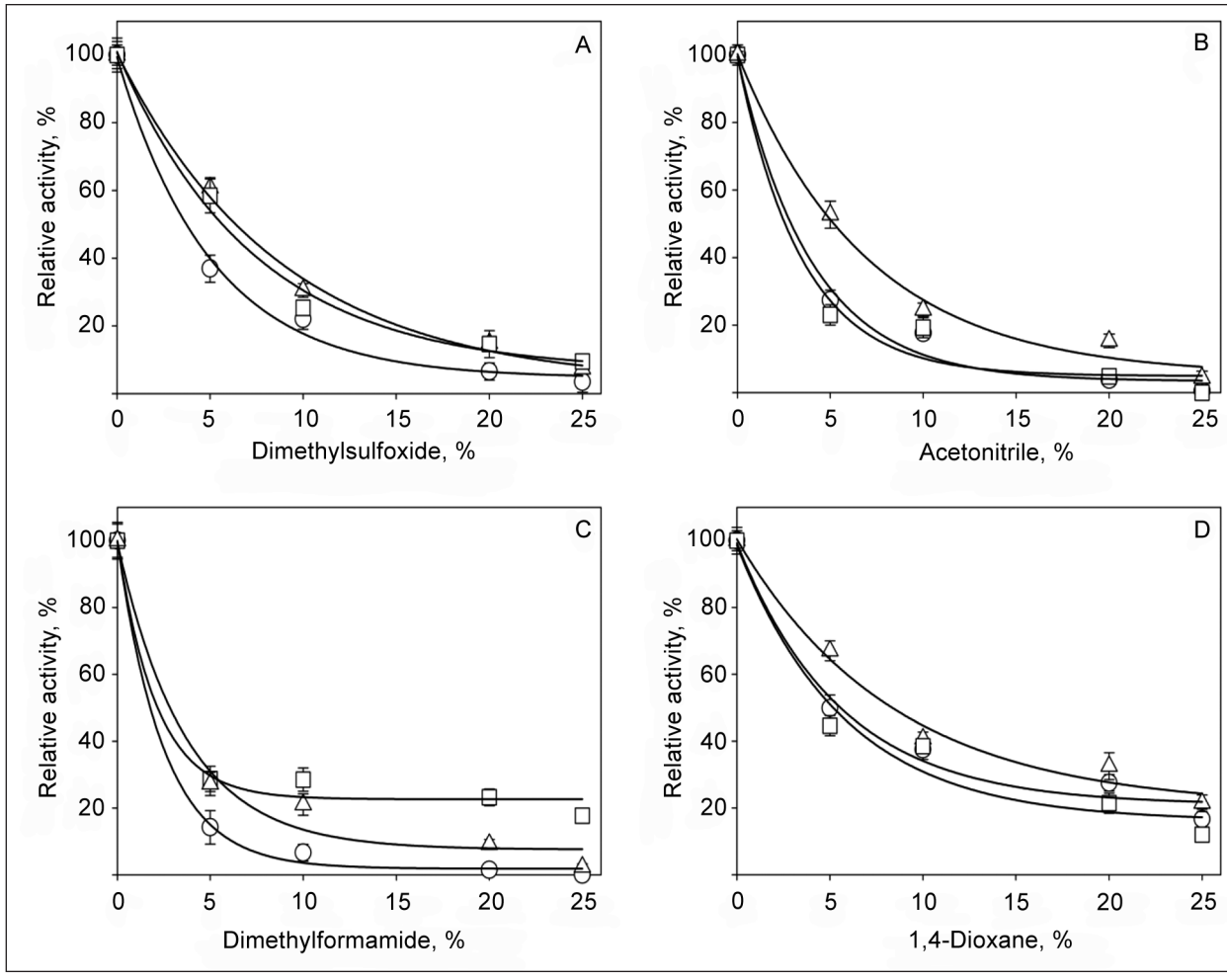


Fig. 5. The dependence of activity of free and immobilized ADH IIG on the concentration of organic solvent in the reaction mixture. A – DMSO, B – AN, C – DMF, D – DO. Open circle – free ADH IIG, open square – ADH IIG-CDI-SS, open triangle – ADH IIG-GA-SS. The activity of free and immobilized ADH IIG was determined as described in Experimental. 100% is equal to the ADH IIG activity measured in the absence of organic solvent

tively. In addition, the presence of DMSO had minor influence on K_M values of the covalently immobilized ADH IIG (2.18 mM – ADH IIG-GA-SS and 1.73 mM – ADH IIG-CDI-SS), but caused the three-fold decrease in the rate of glycerol oxidation. Therefore, it can be stated that neither the addition of DMSO to the reaction mixture nor the immobilization of ADH IIG changed the affinity of the substrate to the active site substantially.

