Journal of Molecular Catalysis B: Enzymatic 67 (2010) 208–213

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Systematic assessment of the stability of benzaldehyde lyase in aqueous–organic biphasic systems and its stabilization by modification with methoxy-poly(ethylene) glycol

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ARTICLE INFO

Article history:
Received 20 March 2010
Received in revised form 7 August 2010
Accepted 12 August 2010
Available online 18 August 2010

Keywords:
Benzaldehyde lyase
Organic solvent stability
Process stability
mPEG modification

ABSTRACT

Benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I [BAL; E.C.4.1.2.38] catalyzes the stereoselective formation of C–C bonds coupling aldehydes to generate alpha-hydroxy ketones. A broad range of poorly water-soluble substrates are accepted in forward and reverse reactions. In this study, the stability of BAL in aqueous–organic biphasic systems as promising reaction media was systematically investigated using methyl-*tert*-butylether, 2-octanone, and toluene as the organic phase. Surprisingly, a strong individual molecular toxicity of these water-immiscible solvents was observed along with the interfacial toxicity exerted by the aqueous–organic interfaces. They could be considerably reduced by covalent attachment of methoxy-poly(ethylene) glycol ($mPEG_{750}$ and $mPEG_{2000}$) to the enzyme surface increasing the half-life by a factor of up to 18. However, under reactive conditions solvent effects were strongly superimposed by an additional deactivating effect, possibly caused by the aldehyde substrate, and no differences between unmodified and modified BAL were detectable. For technical application of the enzyme in aqueous–organic biphasic media additional strategies for stabilization will therefore be desirable.

1. Introduction

Benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I [BAL; E.C.4.1.2.38] catalyzes the stereoselective cleavage and formation of C–C bonds on a broad range of α-hydroxy ketones and aldehydes, respectively [1–4]. This is of utmost synthetic interest as chiral α-hydroxy ketones are important building blocks for various pharmacologically active compounds such as antiallergics, antidepressants, fungicides, or anti-inflammatory drugs [5,6], but access is still limited due to low yields and enantiomeric excess of alternative chemical routes [7–9].

Most substrates of BAL are characterized by a very low solubility in water, i.e. organic solvents have to be employed to achieve relevant concentrations. This can either be done by addition of water-miscible co-solvents to a homogeneous aqueous medium [10,11] or by introduction of water-immiscible solvents to form a biphasic system [12] in which the enzymes are dissolved in the aqueous phase, while substrates are provided and products are extracted via the solvent phase [13]. The use of biphasic systems provides a couple of advantages over the use of homogeneous media such as the constant supply of substrates [14], the increase of product yields [15–17] and the facilitation of downstream processing [17]. It is even envisaged, that biphasic systems will in future be the clue to the direct integration of enzyme catalyzed reactions in existing chemical processes [18]. Nevertheless, the transfer of biphasic media to enzyme catalyzed reactions is still limited [13] because of their often detrimental effects on the catalytic activity [19,20]. It is generally assumed that deactivation in these systems mainly results from the interaction of the protein structure with the interface ('interfacial toxicity') [14], whereas deactivation by direct interactions between enzyme and solvent molecules in the aqueous phase ('molecular toxicity') only plays a minor role [21,22].

Many reports have already mentioned the response of BAL stability to aqueous–organic biphasic systems [11,12], but a systematic investigation has not been performed to date. Consequently, a rational approach to the stabilization of this versatile catalyst has not been possible yet, though it has been found in previous works that an improvement of stability can be achieved by

Abbreviations: BAL, benzaldehyde lyase; DMBA, 3,5-dimethoxybenzaldehyde; FA, 2-furaldehyde; FO, 2,2′-furoin; mPEG, methoxy-poly(ethylene) glycol; MTBE, methyl-*tert*-butylether; ThDP, thiamin diphosphate; TMB, 3,3′,5,5′-tetramethoxybenzoin; TNBS, trinitrobenzene sulfonate.

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doi:10.1016/j.molcatb.2010.08.007

the entrapment of the enzyme in an aqueous gel matrix [23–25]. This indicates that a direct contact between the enzyme and the interface must indeed have an impact on stability.

