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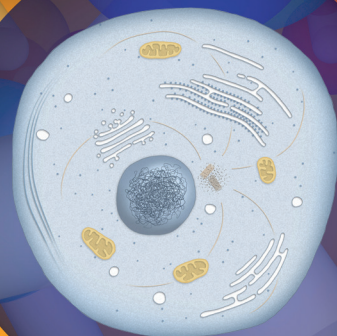
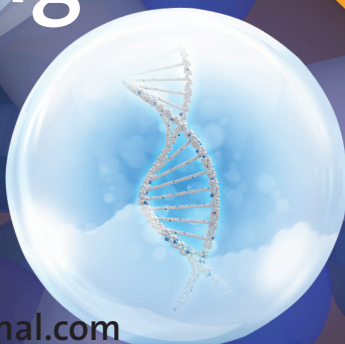
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Research Article

Organic co-solvents affect activity, stability and enantioselectivity of haloalkane dehalogenases

Veronika Stepankova^{1,2}, Jiri Damborsky^{1,2,3} and Radka Chaloupkova^{1,3}

¹ Loschmidt Laboratories, Department of Experimental Biology and Research Centre for Toxic Compounds in the Environment, Masaryk University, Brno, Czech Republic

² International Clinical Research Center, St. Anne's University Hospital, Brno, Czech Republic

³ Enantis Ltd., Brno, Czech Republic

Haloalkane dehalogenases are microbial enzymes with a wide range of biotechnological applications, including biocatalysis. The use of organic co-solvents to solubilize their hydrophobic substrates is often necessary. In order to choose the most compatible co-solvent, the effects of 14 co-solvents on activity, stability and enantioselectivity of three model enzymes, DbjA, DhaA, and LinB, were evaluated. All co-solvents caused at high concentration loss of activity and conformational changes. The highest inactivation was induced by tetrahydrofuran, while more hydrophilic co-solvents, such as ethylene glycol and dimethyl sulfoxide, were better tolerated. The effects of co-solvents at low concentration were different for each enzyme-solvent pair. An increase in DbjA activity was induced by the majority of organic co-solvents tested, while activities of DhaA and LinB decreased at comparable concentrations of the same co-solvent. Moreover, a high increase of DbjA enantioselectivity was observed. Ethylene glycol and 1,4-dioxane were shown to have the most positive impact on the enantioselectivity. The favorable influence of these co-solvents on both activity and enantioselectivity makes DbjA suitable for biocatalytic applications. This study represents the first investigation of the effects of organic co-solvents on the biocatalytic performance of haloalkane dehalogenases and will pave the way for their broader use in industrial processes.

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1 Introduction

The potential of enzymes for stereoselective synthesis and decomposition of environmentally hazardous chemicals is well known [1–3]. For both applications, it is highly desirable that biocatalysts tolerate the presence of organic co-solvents, required to dissolve hydrophobic substrates that are usually only sparingly soluble in aqueous reaction media [4–6]. Moreover, it has been found out that the exposure of specific enzymes to different organ-

ic co-solvents can advantageously modulate their catalytic performance [1, 4, 5]. Medium engineering has been proven to serve as a very effective technique for improving enzyme enantioselectivity toward various target substrates, which is particularly useful for the enzymes employed in organic synthesis [7–11]. However, when an enzyme is placed in organic co-solvent, its activity and stability can be negatively affected by denaturation, conformational rigidity, or inhibition [12–16]. It is, therefore, crucial to select a reaction medium appropriate for dissolution of the reagents and compatible with the biocatalyst. In order to provide a knowledge base for the selection of suitable protein-solvent pair, studies on the effects of organic co-solvents on enzymes have been extensively carried out over the last two decades.

Hydrolases represent the predominant species employed in biotransformations carried out in organic solvents. The merits of these enzymes arise from their avail-

Correspondence: Dr. Radka Chaloupkova, Loschmidt Laboratories, Masaryk University, Kamenice 5/A13, 625 00 Brno, Czech Republic
E-mail: radka@chemi.muni.cz

Abbreviations: CD, circular dichroism; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HLD, haloalkane dehalogenase; PEG, polyethylene glycol; THF, tetrahydrofuran

ability, stability, selectivity, and versatility of action [4]. Haloalkane dehalogenases (HLDs, EC 3.8.1.5) make up an important class of enzymes that are attractive for industrial applications, which catalyze the hydrolytic cleavage of the carbon–halogen bonds in a broad range of halogenated aliphatic compounds [17]. Biodegradation is one of the most promising fields to apply HLDs [18]. They have already been successfully used for bioremediation of 1,2-dichloroethane and hexachlorocyclohexane and to neutralize sulfur mustard [19–21]. Besides, both the substrates (haloalkanes or haloamides) and the products (haloalcohols, alcohols, diols, or hydroxyamides) of HLDs are valuable building blocks in organic and pharmaceutical synthesis [22, 23], making this group of enzymes attractive also in biocatalysis [18]. Recently, the enzymes DbjA and LinB, showing excellent enantioselectivity toward β -bromoalkanes or α -bromoamides, have been used for the kinetic resolution of these compounds on a preparative scale [24, 25]. However, the broader use of HLDs is limited by poor solubility of their substrates in water, evoking the necessity of introduction of organic co-solvents to the reaction media. Despite a number of reports dealing with the effects of organic co-solvents on enzymes, no study using aqueous-organic media has been performed with HLDs.

