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Effect of various water-miscible solvents on enzymatic activity of fungal laccases

J. Rodakiewicz-Nowak, S.M. Kasture, B. Dudek, J. Haber *

Institute of Catalysis and Surface Chemistry, Polish Academy of Sciences, ul. Niezapominajek, 30 239 Cracow, Poland

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Abstract

The effect of acetonitrile, acetone and dimethyl sulfoxide (DMSO) on the catalytic activity of laccase from *P. radiata* and the effect of DMSO on the catalytic activity of laccase from *P. oryzae* in the oxidation of 2,6-dimethoxyphenol were studied. The results obtained in the studied solvent–water mixtures were satisfactorily described using the equations equivalent to the mixed enzyme inhibition. The fitted inhibition parameters were correlated with various physical and chemical parameters characterizing the mixed systems or used solvents, such as: effective pH of the reaction mixture, $\log P$ and E_T^N of the solvent, solubility of the substrate and thermodynamic activity of water in mixed systems. Moreover, a good correlation between the inhibitory effects and the denaturation capacity of the studied solvents was observed.

Keywords: Laccase; Organic solvents; Activity; Inhibition

1. Introduction

Fungal laccases are multi-copper oxidases that catalyze the oxidation of a wide variety of organic compounds, including methoxy-substituted monophenols, *o*, *p*-diphenols, aminophenols, polyphenols, polyamines, aryl amines and lignin, using molecular oxygen as an oxidant, reduced directly to water. They can also catalyze the dimethylation of lignin and methoxyphenol acids [1,2]. They have been shown to catalyze the lignin polymerization and co-polymerization of lignin with phenols and acrylamide [3,4]. With the use of proper mediators, laccases are able to delignificate pulp [5] and oxidize compounds of high redox potentials, such as aromatic alcohols [6].

For all these reasons, these enzymes are a very prospective class of enzymes for biotechnological applications, such as biobleaching, detoxification, organic synthesis and for biosensing [7–9]. However, in many cases the interesting substrates are poorly soluble in water and the knowledge of the behaviour of laccases in various water-restricted media is necessary. The available data show that these extracellu-

* Corresponding author.

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lar and highly glycosylated enzymes retain their activity both in organic solvents of various water content [3,4,9–16] and in reverse micelles [9,17]. A specific feature of these enzymes is, however, the fact that the organic substrate binding site is very close to the surface of the enzymes and it is easily accessible to the solvents [18], which may become an important factor in the effective catalysis in the presence of water-miscible solvents.

The curves representing the profiles of variations of the maximal reaction rate, V_{max}, with concentrations of the added solvents, can be divided into three regions: 1 — a region of a little, if any variation of V_{max} with the content of a solvent; 2 — a region of a sharp decrease in the enzymatic activity, caused by the denaturation of the enzyme by a solvent; 3 — a region of a very low enzyme activity. Mozhaev et al. [10] and Khmelnitsky et al. [19] analyzed the enzyme dehydration and solvation by the solvent processes proceeding in region 2. They correlated the C_{50} values (the solvent concentration needed for the 50% reduction of the V_{max} value observed in the absence of the solvent) with the thermodynamic parameters determining the above processes. The analysis lead to the denaturation capacity (DC) scale of organic solvents, which was proposed as a tool for predicting the denaturation of various enzymes by organic solvents [19]. Luterek et al. [12] and Rogalski et al. [13] compared the effect of organic solvents on the native and immobilized laccases, finding the protective effect of the immobilization in the region of high solvent concentrations.

van Erp et al. [11] also studied the effect of various solvents on the activity of the immobilized laccase from *C. versicolor* at low water content (region 3). They showed that the values of the apparent binding constant, K_m, increased exponentially with water concentration and the apparent values of V_{max}, two orders of magnitude lower than in the respective aqueous solutions, increased linearly with water content in the systems. This increase was again described in terms of the enzyme hydration number and hydration equilibrium constant.

The hydration of enzymes is related to the water thermodynamic activity, a_w [20,21]. Bell et al. [22] re-analyzed the data of van Erp et al. [11] using a_w values instead of the water content in the system. They found that the changes of water thermodynamic activity and the hydration of the enzyme are not sufficient to explain the observed reduction of laccase activity.