In this study the stability of BAL in aqueous–organic biphasic reaction systems was for the first time systematically investigated taking into account the lifetime in reactive and non-reactive biphasic systems, and upon incubation in solvent-saturated aqueous phases. For optimal reproducibility and comparability an experimental set-up was developed that enabled the measuring of all three systems with high efficiency and minimum variation. Finally, a stabilization of the enzyme by the covalent attachment of methoxy-poly(ethylene) glycol (mPEG) to accessible lysine residues on the protein surface was performed and evaluated with regard to enhanced stability in the different systems.

2. Experimental

2.1. Chemicals and enzyme

DMBA was purchased from Alfa Aesar (Karlsruhe, Germany), all other reagents and solvents were from Sigma–Aldrich (Steinheim, Germany), of highest available purity and used without further purification. BAL was expressed in *Escherichia coli* SG13009 $pRep_4$ (Pharmacia, Sweden) from expression vector $pBAL\text{-}his_6$ [26]. Cell disruption was done by sonication of a cell solution (4 mL lysis buffer per g cell wet weight; 0.05 M potassium phosphate, pH 8, 40 mM imidazole, 300 mM NaCl). Enzyme was purified from crude extract supernatant via immobilized metal-ion affinity chromatography on Ni-Sepharose (GE Healthcare, 25 mL matrix, flow: 1 mL/min). Elution of enzyme from affinity matrix was achieved by increasing imidazole concentration from 40 to 250 mM (in 0.05 M potassium phosphate, pH 8, 300 mM NaCl). Eluted protein was desalted using a G25-desalting column (GE Healthcare; 0.05 M potassium phosphate buffer, pH 8, 10 mM NaCl), lyophilized and stored at −20 °C until use.

2.2. Determination of activity and stereoselectivity

All activity measurements were performed in potassium phosphate buffer (0.05 M; pH 8; 0.25 mM ThDP; 2.5 mM $MgSO_4$; 25% (v/v) dimethylformamid (DMF) was added to reactions with 3,5-dimethoxybenzaldehyde (DMBA)) using either UV–vis- or fluorescence-spectrometry at 25 °C. Substrate concentrations were 2 mM 2-furaldehyde (FA) and 2.5 mM DMBA, respectively. UV–vis spectrometry was performed using a Varian Cary 50 (Varian, USA) with UV-Quartz cuvettes of 0.5 mm path length for measurement of both model reactions: (1) 2-furaldehyde to 2,2′-furoin (FO) (277 nm) and (2) 3,5-dimethoxybenzaldehyde to 3,3′,5,5′-tetramethoxybenzoin (TMB) (325 nm). Concentrations of all reactants were calculated from the extinction data using Lambert–Beer's law with extinction coefficients determined beforehand ($\varepsilon_{FA} = 14.883\ mM^{-1}\ cm^{-1}$; $\varepsilon_{FO} = 12.484\ mM^{-1}\ cm^{-1}$; $\varepsilon_{DMBA} = 2.434\ mM^{-1}\ cm^{-1}$; $\varepsilon_{TMB} = 2.26\ mM^{-1}\ cm^{-1}$) and the mass balance of the reaction. Reaction of DMBA to TMB was also measured using fluorescence spectrometry (LS 55 Fluorescence Spectrometer, Perkin Elmer, USA) with an excitation wavelength of 360 nm and an emission wavelength of 470 nm. Determination of residual activities was performed as a relative measure compared to starting values.

Enantioselectivity was determined in batch reactions (2 mL) at 25 °C in potassium phosphate buffer (0.05 M; pH 8; 0.25 mM ThDP; 2.5 mM $MgSO_4$; 25% DMF) with DMBA (10 mM) as substrate. After reaction samples were taken and extracted with an equal volume of dichloromethane. The organic phase was dried with $MgSO_4$ and analyzed via supercritical fluid chromatography (SFC). SFC-analysis was performed using an analytical SFC (Jasco, USA) equipped with a Chiralpak OD-H column (J.T. Baker, Deventer, Netherlands) with supercritical CO_2 ($scCO_2$) as mobile phase. Details of analysis: pressure 120 bar, eluent $scCO_2$:isopropanol (5:1), flow 3 mL/min, temperature 40 °C, UV-detector 220 nm, typical retention times: (*R*)-TMB 4.4 min, (*S*)-TMB 6.0 min.