Here we focused on the effects of several organic co-solvents on activity, structure, thermal stability, and enantioselectivity of DbjA from *Bradyrhizobium japonicum* USDA110 [24, 26], DhaA from *Rhodococcus rhodochrous* NCIMB13064 [27], and LinB from *Sphingobium japonicum* UT26 [28]. The organic co-solvents were selected for their different physico-chemical characteristics: polar protic – glycerol, ethylene glycol, polyethylene glycols (PEGs); polar protic – formamide, methanol, ethanol, isopropanol; polar aprotic – dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetonitrile, acetone, tetrahydrofuran (THF); and non-polar – 1,4-dioxane. They are also representatives of co-solvents most likely employed in the biocatalytic applications. We present the ability of HLDs to carry out reaction in organic-aqueous media and identify the organic co-solvent most appropriate for the particular enzyme. This constitutes the first study of HLDs' tolerance to organic solvents and provides an essential knowledgebase for broader use of these enzymes in the industrial processes.

2 Materials and methods

2.1 Organic solvents

All chemicals were of analytical grade and were purchased from Sigma–Aldrich.

2.2 Protein expression and purification

The His-tagged LinB, DhaA, and DbjA were over-expressed in *Escherichia coli* BL21 cells using a previously described method [26, 29, 30]. Proteins were purified using the Ni-NTA Superflow Cartridge (Qiagen). The His-tagged enzymes were bound to the resin in an equilibrating buffer (20 mM potassium phosphate buffer, pH 7.5 containing 0.5 M sodium chloride, and 10 mM imidazole), unbound and weakly bound proteins were washed out. The His-tagged enzymes were eluted by a buffer containing 300 mM imidazole. The active fractions were pooled and dialyzed overnight against 50 mM potassium phosphate buffer (pH 7.5) and then stored at 4°C. Protein concentration was determined by the Bradford reagent (Sigma–Aldrich). Purity of purified proteins was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2.3 Activity assay

HLDs activity was assayed at 37°C by the previously described method [31]. The released halide ions were analyzed spectrophotometrically at 460 nm after a reaction with mercuric thiocyanate and ferric ammonium sulfate. The reaction mixture was composed of 100 mM glycine buffer (pH 8.6), 2 μ L of substrate 1-iodohexane and an appropriate amount of organic co-solvent. Co-solvent concentration was increased until the enzyme activity dropped below 50%. The reaction was initiated by the addition of enzyme in a final concentration of 0.005 mg/mL. The reaction was monitored by withdrawing samples at periodic intervals from the reaction mixture. The samples were immediately mixed with 35% nitric acid to terminate the reaction. Dehalogenating activity was quantified as a rate of product formation in time. Each activity was measured in 3–5 independent replicates and expressed as the mean values of relative activity, representing percentage of activity in a pure buffer.

2.4 Circular dichroism spectroscopy

Circular dichroism (CD) spectra of enzymes were determined at 37°C using the Jasco J-810 spectropolarimeter equipped with the Peltier thermostat (Jasco). Data were collected from 185 to 260 nm in a buffer and from 200 to 260 nm in the presence of organic co-solvents, at 100 nm/min, 1 s response time and 2 nm bandwidth. Spectra were recorded in a 0.1 cm quartz cuvette containing 0.25 mg/mL enzyme in 50 mM phosphate buffer (pH 7.5) and defined amount of organic co-solvent. Each spectrum shown is the average of 10 scans and baseline corrected. CD spectra were expressed as the mean residue ellipticity (θ_{MRE}) calculated according to Equation (1):

$$\theta_{\text{MRE}} = \frac{\theta_{\text{obs}} M_w 100}{ncl} \quad (1)$$

where θ_{obs} is the observed ellipticity in degrees, M_w the protein molecular weight, n the number of residues, l the cell path length, c the protein concentration, and the factor 100 originates from the conversion of the molecular weight to mg/dmol.

2.5 Fluorescence steady-state spectroscopy

The fluorescence emission spectra were determined using the FluoroMax-4P spectrometer (HORIBA Jobin Yvon Inc.). The fluorescence data of the intrinsic fluorophores, tryptophan, and tyrosine, were collected from 290 to 450 nm with an excitation wavelength of 280 nm, at 50 nm/min and band-passes set to 1 nm. The spectra were recorded in a 0.5 cm quartz cuvette containing 0.25 mg/mL enzyme in 50 mM phosphate buffer (pH 7.5) and a defined amount of organic co-solvent. All samples were incubated at 37°C for 10 min prior to the measurement. Each spectrum was baseline corrected and expressed in terms of arbitrary units of the instrument.

