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Biochemical peculiarities of benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I in the dependency on pH and cosolvent concentration

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ABSTRACT

Benzaldehyde lyase from *Pseudomonas fluorescens* (BAL, EC 4.1.2.38) is a versatile catalyst for stereoselective carboligations. Nevertheless, rather inconsistent data about its biochemical properties are reported in literature. In this study, the dependency of BAL activity on ionic strength, pH, and concentration of DMSO was for the first time systematically investigated and interpreted. It was found that the activity of BAL strongly depends on all three parameters, and a correlation exists between the dependency on pH and DMSO concentration. This correlation could be explained by an interaction of DMSO with an ionic amino acid in the catalytic site. A model-based analysis indicated that the pK_a of this residue shifts to the alkaline milieu upon addition of DMSO. Consequently, the optimum pH also shifts to alkaline values when DMSO is present. Potentiometric experiments confirmed that the pK_a can most probably be attributed to Glu50 which governs the activity increase of BAL on the acidic limb of its pH-activity profile. With these findings, the apparently contradicting data from literature become comprehensible and optimal reaction conditions for synthesis can easily be deduced.

1. Introduction

Benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I (BAL[1], EC 4.1.2.38) is a synthetically valuable biocatalyst for the generation of enantiomerically pure building blocks. It catalyses the stereoselective carboligation of various substituted aromatic and aliphatic aldehydes to the corresponding hydroxy ketones [1–4] by using one of the aldehydes as electron donor and the other as electron acceptor [5]. The reaction probably proceeds at the covalently bound cofactor thiamine diphosphate (ThDP) [2], and was assigned to be a reversible *Bi–Uni* mechanism according to Cleland [6].

Interestingly, reported data about technically relevant biochemical properties of BAL considerably vary or even contradict each other. Thus, no distinct pH optimum of enzyme activity can be identified, since values ranging from pH 6.5 and 7.5 [7] over 8.0 [8] and 9.0 [9] to 9.5 [10] have been observed. Additionally, an activity increase upon addition of the cosolvent dimethyl sulfoxide (DMSO), which is often employed with BAL to enhance substrate solubility [8,9,11–13] has been reported by Domínguez de María et al. [10], whilst Janzen found that the presence of DMSO decreased BAL activity by almost 90% [14]. In contrast, both authors determined a stabilisation of the biocatalyst by the solvent. In particular the considerably lower pH optimum reported by Nemeria et al. [7] might be attributed to differences in the determination of BAL activity, which in contrast to all other studies [8–10] involved a coupled enzyme assay monitoring benzoin cleavage instead of direct quantification of benzoin formation. However, a distinct explanation for the findings has not been offered to date.

In this study, a systematic theoretical and experimental investigation of the interactive influences of pH, ionic strength, and DMSO concentration on the activity of BAL was performed. A model-based mathematical analysis of dissociation constants was used to elucidate effects of these parameters on the behaviour of the two catalytically important amino acids Glu50 and His29, and potentiometric experiments were applied to evaluate the impact of the two residues on the biochemical properties of BAL. Results are validated against available data on pH-dependency, structure, and kinetics of the enzyme.

2. Materials and methods

All chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany) or Fluka (Neu-Ulm, Germany) and used as obtained.

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[1] *Abbreviations used:* BAL, benzaldehyde lyase; DMBA, 3,5-dimethoxy benzaldehyde; DMSO, dimethyl sulfoxide; ThDP, thiamin diphosphate; TMB, (R)-3,3′,5,5′-tetramethoxy benzoin.

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Nomenclature

Symbols

a	model parameter (–)
c	concentration (mM)
C_I	model parameter ($mM^{-0.5}$)
d	optical pathlength (cm)
DMBA	concentration of DMBA (mM)
DMSO	concentration of DMSO (v/v)
ε	extinction coefficient (mM^{-1} cm^{-1})
Ext	extinction (–)
I	ionic strength (mM)
i	chemical species (–)
λ	wavelength (nm)
pH	pH value (–)
pH_{opt}	pH optimum (–)
pK_a	dissociation constant (–)
t	time (s)
TMB	concentration of TMB (mM)
k_{cat}	turnover number (s^{-1})
$k_{cat,opt}$	turnover number at pH optimum (s^{-1})
z	charge (–)

Benzaldehyde lyase from *P. fluorescens* Biovar I (BAL) was prepared from *Escherichia coli* $SG13009_{prep4}$ [pBAL-his_6] according to Iding et al. [15], lyophilised and stored at −20°C.