2.3. Determination of water solubility of solvents

Solvent concentrations within the aqueous phase were determined from solvent-saturated buffer samples via GC-analysis using a HP5890 Series II (Agilent, Germany) equipped with a FS-FFAP-CB-0.5 column (CS-Chromatography, Germany). 1-Butanol was used as internal standard and was added after sampling. Details of analysis: N_2 0.25 bar; injector/detector (FID) 200 °C; 45 °C for 3.5 min, 200 °C (20 K/min); typical retention times: methyl-*tert*-butylether (MTBE) 2.1 min, toluene 3.7 min, 1-butanol 6.6 min, 2-octanone 8.2 min.

2.4. Stability measurement under non-reactive conditions

Residual activities upon incubation in solvent-saturated monophasic systems and respective biphasic systems were measured using the standard activity measurements as mentioned above. BAL variants were incubated in the different mono- and biphasic systems at 25 °C in potassium phosphate buffer (0.05 M; pH 8; 0.25 mM ThDP; 2.5 mM $MgSO_4$). To prevent evaporation of solvents from the saturated monophasic systems, incubation was done in sealed glass vessels. To avoid dilution of protein due to solvent partition, buffer and solvents were saturated with the respective other prior to use. Saturation was performed in a separating funnel, separated phases were stored in sealed, unshaken vessels.

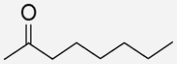
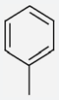
2.5. Stability measurements under reactive conditions

Reactions were run continuously with respect to the organic phase at 25 °C. Both phases had a volume of 5 mL and were saturated with the respective other prior to use. Solvent-saturated aqueous phase contained the enzyme and cofactors (0.05 M potassium phosphate buffer; pH 8; 2.5 mM $MgSO_4$; 0.25 mM ThDP). Substrate (100 mM FA) was supplied via the continuously fed buffer-saturated organic phase using a dosing pump (BF414S32, Telab, Germany). Flow of the organic phase through the system was accomplished by an overflow mechanism (pressurized with solvent-saturated nitrogen). Further details on the reaction set-up are given in [27]. Samples were taken from the organic phase outlet and analyzed via GC using a HP5890 Series II (Agilent, Germany) equipped with a FS-Supreme-5 column (CS-Chromatography, Germany) and dodecane as internal standard. Details of analysis: N_2 0.35 bar; injector/detector (FID) 275/290 °C; 60 °C for 2.2 min, 280 °C (30 K/min); typical retention times: FA 2.7 min, FO 7.6 min, dodecane 5.8 min.

2.6. Determination of free amino groups (TNBS reaction)

Free amino groups were detected by reaction with 2,4,6-trinitrobenzene (TNBS) sulfonate according to Snyder and Sobocinski [28]. Samples were dissolved and diluted to give different protein concentrations in 0.1 M sodium borate buffer pH 9.3. The reaction was started by addition of 15 μL 0.03 M TNBS-solution (0.1 M sodium borate buffer pH 9.3) to 600 μL of these protein samples with and without addition of 10 mM 2-furaldehyde and followed spectrophotometrically at 420 nm.

Table 1
Physicochemical properties of methyl-*tert*-butylether (MTBE), 2-octanone, and toluene.

Solvent	Structure	log *P* [–]	Interfacial tension (mN/m)	Solubility in water (mol/L)[a]
MTBE		0.94[b]	10.5[c]	0.668 ± 0.153
2-Octanone		2.37[b]	14.1[c]	0.006 ± 0.0007
Toluene		2.73[b]	36.1[c]	0.003 ± 0.0012

[a] Determined as described under Section 2.3.
[b] Lide [31].
[c] Freitas et al. [32] (in pure water systems).

2.7. Chemical modification of BAL with methoxy-poly(ethylene) glycol (mPEG)

Activation of mPEG derivatives was performed following the procedure of Hernáiz et al. [29] using *p*-nitrophenylchloroformate as activating agent. The final precipitate was dried under vacuum and stored at −20 °C. The yield of precipitated mPEG in respect to the initially applied mPEG amount was >95%. The activation degree of the purified mPEG derivatives (>99%) was determined spectrophotometrically by measuring *p*-nitrophenol (λ = 400 nm) released upon alkaline hydrolysis. *p*-nitrophenyl-mPEG$_{2000}$-carbonate ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) = 8.32–8.24 (m, 2H, Ar-H), 7.43–7.35 (m, 2H, Ar-H), 3.9–3.4 (m, all other H), 3.36 (s, 3H, OMe); *p*-nitrophenyl-mPEG$_{750}$-carbonate ^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm) = 8.32–8.24 (m, 2H, Ar-H), 7.43–7.35 (m, 2H, Ar-H), 3.9–3.4 (m, all other H), 3.36 (s, 3H, OMe).