2.6 Thermal unfolding

Thermal unfolding of 0.2 mg/mL enzyme solutions in 50 mM phosphate buffer (pH 7.5) and in the presence of 20% v/v concentrations of methanol, 1,4-dioxane, acetonitrile, ethanol, acetone, isopropanol, THF, and ethylene glycol was followed by monitoring the ellipticity at 221 nm using the Jasco J-810 spectropolarimeter equipped with the Peltier thermostat (Jasco). The measurements were conducted over the temperature range of 20–80°C, with a resolution of 0.1°C, at a heating rate 1°C/min. Recorded thermal denaturation curves were roughly normalized to represent signal changes between approximately 1 and 0 and fitted to the sigmoidal curves using the software Origin 6.1 (OriginLab Corporation). The melting temperatures (T_m) were evaluated as a midpoint of the normalized thermal transition.

Thermal unfolding of 1 mg/mL enzyme solutions in 50 mM phosphate buffer (pH 7.5) and in the presence of 20% v/v concentrations of DMF and DMSO was followed by monitoring the heat capacity using the VP-capillary differential scanning calorimetry system (MicroCal). The measurements were performed at the temperatures from 20 to 80°C at 1°C/min heating rate. All samples were degassed before the measurements. The melting point was determined as the temperature at which the heat capacity curve reached the maximum value [32].

2.7 Enantioselectivity assay

Kinetic resolution experiments were performed at room temperature (21°C) as follows. Racemic substrates were

added to a final concentration of approximately 0.5 mM, to screw-capped reaction vessels containing 25 mL Tris-sulfate buffer (50 mM, pH 8.2) and a defined amount of organic co-solvent. Enzymatic reactions were initiated by addition of an enzyme in a final concentration of 0.02–0.08 mg/mL (depending on its specific activity). The progress of each reaction was monitored by periodically withdrawing samples from the reaction mixture, extracting them with diethyl ether, drying them on a column containing anhydrous Na_2SO_4 , and analyzing them using the Hewlett-Packard 6890 gas chromatograph (Agilent) equipped with the flame ionization detector and the Chiraldex B-TA and G-TA chiral capillary columns (Alltech). The enantiomeric ratio was calculated according to the Eq. (2) [33]:

$$E = \frac{(k_{\text{cat}}/K_m)_R}{(k_{\text{cat}}/K_m)_S} \quad (2)$$

where k_{cat}/K_m represents the specificity constant. To estimate E -values, the equations describing competitive Michaelis–Menten kinetics were fitted by numerical integration to time courses of changes in the substrate concentrations obtained from the kinetic resolution experiments using the MicroMath Scientist for Windows (ChemSW).

3 Results and discussion

3.1 Catalytic activity of HLDs in organic co-solvents

The effects of the increasing concentration of 14 organic co-solvents, with different physico-chemical characteristics, on the dehalogenase activities of DbjA, DhaA, and LinB were investigated with the substrate 1-iodohexane (Fig. 1, Supporting information, Tables S1–S3). The studied HLDs exhibited different responses to the co-solvents even though they all belong to the same protein family. The enzymes can be ordered in term of their tolerance toward organic co-solvents as follows: DbjA > DhaA > LinB. The catalytic efficiency of DbjA was preserved in most of the co-solvents tested up to concentrations of 20% v/v. Furthermore, this enzyme was activated by up to 1.8-fold in glycerol 30% v/v, PEG 1000 30% v/v, ethylene glycol 30% v/v, DMF 10% v/v, methanol 20% v/v, 1,4-dioxane 10% v/v, acetone 10% v/v, isopropanol 10% v/v, and THF 5% v/v. Formamide was the only co-solvent which inhibited DbjA at all concentrations tested. In contrast, the activities of other two enzymes, DhaA and LinB, sharply decreased with increasing co-solvent concentration. LinB was inactivated by the majority of co-solvents, even at low concentrations (less than 10% v/v). Similar effects were observed for DhaA, except for a 1.3-fold activation in the presence of formamide 5% v/v and methanol 5% v/v.