Enzyme activity was determined for (R)-3,3′,5,5′-tetramethoxy benzoin (TMB) formation from 3,5-dimethoxy benzaldehyde (DMBA) (Fig. 1) using cofactor concentrations of 0.25 mM $MgCl_2$ and 0.25 mM ThDP. KP_i at a pH of 5.0 to 11.5 and an ionic strength of 25–500 mM was used as buffer, and 5–40% (v/v) DMSO were added as cosolvent. The turnover number was defined as the TMB concentration that was formed per second related to the number of BAL active sites in the assay. The molecular concentration of active sites was calculated by dividing the weight of purified enzyme by the molecular weight of one subunit (59,800 Da) [8].

Kinetic measurements were conducted at 25 °C and an initial substrate concentration of 5 mM. The reaction was initiated by adding 8.3×10^{-2}–3.3×10^{-1} μM BAL and recorded over a time period of ca. 120 s at 325 nm on an UV–Vis spectrometer (Varian, Cary 50, Palo Alto, USA). Maximum conversion within this time was 2%. Initial rates were calculated from the slope of the extinction (*Ext*) against time according to Lambert–Beer's law (Eq. (1)). Extinction coefficients of DMBA (ε_{DMBA}) and TMB (ε_{TMB}) were 2.434 mM^{-1} cm^{-1} and 2.260 mM^{-1} cm^{-1}, respectively.

$$Ext = (\varepsilon_{DMBA} \cdot DMBA + \varepsilon_{TMB} \cdot TMB) \cdot d \quad (1)$$

The reaction was balanced according to Eq. (2).

$$DMBA_0 = DMBA(t) + 2 \cdot TMB(t) \quad (2)$$

In these equations, d denotes the optical pathlength. $DMBA_0$ is the initial DMBA concentration and DMBA(t) and TMB(t) are DMBA and TMB concentration measured at time point t.

Determination of pH was accomplished with a conventional pH electrode (Digital-pH-Meter 646, Knick, Berlin, Germany). This was calibrated with 50 mM potassiumhydrogenphthalate and 50 mM KP_i buffer according to Taylor [16]. The measured values were corrected according to Mukerjee and Ostrow [17] when the DMSO concentration in the aqueous solution exceeded 30%.

Titration curves were determined at 25 °C using an auto-titrator (Titro-line, Fa. Schott, Mainz, Germany). 5 mM L-glutamate or L-histidine was dissolved in deionised water with or without 30% (v/v) DMSO. The ionic strength was adjusted to 100 mM with KCl. The solution was titrated from both the acidic and alkaline milieu, i.e. the initial pH value was set to 2.5 and 12 with 5 M HCl and 5 M KOH, respectively. The solution was titrated to a final pH value of 12 and 2.5, respectively, using 100 mM KOH and HCl, respectively. The consumption of KOH and HCl, and the corresponding pH value were recorded. The obtained data were smoothed and subsequently differentiated with the software Matlab 2006R (The MathWorks Inc., Natick, USA). Dissociation constants were determined from the minima of the pH value against a dpH/dV plot.

3. Results and discussion

Literature survey: Published data on the pH optimum (pH_{opt}) of BAL activity towards synthesis reveal remarkable discrepancies of up to three units. However, upon a closer look to the experimental conditions underlying the published data it becomes obvious that various types of buffer, different buffer strengths and diverse DMSO concentrations were used for the respective studies (Table 1).