Many authors (see Refs. [23–25]) showed that the analysis of the effect of solvents on enzymatic reactions also needs to take into consideration their influence on substrates and intermediates of the reaction. Moreover, there are no detailed data on the influence of organic solvents on laccases under practically non-denaturating conditions (region 1), where the enzymes retain their native conformation and a high catalytic activity.

Therefore, it seemed of interest to compare the effect of various organic solvents in region 1 on fungal laccases isolated from various sources, using the same two substrates, chosen from the substrates studied before. We used syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine, SYR) and 2,6-dimethoxyphenol (2,6-DMOP) as examples of substrates of a considerably different hydrophobicity.

Our earlier studies showed that under non-denaturating conditions, the short chain alcohols (C_1–C_3) may be treated as weak competitive inhibitors of laccase from *P. radiata*, for both studied substrates [14], whereas ethanol may be treated as a weak mixed (competitive and uncompetitive) inhibitor of laccases from *C. versicolor* and *P. tigrinus*, cultivated in liquid submerged cultures [15].

The aim of the present study was to compare the effect of alkanols with the effect of the solvents containing other functional groups in the molecule on the catalytic activity of one selected laccase (from *P. radiata*) and to extend the study to the effect of one selected solvent on various enzymes. Dimethyl sulfoxide (DMSO) has been chosen because it is being used as a cryprotectant of proteins, has a high solubilization capacity both for organic molecules and proteins and it is also known as a reversible denaturant of many proteins. We were interested in the limits of practical application of DMSO in our further studies.

We asked two questions: (i) whether we would be able to separate the effect exerted by the studied solvents on the enzymes from that exerted on the substrates; and (ii) whether the data obtained under the non-denaturating conditions may give some information on denaturation of laccases by more concentrated solutions of these solvents.

2. Experimental

2.1. Materials

2.1.1. Enzymes

2.1.1.1. P. Radiata laccase (PRL). The white-rot fungus, *P. radiata* Fr, strain 79, immobilised on a polypropylene carrier, was cultivated in a laboratory fermentor under semi-continuous conditions, on the Kirk medium [26], modified by Hatakka and Uusi-Raava [27], as described earlier [28].

The culture medium was filtered and the enzyme was purified by the double exchange ionic chromatography on DEAE Toyopearl 650 M. Enzyme preparations were dialysed against 25-mM tris–HCl buffer, pH 7.0 and stored at −70°C. Laccase activity during cultivation and purification was checked by the rate of oxidation of SYR and diammonium 2,2′-azino-bis(-3-ethylbenzthiazoline-6-sulfonate) (ABTS), as described earlier [28].

P. oryzae laccase (POL) was a commercial preparation of Sigma, used without further purification.

2.1.2. Chemicals

SYR and ABTS were products of Sigma. Acetic acid was of a.g. purity and a product of Fluka. 2,6-DMOP was produced by Aldrich. Sodium acetate and bromocresol purple (BCP) were produced by POCh, Poland. Disodium hydrogen phosphate, potassium dihydrogen phosphate and DMSO, the latter of spectral UV purity, were products of Merck. Acetonitrile and acetone were of a.g. purity, and products of Fluka and POCh, respectively.

2.2. Measurements

pH was determined electrochemically and/or spectrophotometrically, using the pH meter, Elwro, and Shimadzu PC2101 UV/Vis spectrophotometer (with BCP as an indicator).

Kinetic measurements of 2,6-DMOP oxidation rates were carried out spectrophotometrically, at 30°C ($\varepsilon_{468} = 14\,800\ M^{-1}\ cm^{-1}$ [29]), using Shimadzu PC 2101 spectrophotometer. Concentration of substrates and syringaldazine solubility in ethanol solutions were checked spectrophotometrically. Enzyme specific activity was verified everyday.

Two kinds of kinetic experimental series were determined: (i) at constant solvent content, leading to the apparent values of the Michaelis–Menten parameters and (ii) at constant substrate concentration and varying addition of the respective solvent, leading to the Dixon-like relationships. The first type of experimental data was analysed graphically and numerically using the Michaelis–Menten equation. Then all data obtained for a particular system (consisting of 100 experimental points, on average) were analysed using various models of enzyme inhibition, described below. The numerical fittings were carried out using the Sigma Plot 5.0 software.