Modification of BAL with mPEG was performed for up to 24 h at 25 °C in potassium phosphate buffer (0.05 M; pH 9; 0.25 mM ThDP; 2.5 mM $MgSO_4$) with a 500-fold molar excess of activated mPEG$_{750}$ or mPEG$_{2000}$, respectively. Modified protein was purified from the reaction mixture with desalting columns (PD-10, GE Healthcare) allowing simultaneous buffer exchange (0.05 M; pH 8; 0.25 mM ThDP; 2.5 mM $MgSO_4$). Samples of purified mPEG-variants were applied to activity measurements, SDS-PAGE and size exclusion chromatography for characterization.

2.8. SDS-PAGE and size exclusion chromatography of mPEG-BAL

Denaturating polyacrylamide gel electrophoresis was performed according to Lämmli [30]. Separating gels had a concentration of 10% (w/v) polyacrylamide, stacking gels a concentration of 5% (w/v). Size exclusion chromatography was performed on a fast protein liquid chromatography system equipped with an UV-detector (Pharmacia, Sweden) using 240 mL Sephacryl HR-300 matrix in a 102 cm × 0.8 cm (height/width) column at a flow rate of 0.5 mL/min (0.1 M TEA/HCl pH 8; 0.15 M sodium chloride). Exclusion volume was determined using Blue Dextran (2000 kDa) and calibration was done with carboanhydrase from erythrocytes (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase from *Saccharomyces cerevisiae* (150 kDa) and apoferritin from horse liver (443 kDa).

3. Results and discussion

3.1. Investigation system

As a general set-up for measuring stability a purpose-built biphasic mini-reactor allowing the consumption of minimum amounts of solvent and catalyst was chosen [27]. The interfacial area in this reactor was kept constant and independent stirring of both phases ensured optimum mixing and mass transfer. The evaporation of volatile organic solvents was prevented by use of airtight constructions; operation was equally possible in continuous and batch mode.

Mixing, reaction temperature, pH and buffer type and strength were identical in all experiments. Methyl-*tert*-butylether (MTBE), 2-octanone, and toluene, respectively, were selected to form the organic phase because of their suitable solvation capacity for substrates and products and their different polarity, hydrophobicity and functionality. The relevant physicochemical properties of these solvents are summarized in Table 1.

Enzyme activity and stereoselectivity were measured with regard to the carboligation of either 2-furaldehyde (Fig. 1A) or 3,5-dimethoxybenzaldehyde (Fig. 1B).

3.2. Stability of unmodified BAL under non-reactive conditions

The half-life of BAL in non-reactive systems was determined by measuring the residual carboligation activity after different times of incubation in the respective mono- and biphasic systems in the absence of substrate. The corresponding deactivation rates and half-lives were estimated assuming a time-dependent first order rate law.

The half-life of BAL in the presence of the investigated solvents, but without formation of distinct solvent phases was found to be 17.3 h (±6.3), 5.6 h (±2.5) and 3.2 h (±0.8) for saturation with MTBE, 2-octanone, and toluene, respectively (Table 2). Control experiments in pure aqueous phase without solvent confirmed that deactivation in such systems did not result from stirring and/or contact with the aqueous–gaseous interface (half-life 82.1 ± 6.8). In the respective non-reactive biphasic systems half-lives of 14.9 h (±2.9), 0.6 h (±0.5) and 0.2 h (±0.1) were calculated for MTBE, 2-octanone and toluene as organic phase (Table 2).

3.3. Stability of unmodified BAL under process conditions

The half-life of BAL under process conditions in biphasic media, i.e. during reaction was determined under steady-state conditions in continuous operation mode. Substrate and product were continuously supplied and extracted, respectively, via the organic phase while keeping the phase volume and the substrate concentration constant; the enzyme was kept in the discontinuous aqueous phase. The activity decrease of BAL in the presence of MTBE, 2-octanone and toluene as organic phase is illustrated in Fig. 2. The corresponding deactivation rates were estimated via non-linear regression assuming a time-dependent first order rate law. With all three solvents, MTBE, 2-octanone, and toluene, very similar half-lives of 1.5 h (±0.1), 1.4 h (±0.1) and 2.0 h (±0.1), respectively, were found.