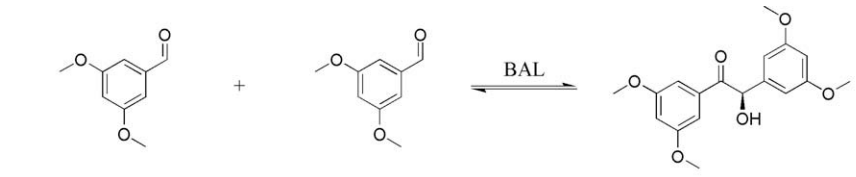
To date, little is known about the effects of buffer type and DMSO concentration on the optimal pH of enzyme catalysed reactions. The use of different buffer strengths, however, can certainly affect pH-activity via concomitant changes in the ionic strength [18,19]. For KP_i buffer this can briefly be explained by Eq. (3) (Henderson–Hasselbalch) and Eq. (4).

$$pH = pK'_a + \frac{c(HPO_4^{2-})}{c(H_2PO_4^-)} \quad (3)$$

$$I = 0.5 \cdot \sum_i c_i \cdot z_i^2 = 3 \cdot c(K_2HPO_4) + c(KH_2PO_4) \quad (4)$$

Table 1
Literature data on pH optima of BAL activity towards synthesis.

pH_{opt}	Buffer	Type DMSO (%, v/v)	Reference
9.5	50 mM KP_i	30	[10]
9.0	10 mM KP_i	30	[9]
6.5–7.5	50 mM acetic acid/MES/Tris	17	[7]
8.0	50 mM KP_i	0	[8]



3,5-dimethoxy benzaldehyde (DMBA) 3,5-dimethoxy benzaldehyde (DMBA) (*R*)-3,3',5,5'-tetramethoxy benzoin (TMB)

Fig. 1. BAL-catalysed carboligation of DMBA to TMB.

In these equations, pK'_a denotes the apparent dissociation constant of the buffer, c the molar concentration of dissociated buffer species, z_i their charge in solution, and I the ionic strength. Conversely, these relations also imply that changes in the ratio of differently charged dissociated buffer species cause a drift of ionic strength. As a consequence, the ionic strength also changes when the pH is varied at constant buffer strength like in the studies cited above [7–10]. Nevertheless, no data considering the concrete influences of ionic strength on BAL activity is available from published literature.

In the following, the dependency of synthetic BAL activity on ionic strength, and additionally the influence of DMSO concentration on pH-dependency were thoroughly studied. As a model reaction, the symmetrical carboligation of 3,5-dimethoxy benzaldehyde (DMBA) yielding 3,3′,5,5′-tetramethoxy benzoin (TMB) was used (Fig. 1) instead of the condensation of unmodified benzaldehyde yielding benzoin reported in former studies [8–10]. This was done for a better solubility of substrates and products in aqueous media and enabled a very precise determination of concentrations by fluorescence spectroscopy. An impact on the comparability of results with reported data [8–10] must not be expected since the mechanism of benzoin condensation will not be altered by a substrate change.

Influence of ionic strength: Investigations of synthetic BAL activity in KP_i buffer (pH 8.5, 30% v/v DMSO) revealed that the ionic strength of the buffer system has a very strong reciprocal effect on the activity of BAL, i.e. in a range between 25 mM and 500 mM, highest initial rates were observed at lowest ionic strength, and activity decreased with increasing ionic strength (Fig. 2). This was calculated with Eq. (5).

$$\frac{k_{cat}}{k_{opt,I}} = 10^{C_I\cdot(\sqrt{I}-\sqrt{I_{opt}})} \tag{5}$$

In this equation, C_I is a model parameter and $k_{opt,I}$ the turnover number at the optimal ionic strength I_{opt} (25 mM), as formerly proposed [19–21]. Eq. (5) was fitted to the experimental data in order to estimate the slope of the linearised curve (C_I). The distinct value of C_I was $-0.0296 \pm 0.0016\ mM^{-0.5}$.