3. Results and discussion

3.1. Stability of PRL in DMSO and acetonitrile solutions

First, the effect of laccase incubation in DMSO-buffer solutions on the effective initial reaction rate, V_{in}, was studied (Fig. 1). The observed initial rate

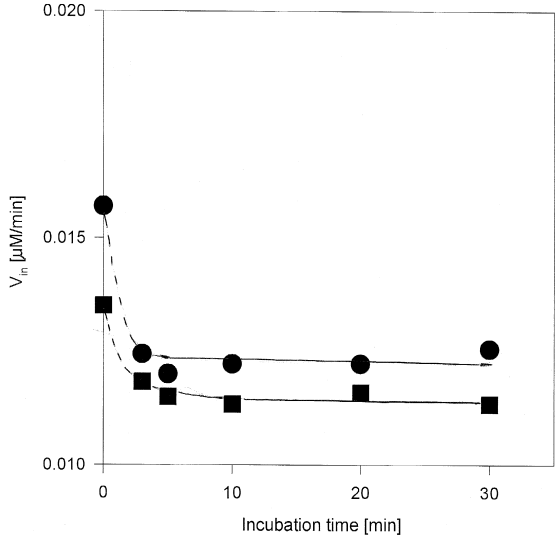

Fig. 1. Effect of enzyme incubation in DMSO–acetate buffer solution on the initial reaction rate of 2,6-DMOP oxidation in the presence of PRL. 1.54 mM 2,6-DMOP, 1.41 M (●) and 2.82 M (■) DMSO.

was lower than in the respective aqueous buffer solutions and dropped down during the first minutes. Then, it was practically independent of the incubation period. A 60-min long incubation in 1.9 and 3.8 M acetonitrile did not reduce the initial reaction rate.

3.2. Organic solvents as weak inhibitors of fungal laccases

3.2.1. Kinetic measurements

Enzymatic activity of laccase from *P. radiata* was investigated in 25 mM acetate buffers, pH 5.0, with addition of 0–3.5 M DMSO, acetone and acetonitrile. The studies of POL were limited to DMSO-buffer solutions only (25 mM acetate buffer, pH 5.0).

Our earlier studies of the effect of short chain alkanols on catalytic activity of PRL showed that methanol, ethanol and *n*-propanol did not affect the effective V_{max} values over a relatively wide range of alcohol concentrations, and increased linearly the apparent K_m values, behaving like weak competitive inhibitors of the laccase [14]. Moreover, it was found that ethanol behaved like a weak mixed inhibitor of blue laccases from *C. versicolor* (CBL) and *P. tigrinus* (PBL) [15], over a wide range of ethanol concentrations. None of the other commonly used models of enzyme inhibition: hyperbolic, non-competitive, or uncompetitive, satisfactorily described the experimental data. The same was observed for the data reported here. All the studied systems could be satisfactorily described using the mixed inhibition (MI) model (Scheme 1 and Eq. 1):

$$V_{in} = V[S]/\left(K_m(1 + [I]/K_I) + [S](1 + [I]/K_{IS})\right), \quad (1)$$

where: $V = V_{max}[E]$, K_I and K_{IS} are the inhibition constants, describing the reversible binding of inhibitor molecules with enzyme or enzyme–substrate complex, [E], [I] and [S] are the enzyme, solvent and substrate concentrations, and K_m and V_{max} retain their usual meaning of the effective substrate binding constant and enzyme maximum catalytic rate.

It should be mentioned, however, that the same final Eq. (1) may be obtained also by assuming the binding of inhibitor I (solvent) to the substrate molecules, S, and to the ES complex. In that case,

$$\begin{array}{ccccc} & & \mathbf{K_m} & & \mathbf{k} \\ \mathbf{E + S} & & \Leftrightarrow & \mathbf{ES} & \Rightarrow & \mathbf{E + P} \\ \mathbf{K_I} \uparrow\downarrow \mathbf{+ I} & & & \mathbf{+ I} \uparrow\downarrow & \mathbf{K_{IS}} \\ \mathbf{EI} & & \Leftrightarrow & \mathbf{ESI} & & \end{array}$$

Scheme 1.

K_I and K_{IS} would be the inhibition constants, representing the SI and ESI equilibrium constants. The relative positions of the equilibria: E + S ⇔ ES and E + I ⇔ EI (see Scheme 1) must depend on the relative values of the solubility of the substrate and the inhibitor in water.

At constant enzyme and substrate, the ratios of K_I values for the different substrates in solutions of one chosen solvent should vary similarly as K_m values for those substrates. As we see further (Table 3), there is some parallelism between the ratios for SYR and 2,6-DMOP, obtained for three different laccases in ethanol solutions.