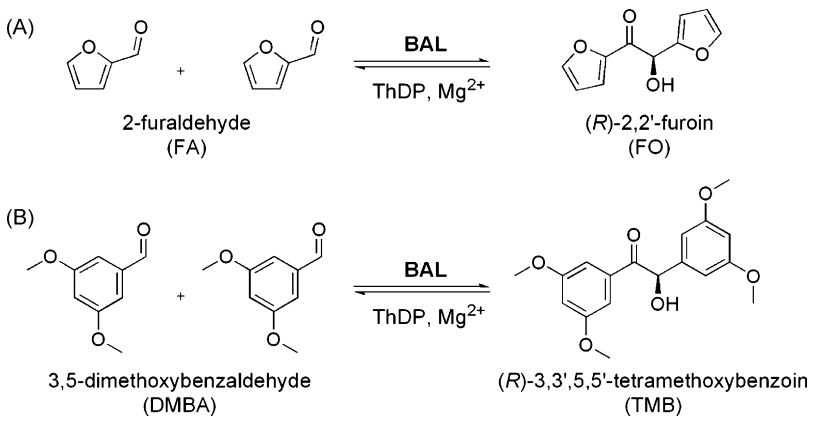


Fig. 1. BAL-catalyzed carboligation of (A) 2-furaldehyde to (*R*)-2,2′-furoin and (B) 3,5-dimethoxybenzaldehyde to (*R*)-3,3′,5,5′-tetramethoxybenzoin.

Table 2
Stability of native BAL, mPEG$_{750}$-BAL and mPEG$_{2000}$-BAL in non-reactive mono- and biphasic as well as reactive biphasic systems with MTBE, 2-octanone and toluene (protein concentration 100 μg/mL). The large deviations of the measurements can be explained by the inhomogeneous modification extent observed for the mPEG-BAL preparations [42].

Solvent	System	Half-life (h)		
		Native BAL	mPEG$_{750}$-BAL	mPEG$_{2000}$-BAL
Without	Non-reactive, monophasic	82.1 ± 6.8	–	–
MTBE	Non-reactive, monophasic	17.3 ± 6.3	44.0 ± 16.8	69.7 ± 1.3
	Non-reactive, biphasic	14.9 ± 2.9	36.3 ± 11.4	6.4 ± 1.4
	Reactive, biphasic	1.5 ± 0.1	2.3 ± 0.2	1.9 ± 0.2
2-Octanone	Non-reactive, monophasic	5.6 ± 2.5	4.6 ± 0.4	10.5 ± 3.8
	Non-reactive, biphasic	0.6 ± 0.5	2.0 ± 0.2	0.7 ± 0.1
	Reactive, biphasic	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
Toluene	Non-reactive, monophasic	3.2 ± 0.8	17.3 ± 2.6	57.8 ± 7.4
	Non-reactive, biphasic	0.2 ± 0.1	0.5 ± 0.1	3.4 ± 0.3
	Reactive, biphasic	2.0 ± 0.1	2.1 ± 0.2	1.8 ± 0.2

This was a decrease of at least 80 h compared to the half-life of the catalyst in non-reactive, purely aqueous medium (see Table 2).

3.4. Conclusions on the stability of unmodified BAL

An overview of the results of all stability measurements, including the half-life of BAL in stirred aqueous phases without addition of any solvent or substrate, is given in Table 2. They clearly demonstrate two things: 1. Any of the investigated solvents, whether dissolved or forming a second phase, has a strong negative effect on the enzyme stability; half-life decreases by a minimum of 65 h (∼79%). 2. The enzyme stability is not only, not even mainly, affected by the formation of interfaces, but reacts on several interrelated influences.

Even very small concentrations of dissolved solvent molecules in the monophasic systems (see Table 1) exert a strong deactivating effect on BAL, which demonstrates that the molecular toxicity of hydrophobic solvents cannot be neglected, as is generally assumed [21,22]. Among the investigated solvents the lowest impact on stability was not even correlated with the lowest solvent concentration in water, as the best stability was found for MTBE, which has a considerably higher solubility in water than 2-octanone and toluene.