Based on this finding it can be assumed that the activity of BAL described in literature is cross-influenced by a drift of ionic strength connected with the use of the respective buffer systems at different pH values. This was confirmed by calculation of the ionic strengths in the studies with Eqs. (3), (4), and (6) (Debye–Hückel equation), and subsequent estimation of the influence of the obtained values on BAL activity using Eq. (5). The pK_a value of phosphate buffer in a 30% DMSO solution was set to 7.7 according to Taylor [16], cofactor concentrations were considered negligible for ionic strengths. Results of this procedure are summarised in Table 2. The so-determined activity fluctuations can be regarded as the impact of ionic strengths on published data formerly attributed to an influence of pH. In the two investigated buffers (KP_i at different concentrations, see Table 2), this impact ranges up to 15% and 30%, respectively.

$$pK'_a = pK_a + (2\cdot z_a - 1)\cdot\left(\frac{0.5114\cdot\sqrt{I}}{1+\sqrt{I}} - 0.1\cdot I\right) \tag{6}$$

In Eq. (6), pK_a denotes the thermodynamic dissociation constant of KP_i and z_a is the charge on the conjugate acid species.

Surprisingly, despite this clear influence of the ionic strength on the measured activity of BAL, cross effects on the pH-dependency of this enzyme [7–10] were only neglectable. This was demonstrated by a comparison of an activity profile of BAL measured at varying pH, constant ionic strength (100 mM), and constant DMSO concentration (30% v/v) to activity profiles derived from tagging this curve with the probable cross effects of the ionic strength in the studies of Domínguez de María et al. [10] and Stillger et al. [9] (Fig. 3).

Eq. (7) was fitted to each profile in order to determine the respective pH optima as proposed by others [7,22–25]. The resulting values were 9.41 in 50 mM KP_i [cf. 10], 9.44 in 10 mM KP_i [cf. 9], and 9.46 (±0.07) for the curve measured in this study. The deviations were insignificant and not nearly in the range of the reported differences in pH optima.

Subsequently, a recalculation of the pH optimum from the uncorrected raw data of Domínguez de María et al. [10] revealed that the discrepancy between the values reported by this author, Stillger et al. [9] and our own findings (Fig. 3) is most probably due to differences in the evaluation of experimental results. The pH optimum found by application of Eq. (7) according to Buchholz et al. [26] and Marangoni [24] to the data of Domínguez de María et al. [10] was 9.27 ± 0.19. This is statistically almost equivalent to the value of 9.0 reported by Stillger et al. [9] and complies well with the pH optimum of 9.46 (±0.07) determined in this study (see previous paragraph). A similar processing of the data reported by Stillger et al. [9] was impossible, because the number of available data points was too small. An explanation for the residual differences in the pH optima of Stillger et al. [9] and Domínguez de María et al. [10] can probably be found in the different determination of pH in both studies. While Stillger et al. [9] determined pH before addition of DMSO (30% v/v), Domínguez de María et al. [10] used the mixture of buffer/DMSO DMSO (30% v/v) for pH

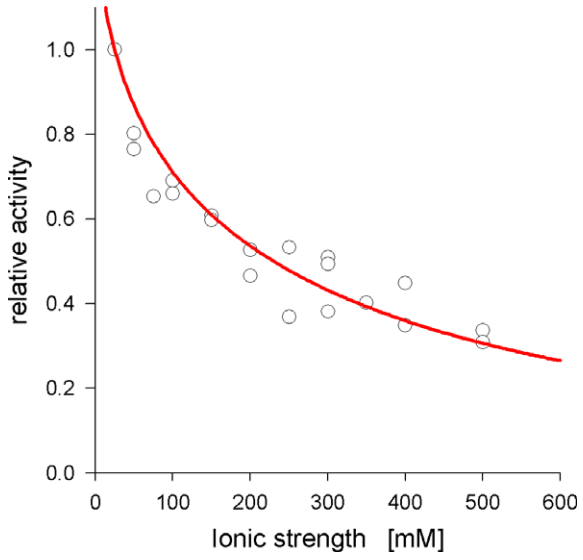


Fig. 2. Influence of ionic strength on the synthetic activity of BAL in KP_i (pH 8.5, 30% v/v DMSO) calculated according to Eq. (5). Ionic strength was determined according to Eqs. (3) and (4).