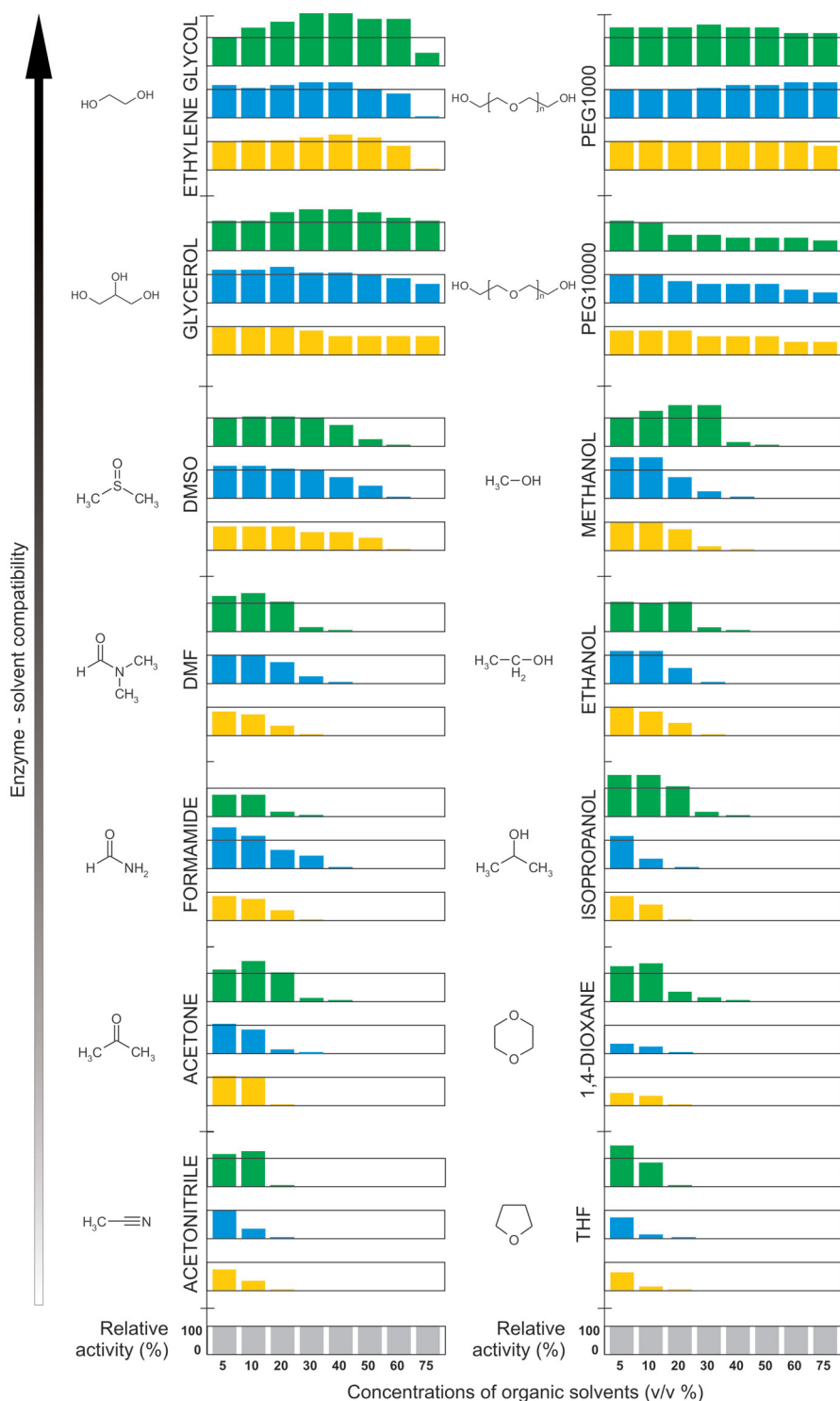


Figure 1. The relative activities of DbjA (green), DhaA (blue) and LinB (yellow) in the presence of different concentrations of organic co-solvents. The relative activities were measured at 37°C and are expressed as a percentage of the specific activity in glycine buffer (100 mM, pH 8.6). The specific activities (in $\mu\text{mol s}^{-1} \text{mg}^{-1}$ of enzyme) of DbjA, DhaA, and LinB in glycine buffer were 0.0213, 0.0355, and 0.0510, respectively. DMF, dimethylformamide; DMSO, dimethyl sulfoxide; PEG, polyethylene glycol; THF, tetrahydrofuran. Each activity was measured in three to five independent replicates.

The polyols – glycerol, ethylene glycol, and PEGs – were found to be the most compatible with all HLDs tested. This result is consistent with earlier reports, showing that polyols are excellent protein stabilizers [34]. Yet, their stabilizing effect cannot be fully generalized because ethylene glycol has also been reported as thermal destabiliz-

er [35]. Among the other studied co-solvents, the least deleterious to catalytic activities of DbjA, DhaA, and LinB was DMSO, followed by methanol and DMF, whereas co-solvents with hydrophobicity parameter $\log P$ higher than -0.35 (especially THF, 1,4-dioxane and acetonitrile) were weakly tolerated (Supporting information, Table S4 and

Fig. S1). These data agree with the criterion proposed earlier, that the best organic solvents used in the water-organic co-solvent mixtures are the most hydrophilic ones due to their lower binding to the hydrophobic residues of proteins [36]. On the contrary, no correlation between deactivation efficiency of organic co-solvents and their physical parameters, including polarity index, dipole moment, dielectric constant, and denaturation capacity was observed (Supporting information, Fig. S1).