It should be noted here that the catalytic cycle of laccases comprises a number of steps: the binding of the reductant substrate, binding the second substrate–oxygen, intramolecular electron transfer, oxygen reduction, proton transfer, and release of the reaction products as the most important events [30,31]. The oxygen binding constant is low, of the order of about 20 μM and laccases usually work under enzyme saturation with oxygen, even in organic solvents [11], but many reaction steps are irreversible. Therefore, even in homogeneous aqueous solutions, the Michaelis–Menten equation, although it describes the enzyme kinetics satisfactorily and is used by laccases researchers, gives only the apparent K_m and V_{max} values. The interpretation of global kinetic data is more complex in the presence of solvents. Although in water-rich solutions of organic solvents, the change in water thermodynamic activity should not shift the reaction equilibrium, the reductant substrate binding constant, as well as the oxygen binding constant and the particular kinetic constants for single catalytic events, may be affected by the presence of the solvents. Irrespective of the complex real physical meaning of the inhibition pa-

Table 1
Enzymatic activity of investigated laccases in aqueous buffered solutions

Enzyme and substrate	V_{max} (μmole/min/[E])	K_m (μM)
PRL + 2,6-DMOP	0.687[a]	246/440
CBL + 2,6-DMOP [16]	145[b]	20.3
PBL + 2,6-DMOP [16]	100[b]	16.6
POL + 2,6-DMOP	0.0099[c]	27.1
PRL + SYR [14][d]	0.381[a]	3.34
CBL + SYR [16][d]	123[b]	2.2
PBL + SYR [16][d]	72.6[b]	2.0

Enzymes: PRL — *P. radiata laccase*; POL — *P. oryzae laccase*; CBL — *C. versicolor laccase*; PBL — blue lacasse from *P. tigrinus*. Substrates: 2,6-DMOP — 2,6-dimethoxyphenol; SYR — syringaldazine.

[a][E]: ml of protein solution.

[b][E]: mg of purified protein.

[c][E]: mg of solid.

[d]For water SYR, the values given in the table were extrapolated from measurements carried out in aqueous–ethanol mixtures.

rameters, K_I and K_{IS}, they are the useful tools for comparing the effective influence of various solvents on the studied kinetic catalytic effects, independent of the composition of the investigated mixture.

Table 1 compares the apparent Michaelis–Menten parameters obtained for various laccases studied in oxidation of the selected substrates. All laccases strongly bind SYR, which has very poor solubility in water. Laccases from *P. tigrinus*, *C. versicolor* and *P. oryzae* bind 2,6-DMOP (quite hydrophilic) also relatively strongly. Laccase from *P. radiata* has lower affinity towards this substrate [13,14]. In terms of the maximum reaction rates, V_{max}, 2,6-DMOP is, however, a better substrate than SYR for all studied laccases.

Table 2 presents the results of fitting Eq. (1) to the experimental data. The following conclusions may be drawn directly from this table.

- The simple competitive (CI) model of inhibition (a limiting case of the MI model at $1/K_{IS} = 0$) describes only the effect of alkanols on laccase from *P. radiata*. Other systems are described using the MI model, although in the studied range of concentrations of the inhibitors, $1/K_{IS}$ values for all studied solvents, except of DMSO, are rather low.

- The inhibitory effects depend both on the particular enzyme studied and the substrate, being higher for SYR than for 2,6-DMOP.

- These effects increase with solvent hydrophobicity (except for DMSO), for the studied solvents (cp. methanol, ethanol and propanol).

The experiments have been planned so as to pick up some effects directly from the results.

3.2.2. Effect of the solvent hydrophobicity (logP) on the inhibitory effect

The results indicate a predominant effect of solvent hydrophobicity.