Table 2
Cross influences of changes in the ionic strength at different pH on activity determinations in 50 mM KP_i [cf. 10] and 10 mM KP_i [cf. 9]. Ionic strength was determined according to Eqs. (3), (4).

pH	50 mM KP_i Cross influence		10 mM KP_i Cross influence	
	I (mM)	(%)	I (mM)	(%)
5.0	50.3	0	10.1	0
6.0	52.6	1	10.5	1
7.0	71.2	9	14.2	4
8.0	122.9	24	24.6	11
9.0	146.4	29	29.3	14
10.0	149.6	30	29.9	15
11.0	150.0	30	30.0	15

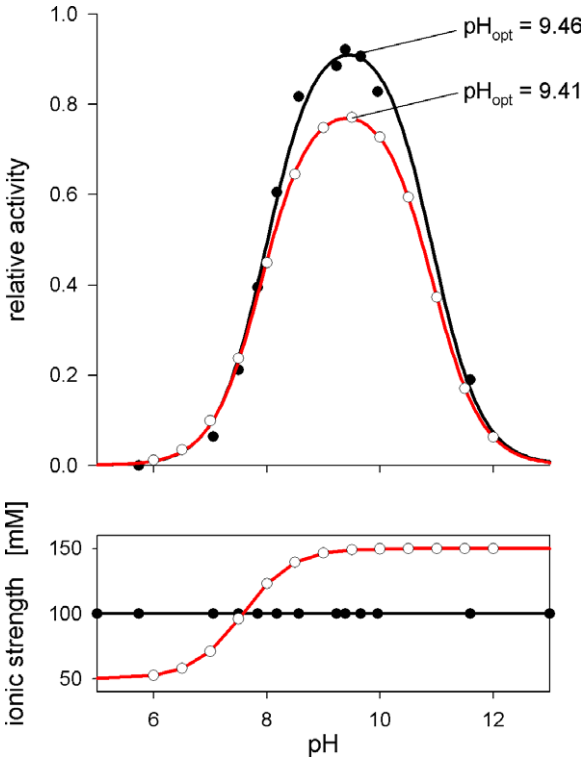


Fig. 3. Upper graph: synthetic activity of BAL at various pH values, constant ionic strength (100 mM) and 30% (v/v) DMSO (●). *In silico* experiments with constant buffer strength of 50 mM (○) [cf. 10]. Fit of Eq. (7) to experiments to determine pH_{opt} (--). Lower graph: development of ionic strength related to the upper graph.

determination. This must have caused a shift of the apparent pH as described in detail in the following section.

In summary, the findings clearly demonstrate that ionic strength, though different in the reported experiments, cannot be the reason for the peculiar pH-dependency observed for BAL catalysed reactions. This is in accordance with the fact that even upon recalculation of the pH optimum from the raw data of Janzen et al. [8] (Table 1) by application of Eq. (7) (pH_{opt}: 8.24 ± 0.24), a clear discrepancy to the value of Domínguez de María et al. [10] can be observed (pH_{opt}: 9.27 ± 0.19), although the same buffer was applied (50 mM KP_i).

$$\frac{k_{cat}}{k_{opt,pH}} = \frac{a}{1 + 10^{pKa_1 - pH} + 10^{pH - pKa_2}} \quad (7)$$

In Eq. (7), k_{cat} and $k_{opt,pH}$ denote the turnover numbers at investigated and optimal pH, respectively, a is a model parameter without physical meaning, and pK_{a1} and pK_{a2} mediate the acidic and alkaline limb in the pH-activity profiles.

Influence of DMSO: Records of pH-activity profiles at different DMSO concentrations and constant ionic strength (100 mM) revealed a strong influence of the cosolvent on the activity of BAL, and indicated a cross effect on the optimal pH with a shift of profiles to the alkaline milieu (Fig. 4). Subsequent initial rate analysis at pH_{opt} gave the highest activity between 20% and 30% DMSO (v/v) with a peak at 25%, which is in accordance with the activating effect of DMSO observed by Domínguez de María et al. [10].