The results showed that the effects of organic co-solvents on activity of HLDs are complex and specific to each case. The activities of studied enzymes were increased, decreased, or unaffected at comparable concentrations of the same organic co-solvent. However, in general, the enzyme activity decreased with increasing concentration of organic co-solvent. Several factors have recently been suggested for activity reduction: (i) changes in enzyme conformation and flexibility, (ii) (de)solvation of the active site, (iii) energetics of substrate desolvation, (iv) steric hindrance restricting the accessibility of the substrate, and (v) competitive inhibition by the organic solvent molecules [5, 14, 37–39]. All these mechanisms may be applied to our systems. Despite the fact that most of the tested organic co-solvents are strong denaturants of proteins [40], increase of DbjA activity was observed in nine different water/co-solvent mixtures at low or moderate co-solvent concentrations. Activation of enzymes by organic solvents has been previously observed with various enzymes, including lipase [41, 42], NADH oxidase [39], α -chymotrypsin [43, 44], glyceraldehyde-3-phosphate dehydrogenase [45], neuraminidase [46], hydrogenase [47], Baeyer-Villiger monooxygenase [48], carboxylesterase [49], and feruloyl esterase [50]. Several molecular mechanisms of enzyme activation by the action of organic solvents have been proposed: (i) small perturbations of the protein molecule induced by binding of co-solvent molecules at the activation sites, (ii) removal of extraneous inhibitors from the active site by the co-solvent, (iii) competition of the co-solvent molecules acting as additional nucleophiles with water, (iv) increased affinity of the enzyme toward its substrates, (v) dissociation of oligomeric enzymes due to weakening of intersubunit interactions by co-solvent, and (vi) the influence of the co-solvent on the catalytic steps of enzymatic reactions [43, 46, 51]. Out of these six mechanisms of activation, (ii) and (iii) are not relevant for DbjA.

3.2 Structure of HLDs in organic co-solvents

Circular dichroism (CD) and fluorescence spectroscopy were used to investigate the relationship between inactivation and structural changes of enzymes in the presence of organic co-solvents. The spectra of DbjA, DhaA, and LinB were measured at the co-solvent concentrations which caused reductions in enzyme activity of $\geq 90\%$, and compared with those measured under native conditions

(50 mM potassium phosphate buffer, pH 7.5, 20°C). The CD spectra of native HLDs (Fig. 2A–C) contained two negative features at 208 and 222 nm, characteristic of α -helical content [52]. Visual inspection of the CD spectra obtained in the presence of the co-solvents revealed changes in the intensity and/or shape in comparison with those recorded under native conditions (Fig. 2A–C). This comparison confirmed that addition of high concentrations of organic co-solvents caused significant variations in the spectra, reflecting a loss of α -helical structures of all the enzymes tested [53].

Fluorescence emission was measured upon excitation at 280 nm to monitor the changes in the environment of tyrosine and tryptophan residues [54]. The recorded emission signal reflects the overall changes in the state of all existing tyrosine and tryptophan residues in the studied molecule. The fluorescence spectra of native DbjA, DhaA, and LinB exhibited emission maxima at 334, 335, and 340 nm, respectively (Fig. 2D–F). The presence of organic co-solvents at the concentrations that reduce enzyme activity to 10% or less caused a red shift in the wavelength of the emission maximum for all the enzymes, as well as changes in the emission intensity (Fig. 2D–F). We speculate that at least some of the fluorescent amino acid residues moved toward more polar environment [54]. This indicates significant changes in the protein structure, which agree with the results obtained using the CD spectroscopy.

Denaturation of proteins by organic co-solvents is based on the disruption of the hydration shell around a protein molecule or distortion of the hydrophobic interactions, responsible for maintaining a protein native conformation [36, 55]. Structural changes are pronounced particularly in the polar co-solvents that are able to penetrate into the enzyme cavities and strip water off the enzyme surface [16, 40, 56, 57]. In conclusion, it appears that the major reason for loss of dehalogenase activity by higher organic co-solvent concentrations ($\geq 20\%$ v/v), observed in this study, is a change in the conformation of enzyme molecule. This is in accordance with the previous reports, which showed a good correlation between loss of enzyme activity and disruption of the secondary and tertiary structure of enzymes in organic co-solvents [42, 43, 48, 58–61].