Table 2
Effect of solvents on 2,6-DMOP and SYR oxidation by various laccases

Solvent	log *P* [8]	E_T (30) [19] (kJ/mole)	Substrate	Inhibition model	$1/K_I$ [1/M]	$1/K_{IS}$ [1/M]	Enzyme
DMSO	−1.35	188	2,6-DMOP	MI	1.70	0.80	PRL
Methanol	−0.74	232	2,6-DMOP	CI	0.40	–	PRL
Acetonitrile	−0.34	192	2,6-DMOP	MI	1.05	0.16	PRL
Ethanol	−0.32	213	2,6-DMOP	CI	0.9	–	PRL
Ethanol	−0.32	213	2,6-DMOP	MI	1.04	0.06	CBL [16]
Ethanol	−0.32	213	2,6-DMOP	MI	2.44	0.04	PBL [16]
Acetone	−0.24	177	2,6-DMOP	MI	1.41	0.21	PRL
Methanol	−0.74	232	SYR	CI	0.657	–	PRL [14]
Ethanol	−0.32	213	SYR	CI	1.525	–	PRL [14]
Propanol	0.34	212	SYR	CI	3.214	–	PRL [14]
Ethanol	−0.32	213	SYR	MI	1.21	0.12	CBL [16]
Ethanol	−0.32	213	SYR	MI	1.97	0.58	PBL [16]

pK_I values show the linear dependence on $\log P$ (partition coefficient between water and *n*-octanol) values of the investigated solvents. For the data on 2,6-DMOP oxidation in the presence of solvents with various functional groups in the molecule, excluding the value for DMSO (Table 2, Fig. 2), the slope is equal to 0.95 ± 0.11, for the data on SYR oxidation, it has the value of 0.84 ± 0.02 (Table 2, Fig. 2). Both values remain within the typical range [32] observed for biological systems of different complexity.

3.2.3. Effect of the solvent polarity (E_T^N) on the inhibitory effect

We also tried to correlate $1/K_I$ values with the Reichardt–Dimroth constant for the studied solvents (solvent ability to solvate the polar solutes, as measured with pyridinium phenolate dye) (Fig. 3). The corresponding slopes are equal to -0.017 kJ^{-1} ($r = 0.95$) in 2,6-DMOP oxidation (excluding DMSO) and -0.09 kJ^{-1} ($r = 0.62$) in SYR oxidation. It is worth noticing that the Reichardt–Dimroth constant gives a better description of the effect of DMSO on PRL than $\log P$ does.

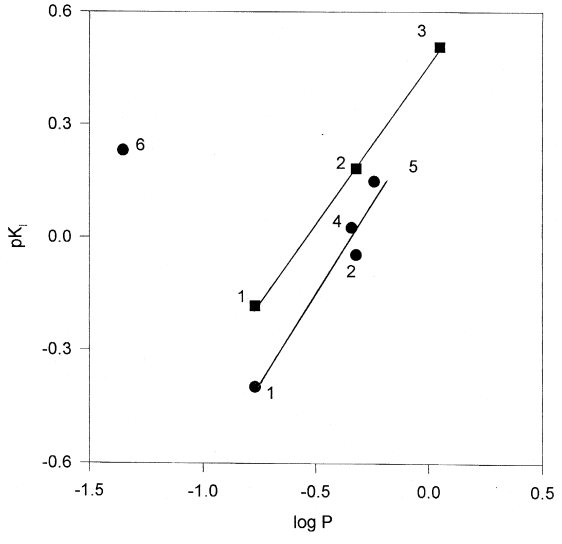

Fig. 2. Effect of solvent hydrophobicity on the effective pK_I values for: ■ PRL–SYR and ● PRL–2,6-DMOP; 1 — methanol; 2 — ethanol; 3 — *n*-propanol; 4 — acetonitrile; 5 — acetone; 6 — DMSO.

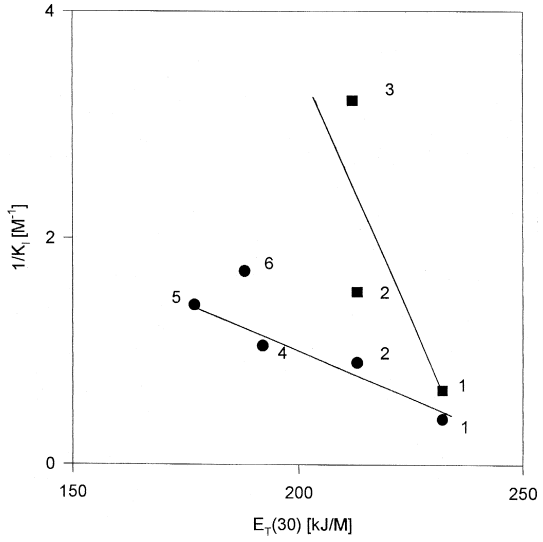

Fig. 3. Effect of solvent polarity on the effective $1/K_I$ values for: ● PRL–2,6-DMOP and ■ PRL–SYR; 1 — methanol; 2 — ethanol; 3 — *n*-propanol; 4 — acetonitrile; 5 — acetone; 6 — DMSO.