Calculation of pH_{opt} by application of Eq. (7) showed a clear increase of this parameter with increasing concentrations of DMSO (Table 3). The calculated values are in good agreement with the recalculated or published pH optima of Domínguez de María et al. [10] and Stillger et al. [9], respectively, who both used a DMSO concentration of 30% (v/v) for their studies. Janzen et al. [8] performed their study without DMSO. For solubility reasons, it was not possible to do this in our study. An extrapolation of the observed relationship between pH optimum and DMSO concentration to 0% DMSO gave 8.60 ± 0.05. This is a statistically relevant difference to the pH optimum calculated from the raw data of Janzen et al. [8] (pH_{opt}: 8.24 ± 0.24), but definitely more in the range of this value than the value of Domínguez de María et al. [10] and Stillger et al. [9]. Considering the usual variations of kinetic measurements performed in different labs, it can be concluded that the discrepancies in pH-dependency of BAL reported by these authors can now be attributed to differences in the cosolvent concentration during analysis. In contrast, a final explanation for the extraordinary low pH optimum observed by Nemeria et al. [7] cannot be offered here. It may result from using a coupled activity assay determining benzoin cleavage instead of formation as stated in the introductory part of this manuscript, indicate cross effects related to the complex buffer system in the experiments, or simply result from an inaccurate assignment of pH in aqueous solutions of DMSO as described in other studies [16,17]. These found that the measured or apparent pH of an aqueous solution can be increased upon addition of DMSO. This increase may either be attributed to a real change of proton activity or merely result from a disturbance of pH determination by the organic solvent. In phosphate buffers the pH increases with increasing amounts of DMSO because of an increasing dissociation constant. The apparent pH resembles the real pH up to about 30% (v/v) DMSO in buffer. Above this threshold real pH and apparent pH increasingly differ. A calibration procedure to rule out mistakes in pH determination in the presence of DMSO is well known in literature [16,17], but it remains unclear wether Nemeria et al. [7] conformed to it in their study.

Relation of DMSO-dependency and amino acid residues: A calculation of the two pKa values, pK_{a1} and pK_{a2}, describing the acidic (pK_{a1}) and alkaline (pK_{a2}) limb of the DMSO-dependent pH-activity profiles (Fig. 4) shows that the observed DMSO-dependent change of pH_{opt} is accompanied by an increase in pK_{a1} whereas pK_{a2} remains almost constant (Table 3). Considering that pH_{opt} comprises the mean value of pK_{a1} and pK_{a2} [24,26], this indicates that the dependency of pH_{opt} is almost exclusively governed by pK_{a1}. Such a dependency of pK_a values on DMSO can frequently be found for acid–base moieties [27], and has recently been reported for amino acid side-chains in proteins [28]. It can be attributed to the formation of strong hydrogen bonds between the alkaline oxygen of DMSO and acidic protons, whereupon the physico-chemical characteristics of surrounding water may be severely affected [29].

With this finding, and considering that according to Buchholz et al. and Marangoni pK_{a1} and pK_{a2} may be assigned to ionisable side-chains in the active site of an enzyme [24,26], an investigation on the relation between DMSO-dependency of BAL activity and structural features of the catalyst was performed. Examination of the active site of BAL for appropriate acid–base side-chains exclusively revealed two residues, namely glutamate at position 50 (Glu50) and histidine at position 29 (His29), as candidates for possible interactions with DMSO. It has been suggested for His29 that the protonation state of this amino acid residue might play an important role in catalysis [30]. A similar role can be assumed for Glu50, as this complies with a highly conserved residue in all ThDP-dependent enzymes [31] which is responsible for the essential binding of the cofactor [32]. As this binding requires the deprotonation of Glu50 [7] both residues can be considered to govern the changes in BAL activity in the acidic limb of the pH-activity profile.

The ability of each residue to interact with DMSO was investigated by potentiometric titrations of isolated L-histidine (L-his) and L-glutamate (L-glu). In the absence of DMSO, the pK_a values of the amino acids were 4.31 for L-Glu and 6.14 for L-His (Fig. 5)