CONCLUSIONS

The covalent immobilization of ADH IIG on silica gel by the GA cross-linking increases the stability of the enzyme against thermal denaturation and inactivation in the presence of organic solvents. ADH IIG from *Pseudomonas putida* HK5 exibits a wide substrate specificity. The enzyme can enantioselectively oxidize different diols and aminoalcohols, hence the biocatalyst is very promising for chemical synthesis of enantiomeric synthons.

ACKNOWLEDGEMENTS

This research was funded by Grant No. MIP-076/2011 from the Research Council of Lithuania. The authors wish to thank Dr. H. Toyama for providing the *Pseudomonas putida* HK5 strain.

Recieved 26 March 2012
Accepted 3 April 2012

References

1. E. Garcia-Urdiales, I. Alfonso, V. Gotor, *Chem. Rev.*, **105**, 313 (2005).
2. C. M. Clouthierzab, J. N. Pelletier, *Chem. Soc. Rev.*, **41**, 1585 (2012).
3. C. H. Barrett, K. S. Dodgson, G. F. White, W. J. Payne, *Biochem. J.*, **187**, 703 (1980).
4. K. Inoue, Y. Makino, N. Iton, *Appl. Environ. Microbiol.*, **71**, 3633 (2005).

5. Z. Findric, D. Vasic-Racki, S. Lutz, T. Daussmann, C. Wandrey, *Biotecnol. Lett.*, **27**, 1087 (2005).
6. M. Amidjojo, D. Weuster-Botz, *Tetrahedron: Asymmetry*, **16**, 899 (2005).
7. D. Metrangolo-Ruiz De Temino, W. Hartmeire, M. B. Ansorge-Schumacher, *Enz. Microb. Technol.*, **36**, 3 (2005).
8. K. Niefind, J. Muller, B. Riebel, W. Hummel, D. Schomburg, *J. Mol. Biol.*, **327**, 317 (2003).
9. K. Matsushita, H. Toyama, M. Yamada, O. Adachi, *Appl. Microbiol. Biotechnol.*, **58**, 13 (2002).
10. E. C. A. Stigter, J. P. Van der Lugt, W. A. C. Somers, *J. Mol. Catal. B: Enzym.*, **2**, 291 (1997).
11. A. Geerlof, J. J. L. Rakels, A. J. J. Straathof, J. J. Heijnen, J. A. Jongejan, J. Duine, *Eur. J. Biochem.*, **226**, 537 (1994).
12. S. S. Machado, U. Wandel, J. A. Jongejan, A. J. J. Straathof, J. A. Duine, *Biosci. Biotechnol. Biochem.*, **63**, 10 (1999).
13. G. Zarnt, T. Schrader, J. R. Andreesen, *J. Bacteriol.*, **183**, 1954 (2001).
14. H. Toyama, F. S. Mathews, O. Adachi, K. Matsushita, *Arch. Biochem. Biophys.*, **428**, 10 (2004).
15. A. S. Vangnai, D. J. Arp, *Microbiol.*, **147**, 745 (2001).
16. H. Toyama, A. Fujii, K. Matsushita, E. Shinagava, M. Ameyama, O. Adachi, *J. Bacteriol.*, **177**, 2442 (1995).
17. I. Bachmatova, L. Marcinkevičienė, R. Meškys, *Biologija*, **2**, 89 (2004).
18. A. Oubrie, H. Roseboom, K. H. Kalk, G. Huizinga, B. W. Dijkstra, *J. Biol. Chem.*, **277**, 3727 (2002).
19. L. Marcinkevičienė, I. Bachmatova, R. Semėnaitė, R. Rudomanskis, G. Bražėnas, R. Meškienė, R. Meškys, *Biologija*, **2**, 24 (1999).
20. A. Jongejan, S. S. Machado, J. A. Jongejan, *J. Mol. Catal. B: Enzym.*, **8**, 121 (2000).
21. K. Matsushita, T. Yamashita, N. Aoki, H. Toyama, O. Adachi, *Biochemistry*, **38**, 6111 (1999).
22. J. Razumiene, R. Meškys, V. Gureviciene, V. Laurinavicius, M. D. Reshetova, A. D. Ryabov, *Electrochem. Commun.*, **2**, 307 (2000).
23. V. Laurinavicius, J. Razumiene, B. Kurtinaitiene, et al., *Bioelectrochemistry*, **55**, 22 (2002).
24. L. Tetianec, M. Dagys, J. Kulys, A. Ziemys, R. Meskys, *Central Eur. J. Biol.*, **2**, 502 (2007).
25. V. Laurinavičius, J. Razumienė, B. Kurtinaitienė, et al., *Biocybern. Biomed. Eng.*, **21**, 21 (2001).
26. O. Adachi, D. Moonmangmee, E. Shinagawa, H. Toyama, M. Yamada, K. Matsushita, *Biochim. Biophys. Acta*, **1647**, 10 (2003).
27. A. Šetkus, A. Galdikas, V. Laurinavičius, R. Meškys, A. Mironas, J. Razumienė, *Colloids Surf., A*, **249**, 141 (2004).
28. A. Galdikas, V. Bukauskas, S. Kaciulis, et al., *Sens. Actuators, B*, **118**, 60 (2006).
29. W. Promden, A. S. Vangnai, P. Pongsawasdi, O. Adachi, K. Matsushita, H. Toyama, *FEMS Microbiol. Lett.*, **280**, 203 (2008).
30. B. Castillo, Y. Pacheco, W. Al-Azzam, K. Griebenow, M. Dvi, A. Ferrer, G. Barletta, *J. Mol. Catal. B: Enzym.*, **35**, 147 (2005).
31. J. Razumiene, V. Gureviciene, A. Vilkanauskyte, et al., *Sens. Actuators, B*, **95**, 378 (2003).
32. A. Ramanavicius, K. Habermuller, J. Razumiene, et al., *Progr. Colloid Polym. Sci.*, **116**, 143 (2000).
33. L. Marcinkevičienė, I. Bachmatova, R. Meškys, *Biologija*, **3**, 38 (2006).
34. J. Magano, *Tetrahedron*, **67**, 7875 (2011).
35. G. M. Dobrikov, V. Valcheva, M. Stoilova-Disheva, et al., *Eur. J. Med. Chem.*, **48**, 45 (2012).
36. N. Urano, S. Fukui, S. Kumashiro, et al., *J. Biosci. Bioeng.*, **111**, 266 (2011).
37. K. A. Schellenberg, L. Hellerman, *J. Biol. Chem.*, **231**, 547 (1958).
38. T. Eicher, S. Hauptmann, *The Chemistry of Heterocycles: Structure, Reactions, Synthesis, and Applications*, 2nd edn., Ch. 6, Wiley-VCH, Weinheim (2003).
39. Y. L. Khmelnitsky, V. V. Mozhaev, A. B. Belova, M. V. Sergeeva, K. Martinek, *Eur. J. Biochem.*, **198**, 31 (1991).
40. N. Duran, M. A. Rosa, A. D'Annibale, L. Gianfreda, *Enzym. Microb. Technol.*, **3**, 907 (2002).
41. H. Hirakawa, N. Kamiya, Y. Kawarabayashi, T. Nagamune, *Biochim. Biophys. Acta*, **1748**, 94 (2005).