3.3 Thermal stability of HLDs in organic co-solvents

Thermal stability is an important property of enzymes impacting their technological applicability. Thermal stability of DbjA, DhaA, and LinB was measured by incubation of the enzymes over the temperature range of 20–80°C, in the absence and presence of 20% v/v of selected organic co-solvents. As shown in Table 1, the thermal stability of HLDs quantified by T_m was significantly decreased by the majority of tested organic co-solvents. For all enzymes, thermal stability decreased in the

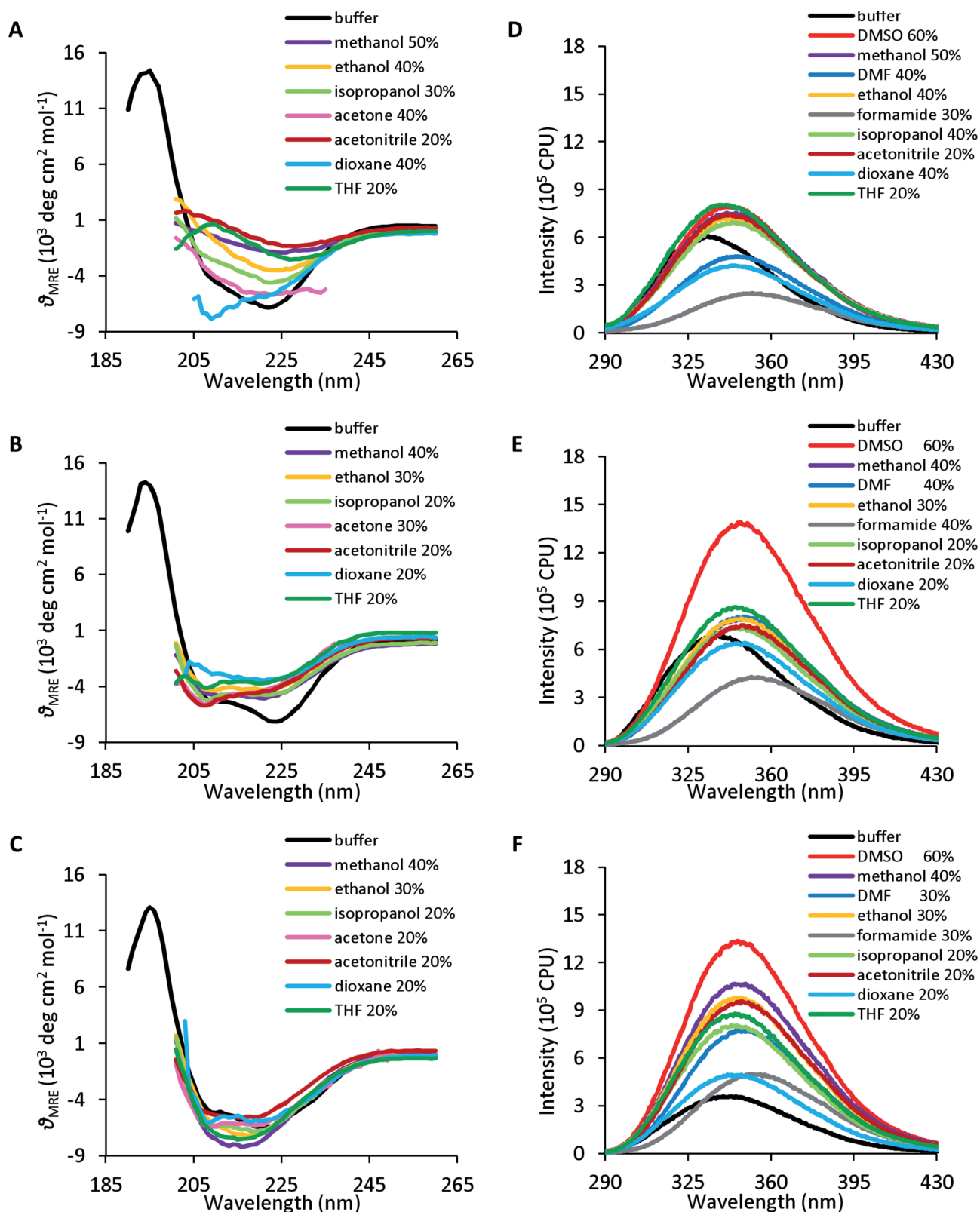


Figure 2. Circular dichroism spectra of (A) DbjA, (B) DhaA, and (C) LinB and fluorescence spectra of (D) DbjA, (E) DhaA, and (F) LinB in the presence of organic co-solvents. The spectra were measured at 37°C in phosphate buffer (50 mM, pH 7.5) and various organic co-solvents at concentrations that caused reductions in enzyme activity of $\geq 90\%$. DMF, dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran. Each spectrum was measured in three independent replicates.

Table 1. The relative activity (a_r) and melting temperature (T_m) of DbjA, DhaA, and LinB in the presence of organic co-solvents.