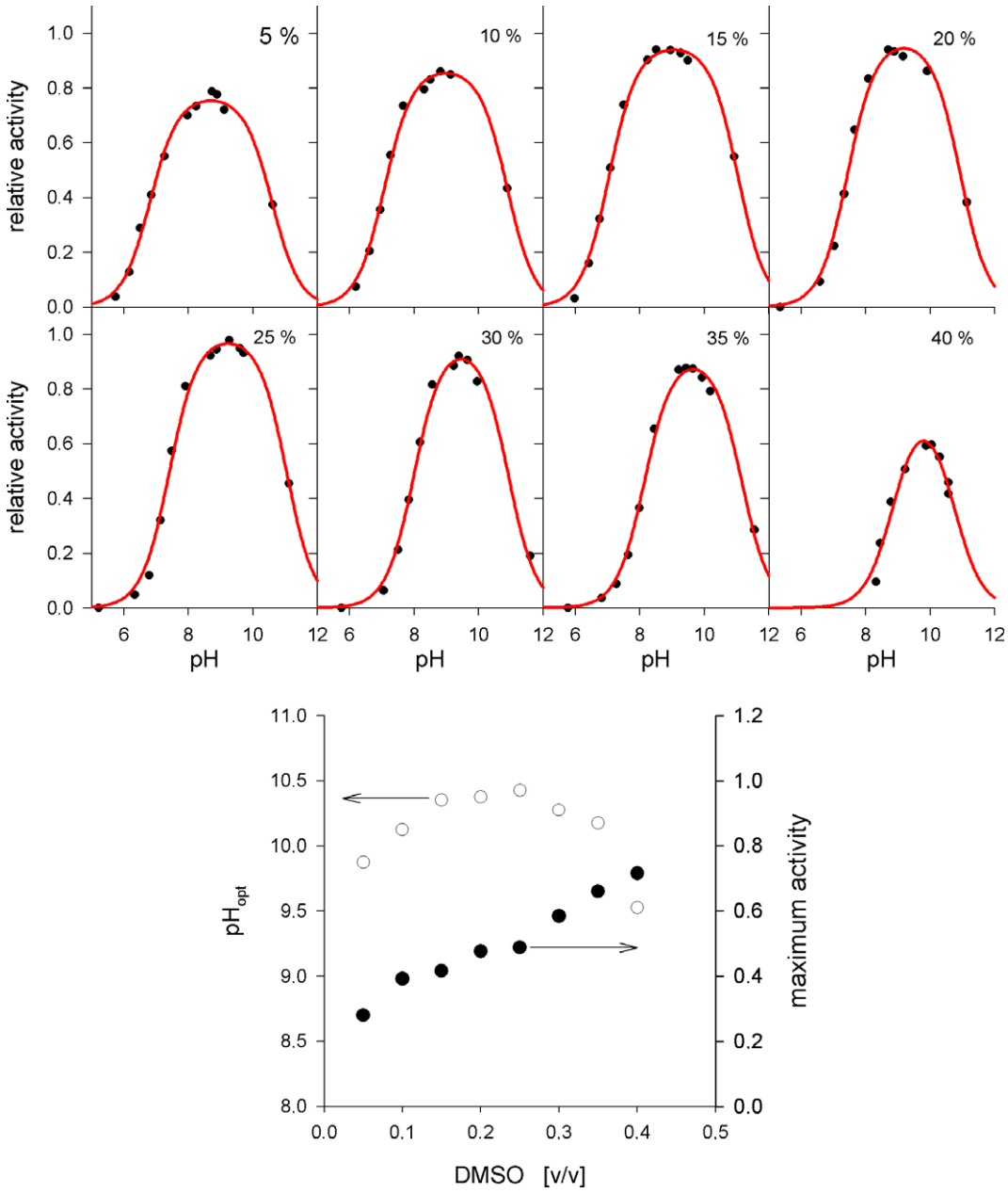


Fig. 4. Upper graphs: pH-activity profiles of BAL at different DMSO concentrations. Lower graph: optimal pH value for carboligation activity and the respective activity as a function of the DMSO content.

Table 3
Influence of DMSO on pH_{opt}, pK_{a1} and pK_{a2}. Values were calculated using Eq. (7).