In the respective non-reactive biphasic systems, which combine the molecular effects of solvent molecules within the aqueous phase and interactions at the interface between aqueous and organic phase, only minor additional impact was found for the MTBE-interface, whereas the half-life of BAL was again strongly affected by the interfaces towards 2-octanone and toluene. This is partly in accordance with the findings of Ghatorae et al. [19], who related the degree of interfacial deactivation in biphasic systems to the interfacial tension. In the investigated systems, the interface

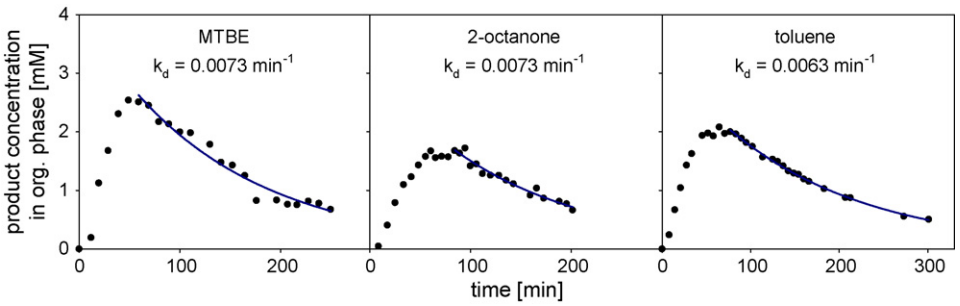


Fig. 2. Formation of (*R*)-2,2′-furoin in continuous reactions using BAL in biphasic systems with MTBE, 2-octanone and toluene as respective organic phases.

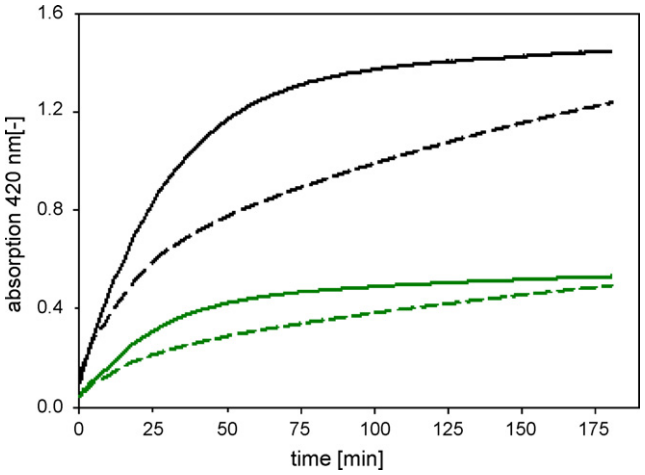


Fig. 3. Course of TNBS reactions with BAL at two different protein concentrations in the absence and presence of 2-furaldehyde (FA): (—) 0.8 mg/mL BAL without FA; (– –) 0.8 mg/mL BAL with 10 mM FA; ( ) 0.3 mg/mL BAL without FA; ( ) 0.3 mg/mL BAL with 10 mM FA. Reactions were followed at a wavelength of 420 nm.

towards toluene exhibits the highest interfacial tension and also results in the highest deactivation of BAL, while the least interfacial effect was measured for MTBE, which also displays the lowest interfacial tension. The results for 2-octanone, however, are not consistent with this theory, which indicates that there must be more influencing factors than the interfacial tension and it also demonstrates that the evaluation of solvent effects is not easily accessed.

Finally, the findings for BAL stability in reactive biphasic systems suggest that this enzyme is not only affected by the solvent system, but also by its substrates. This influence interferes with the observed solvent effects and can even superimpose. It has been described in literature, that aldehydes can form Schiff bases with amino functions of proteins thereby leading to structural rearrangements [34–36]. These Schiff bases are usually not stable, but might induce irreversible unfolding of the protein and thus lead to sustainable deactivation. An interaction of 2-furaldehyde with free amino functions of BAL was indeed observed, i.e. when amino groups of BAL were reacted with trinitrobenzene sulfonate (TNBS) [28] in the absence and presence of 2-furaldehyde, the presence of 2-furaldehyde clearly decelerated the reaction with TNBS (Fig. 3). Formation of Schiff bases with a subsequent denaturation of BAL might therefore account for the observed deactivation of BAL in reactive systems.