Co-solvents (v/v)	DbjA		DhaA		LinB	
	a_r (%)	T_m (°C) ^{a)}	a_r (%)	T_m (°C) ^{a)}	a_r (%)	T_m (°C) ^{a)}
Buffer ^{b)}	100	52	100	50	100	49
Buffer ^{c)}	100	53	100	52	100	49
Ethylene glycol 20% ^{b)}	152	51	111	50	109	49
DMF 20% ^{c)}	105	36	46	34	37	38
DMSO 20% ^{c)}	109	48	107	47	85	49
Methanol 20% ^{b)}	146	46	76	40	76	43
1,4-Dioxane 20% ^{b)}	35	35	0	30	0	36
Acetonitrile 10% ^{b)}	124	41	32	37	38	39
Acetonitrile 20% ^{b)}	10	n.d. ^{d)}	0	n.d. ^{d)}	0	n.d. ^{d)}
Ethanol 20% ^{b)}	102	40	56	35	42	39
Acetone 20% ^{b)}	105	34	11	31	5	36
Isopropanol 20% ^{b)}	101	36	0	33	3	37
THF 10% ^{b)}	78	34	13	32	4	34
THF 20% ^{b)}	0	n.d. ^{d)}	0	n.d. ^{d)}	0	n.d. ^{d)}

^{a)} The melting temperatures were measured by incubation of enzymes over the temperature range of 20–80°C at a heating rate 1°C/min, in 50 mM phosphate buffer (pH 7.5), in three independent replicates with the standard errors of less than 3%;

^{b)} The melting temperatures were measured by monitoring the ellipticity at 221 nm;

^{c)} The melting temperatures were measured by monitoring the heat capacity; n.d. – not determined because of significant structural changes already at 10°C; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran.

order: ethylene glycol < DMSO < methanol < ethanol < DMF < isopropanol < acetone < 1,4-dioxane < acetonitrile < THF. This ranking is, in most cases, in good agreement with the effect of organic co-solvents on activity of HLDs determined at 37°C (Table 1). As long as T_m did not drop below 40°C, a satisfactory enzymatic activity was preserved. This was the case of ethylene glycol, DMSO, and methanol, confirming the compatibility of these co-solvents with HLDs. On the other hand, the decrease of T_m close to or below 37°C led to the decline in enzymatic activity. Among the tested organic co-solvents, the worst impact on thermal stability had acetonitrile and THF, causing significant conformational changes of enzymes even at 10°C.

It is commonly known that enzyme stability toward organic solvents correlates with the thermal stability [62]. Among the studied enzymes, LinB appeared to be the least thermally stable, thus one would expect its destabilization by organic co-solvents to be the most apparent. Comparison of the studied enzymes in organic co-solvents revealed that reduction of the thermal stability follows the order: LinB < DbjA < DhaA. Nevertheless, differences between melting temperatures of individual enzymes are not so significant (1–3°C). Despite the higher structural stability of LinB, deactivation by the co-solvents was more pronounced than those of DbjA and DhaA and the activities of DbjA were not as low, as would be expected from the decrease of T_m below the reaction temperature. These contradictions may be explained by previously observed phenomenon that deactivation is not strictly related to structural instability [63, 64].

The observed negative effects of organic co-solvents on thermal stability of HLDs are in accordance with the previous findings that proteins in the mixtures of water and water-miscible solvents are generally less stable than in pure water [5, 49, 65–67]. An explanation for this effect may be the tendency of hydrophilic solvents to strip off water from the enzyme surface and to compete with the non-covalent interactions, especially at the elevated temperatures [59, 68, 69].

3.4 Enantioselectivity of HLDs in organic co-solvents

It is widely recognized that besides the effect on enzyme activity, the organic co-solvents also influence enantioselectivity [5]. The effect of eleven different organic co-solvents at non-denaturing concentrations on DbjA-catalyzed kinetic resolution of racemic 2-bromopentane was investigated. DbjA was selected for this analysis due to its highest resistance to organic co-solvents and the highest selectivity with the target substrates. The determined E -values are shown in Table 2. It is obvious that DbjA enantioselectivity was affected differently by different organic co-solvents. The obtained E -values varied from approximately 30 to more than 200. The original enantioselectivity (deviation below 10%) was maintained in the presence of DMSO 20% v/v, methanol 20% v/v, DMF 10% v/v, and acetonitrile 10% v/v. The slightly lower E -value was observed in formamide 5% v/v and acetone 10% v/v. Ethanol 20% v/v, isopropanol 10% v/v, and THF 5% v/v decreased E -value more than four times. The significant

Table 2. Enantioselectivity of haloalkane dehalogenase DbjA in the presence of organic co-solvents.

Co-solvents (v/v)	E-value ^{a)}		
	2-Bromo-butane	2-Bromo-pentane	2-Bromo-hexane
Buffer	1	132	8
Ethylene glycol 50%	3	>200	30
Formamide 5%	n.d.	93	n.d.
DMF 10%	n.d.	133	n.d.
DMSO 20%	n.d.	143	n.d.
Methanol 20%	n.d.	124	n.d.
1,4-Dioxane 10%	n.d.	194	n.d.
1,4-Dioxane 20%	1	>200	48
Acetonitrile 10%	n.d.	134	n.d.
Ethanol 20%	n.d.	29	n.d.
Acetone 10%	n.d.	107	n.d.
Isopropanol 10%	n.d.	27	n.d.
THF 5%	n.d.	29	n.d.