(%)	pH_{opt}	pK_{a1}	pK_{a2}
5	8.70 ± 0,05	6.81 ± 0,04	10.58 ± 0.06
10	8.98 ± 0,05	7.08 ± 0,04	10.88 ± 0.05
15	9.04 ± 0,04	7.04 ± 0,03	11.04 ± 0.04
20	9.19 ± 0,05	7.44 ± 0,04	10.93 ± 0.06
25	9.22 ± 0,06	7.40 ± 0,04	11.04 ± 0.07
30	9.46 ± 0,07	7.99 ± 0,04	10.92 ± 0.09
35	9.65 ± 0,05	8.14 ± 0,04	11.16 ± 0.06
40	9.79 ± 0,12	8.85 ± 0,10	10.73 ± 0.13

and thus were in good agreement with the published values of 4.3 and 6.0, respectively [23]. In the presence of 30% (v/v) DMSO, the pK_a of L-His slightly decreased, which does not correspond to the behaviour of pK_{a1} calculated from DMSO-dependent pH-activity profiles of BAL (Table 3). In contrast, the pK_a of L-Glu increased by 0.52 units under the same conditions, resembling the increase of pK_{a1} in the presence of 30% (v/v) DMSO. This suggests that in BAL, Glu50 is affected by DMSO and governs the acidic limb of the pH-activity profile, whereas His29 is without importance. This finding could be challenged with the established knowledge that free amino acids frequently exhibit other pK_a values and behave differently from amino acids in a protein structure [24]. Likewise the catalytic cycle of BAL contains several charged reaction intermediates and DMSO may interact with them or charged residues on the enzyme surface which indirectly influence catalysis. However, it is in line with the sound proposition of Jordan and coworkers that the activity increase of BAL accompanying the deprotonation of Glu50 can thermodynamically be described by the pK_a of this amino acid residue [7,33], and can therefore be strongly supported.

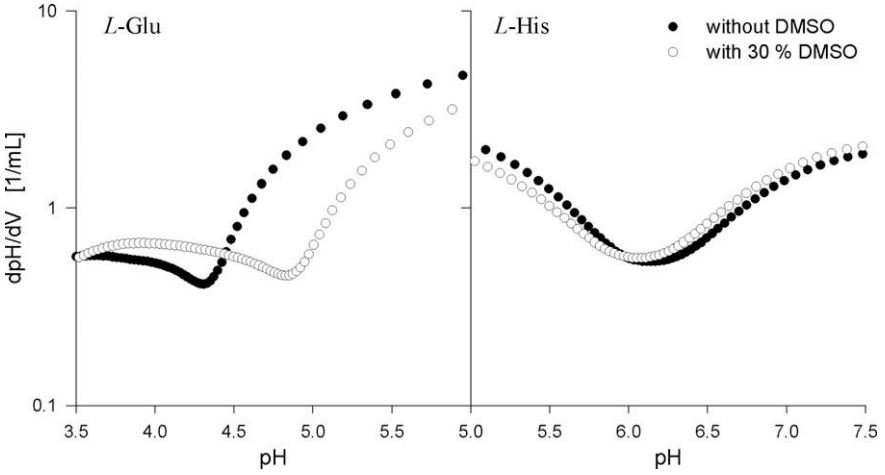


Fig. 5. Progression of the potentiometric titration of L-Glu and L-His without (●) and with (○) 30% (v/v) DMSO.

4. Conclusions

Reported biochemical peculiarities of benzaldehyde lyase from *Pseudomonas fluorescens* Biovar I. (BAL, EC 4.1.2.38) regarding pH optimum and dependence on DMSO can be explained by a direct interaction of DMSO with the amino acid side chain Glu50 in the catalytic site of the biocatalyst. This finding is important since it allows a better understanding of the catalytic mechanism of this enzyme, and consequently a deduction of optimal reaction conditions. Moreover, as Glu50 is an amino acid residue which is highly conserved in the whole class of ThDP-dependent enzymes, e.g. pyruvate decarboxylase [31], it can be expected that the observed relation between enzyme activity, pH-dependency, and DMSO concentration will also apply to these. Thus, the findings provide a novel argument against the use of DMSO as cosolvent in the application of a whole subclass of synthetically valuable biocatalysts, additional to the known complication of downstream processing caused by DMSO. It will have to be further investigated whether similar effects occur with alternative water miscible solvents required to enhance water solubility of substrates and products in the enzyme catalysed reactions.

Acknowledgments

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