Liucija Marcinkevičienė, Jonita Stankevičiūtė, Irina Bachmatova, Regina Vidžiūnaitė, Ana Chaleckaja, Rolandas Meškys

NUO PQQ PRIKLAUSOMOS ALKOHOLIO DEHIDROGENAZĖS IIG IŠ *PSEUDOMONAS PUTIDA* HK5 BIOKATALIZINĖS SAVYBĖS

S a n t r a u k a

Nuo pirolochinolino chinono (PQQ) priklausomos alkoholio dehidrogenazės (ADH) dėl plataus substratinio specifiškumo bei stipriai surišto kofaktoriaus yra patrauklūs katalizatoriai organinėje sintezėje. Šiame darbe tirtas ADH IIG iš *Pseudomonas putida* HK5 gebėjimas stereoselektyviai oksiduoti įvairius pirminius ir antrinius alifatinius bei ciklinius alkoholius ir aminoalkoholius. Parodyta, kad substratais naudojant aminoalkoholius ADH IIG veikimui optimalus pH pasislinkęs į rūgštinę pusę (pH 4–6). ADH IIG buvo kovalentiškai imobilizuota ant silikagelio per glutaro aldehido ir 1-etil-3-(3-dimetilaminopropil)karbodiimido jungtukus. ADH IIG imobilizacija lėmė, kad fermentas galėjo toleruoti aukštesnes temperatūras, išaugo atsparumas organiniams tirpikliams. Imobilizuota ADH IIG gebėjo efektyviau nei laisvas fermentas oksiduoti substratus organinių tirpiklių aplinkoje. Nei tirpikliai reakcijos mišiniuose, nei imobilizacija iš esmės nekeitė fermento aktyvaus centro giminingumo substratams, tačiau mažino katalizuojamų reakcijų greičius.