^{a)} The E-values were measured at 20°C in Tris-sulfate buffer (50 mM, pH 8.2) in three independent replicates and had standard errors of <15%; n.d. – not determined; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran.

enhancement of enantioselectivity (*E*-value higher than 200) was induced by the addition of ethylene glycol 50% v/v and 1,4-dioxane 20% v/v. Visual inspection of the progress curves revealed that the lower enantioselectivity is caused by deceleration of the (*R*)-enantiomer conversion at low concentration and early consumption of unpreferred (*S*)-enantiomer, which starts already before total conversion of preferred (*R*)-enantiomer (Supporting information, Fig. S2B–D). On the contrary, the improvement of DbjA enantioselectivity is clearly connected with almost no consumption of (*S*)-enantiomer, even though (*R*)-enantiomer is completely converted (Supporting information, Fig. S2E and F). No obvious correlation between the enzyme enantioselectivity and the physico-chemical properties of the co-solvents is consistent with previous reports regarding the effects of hydrophobicity and polarity of organic co-solvents on enzyme enantioselectivity [70–72].

Based on these results, the effects of 1,4-dioxane 20% v/v and ethylene glycol 50% v/v were investigated also in DbjA-catalyzed kinetic resolution of 2-bromobutane and 2-bromohexane. For 2-bromobutane, slight enantiodiscrimination was observed in ethylene glycol, whereas 1,4-dioxane appeared to have no effect (Table 2, Supporting information, Fig. S3). On the other hand, the improvement of DbjA enantioselectivity in kinetic resolution of 2-bromohexane was achieved by both selected co-solvents. The *E*-value of 8, obtained in a buffer, was increased fourfold in ethylene glycol and sixfold in 1,4-dioxane (Table 2). Similarly to the case of 2-bromopentane, the higher DbjA enantioselectivity toward 2-bromo-

hexane was brought by reduced consumption of non-preferred enantiomer (Supporting information, Fig. S4).

The enantioselectivity of DhaA and LinB in the presence of ethylene glycol was also tested in the reaction with 2-bromopentane. These enzymes possess excellent enantioselectivity for α -bromoesters, but not β -bromoalkanes [24]. However, neither DhaA nor LinB enantioselectivity was affected by the ethylene glycol (data not shown).

This study demonstrates that the HLD-catalyzed kinetic resolution can be controlled by addition of organic co-solvents to the reaction media, depending on the enzyme, the solvent, and the substrate. The effect of organic solvents on enzyme enantioselectivity has been already described for various types of biocatalysts, including lipase [11, 72–76], Baeyer-Villiger monooxygenase [77], alcohol dehydrogenase [78], carbonyl reductase [8], subtilisin Carlsberg [79], and protease [80]. Several mechanisms have been proposed to rationalize the variability of enzyme enantioselectivity in organic solvents: (i) the interference of the co-solvent molecules with the proper orientation or transformation of one enantiomer more than the other one [81, 82], (ii) the interaction of co-solvent molecules with the surface of the enzyme or its active site, resulting in conformational alterations [83], (iii) co-solvent-induced shift in the racemic temperature [75]. Even though the organic solvents were used at non-denaturing concentrations, causing no structure changes observable by CD and fluorescence spectroscopy, we cannot exclude occurrences of subtle structure alterations that are not detectable by these techniques. Thus, all three mechanisms are possibly valid for changes in DbjA enantioselectivity.

4 Concluding remarks

This study represents the first systematic investigation of the effect of organic co-solvents on structure and function of biotechnologically interesting catalysts HLDs. Polyols, DMSO and methanol were the most tolerated by all of the studied enzymes. On the contrary, the least tolerated were hydrophilic co-solvents, such as 1,4-dioxane, acetonitrile, and THF. At higher concentrations, the organic co-solvents generally decreased enzymatic activity due to the conformational changes. Deleterious effect of organic co-solvents on HLDs' thermal stability indicates that the co-solvents make protein structures more prone to protein unfolding. Furthermore, study of enzymes' enantioselectivity highlights that an enzyme-catalyzed kinetic resolution can be controlled by addition of organic co-solvents to the reaction medium, the so-called "medium engineering." Enhanced enantioselectivity in combination with good resistance toward organic co-solvents makes HLDs interesting biocatalysts for dehalogenation of organic compounds, which are not very soluble in water. Further

research should focus on understanding of the molecular basis of enzyme-solvent interactions and the differences in solvent resistance among related enzymes. This will contribute toward rational design of protein-solvent systems for fundamental studies as well as for industrial applications.

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