3.5. Stabilization of BAL by mPEG modification

In the respective literature it is generally assumed that enzyme deactivation by dissolved solvent molecules results from the dehydration of the protein surface and the concomitant break-down of non-covalent interactions that are essential for maintenance of the correct three-dimensional structure [37], while aqueous–organic interfaces promote the adsorption of protein with concomitant structural rearrangements [19,20]. Consequently, stabilization against both effects should be possible by reducing the surface area susceptible for the interaction with dissolved solvent molecules and/or upon adsorption at the interface. On the other hand, blocking of accessible lysine residues on the surface of the protein should reduce the formation of Schiff bases with aldehydes, i.e. stabilize against the supposed detrimental effects of such reactants. In order to reduce both susceptible surface area and formation of Schiff bases, methoxy-poly(ethylene) glycol (mPEG) was covalently bound to BAL.

The chemical modification of proteins by the attachment of poly(ethylene) glycol (PEG) and derivatives such as methoxy-poly(ethylene) glycol (mPEG) to free amino functions is a well established method, primarily for the increase of solubility in various fluids [38–41]. Applied to BAL, the initial activity decreased to approximately 60%, when $mPEG_{750}$ was employed, and to 70%, when a longer-chain $mPEG_{2000}$ was used; enantioselectivity was unaffected (*e.e.P* > 99%). SDS-PAGE analysis revealed that all enzyme molecules were successfully modified. The modification extent varied between 2 and 4 mPEG molecules per BAL subunit, i.e. 8–16 mPEG molecules were attached to each BAL-tetramer. The tetrameric structure was not affected as verified by size exclusion chromatography.

Interestingly, the half-life of modified BAL in the investigated systems changed rather incoherently; in parts a considerable increase was observed (Table 2).

Stability against dissolved solvent molecules in the aqueous phase, especially against MTBE and toluene, was clearly enhanced; the stabilization was higher with $mPEG_{2000}$ compared to $mPEG_{750}$. It has been described that mPEG forms folded domain structures when interacting with hydrophobic surface regions [43,33]. Such structures could prevent interactions between the solvent molecules and hydrophobic surface regions and thus account for stabilization. They also increase the rigidity of the protein molecule thereby hindering the unfolding events underlying deactivation [42,44–47]. The different effects of the two mPEG species at the same modification extent could then be explained by the larger surface shielding and higher rigidity implied by the longer-chain mPEG.

In the corresponding biphasic systems stability of mPEG-BAL variants varied with mPEG species as well as solvents. This could be explained by two competing effects resulting from mPEG modification: enhanced adsorption onto the interface on the one hand and reduced structural rearrangements on the other hand. Both would be stronger with the longer-chain $mPEG_{2000}$ as explained in the preceding paragraph. In the biphasic system with toluene the increase in adsorption might not be very pronounced since it can be assumed that the unmodified protein is already adsorbed to a great extent. Consequently, the major effect of the modification would be the shielding against structural rearrangements, where the modification with the larger $mPEG_{2000}$ has the more beneficial effect. In contrast, increased adsorption to the interface probably plays a bigger role in the biphasic systems with MTBE and 2-octanone, which leads to an unchanged or decreased stability of $mPEG_{2000}$-BAL variants in these media. Overall, the modification with mPEG led to good stabilization of BAL under non-reactive conditions, particularly against deactivation by dissolved solvent molecules. Nevertheless, the additional deactivation by the interface in the respective biphasic systems was still high. The strongest effect was achieved against toluene, where the stability of $mPEG_{2000}$-BAL surpassed the stability of unmodified BAL by factor 18. In contrast, the modification with $mPEG_{750}$ and $mPEG_{2000}$ had no pronounced effect on BAL stability in reactive biphasic systems, which indicates that amino functions susceptible for or affected by the formation of Schiff bases were not modified by reaction with mPEG.

4. Conclusion

In aqueous–organic biphasic systems the stability of benzaldehyde lyase is affected by solvent molecules present in the aqueous phase (“molecular toxicity”), aqueous–organic interfaces (“interfacial toxicity”), and substrate molecules. Their effects strongly overlap. Molecular toxicity and interfacial toxicity mainly depend on the solvent system and can be reduced by covalent binding of mPEG to the protein surface. However, under reactive conditions

both effects are superimposed by another deactivating effect, most likely by the aldehyde substrate. Stabilization of BAL for technical application will therefore require the development of additional strategies to prevent this deactivation.

Acknowledgements

We gratefully acknowledge the financial support by the German Research Foundation DFG within the DFG research training group GRK 1166 (Biocatalysis in Non-Conventional Media–BioNoCo).

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