3.2.4. Effect of pH variation caused by the presence of DMSO in buffer solutions on reduction of PRL activity

Organic solvents change the pH values of aqueous solutions, thus affecting the dissociation of substrates and enzymes and conformation of the latter. Recent investigations of the influence of pH of the aqueous solutions upon the apparent V_{max} and K_m values of various laccases in oxidation of phenolic and non-phenolic substrates showed that the optimum pH values for phenolic derivatives were dependent on the enzyme studied, and independent of the substrate of the oxidation reaction. Moreover, it was shown that the apparent K_m values were constant at least between pH = 4 and 6, afterwards decreasing with pH, while the apparent V_{max} values showed distinct maxima at pH values dependent on the laccase.

To evaluate the effect of solvents related to variations in pH of the studied mixtures, we measured the pH of the DMSO–acetate buffer mixtures spectrophotometrically (25, 50, 100 and 200 mM acetate and phosphate buffers) (Fig. 4a). As the indicator BCP was chosen, of $pK = 6.1$ The absorption values of BCP were measured at 588 nm, for the buffer,

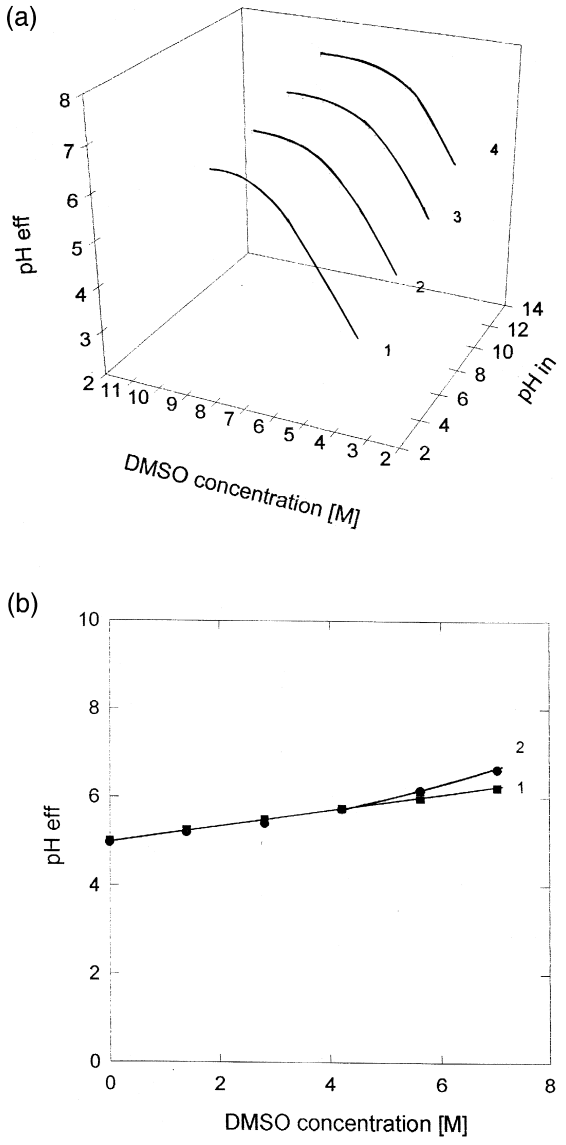

Fig. 4. (a) Effective pH of the investigated buffer–DMSO mixtures: 1 — 25 mM; 2 — 50 mM; 3 — 100 mM and 4 — 200-mM buffer solution. (b) Effect of DMSO on the effective pH of 25 mM acetate buffer, $pH_{in} = 5.0$; spectrophotometrical determination; 2 — electrochemical determination.

initial pH values varying from 3.6–8.2 and for DMSO concentrations, changing between 0 and 12 M.

Fig. 4b presents the influence of DMSO on the effective pH of 25 mM-acetate buffer, $pH_{in} = 5.0$, as measured electrochemically (curve 1) and spectrophotometrically (curve 2). An almost linear increase of the effective pH may be observed, over a wide range of DMSO concentrations. The observed pH shifts indicate that DMSO-derived pH changes should affect mainly the V_{max} values of laccase.

Fig. 5a presents the pH effect on *P. radiata* enzymatic activity in oxidation of 2,6-DMOP, in

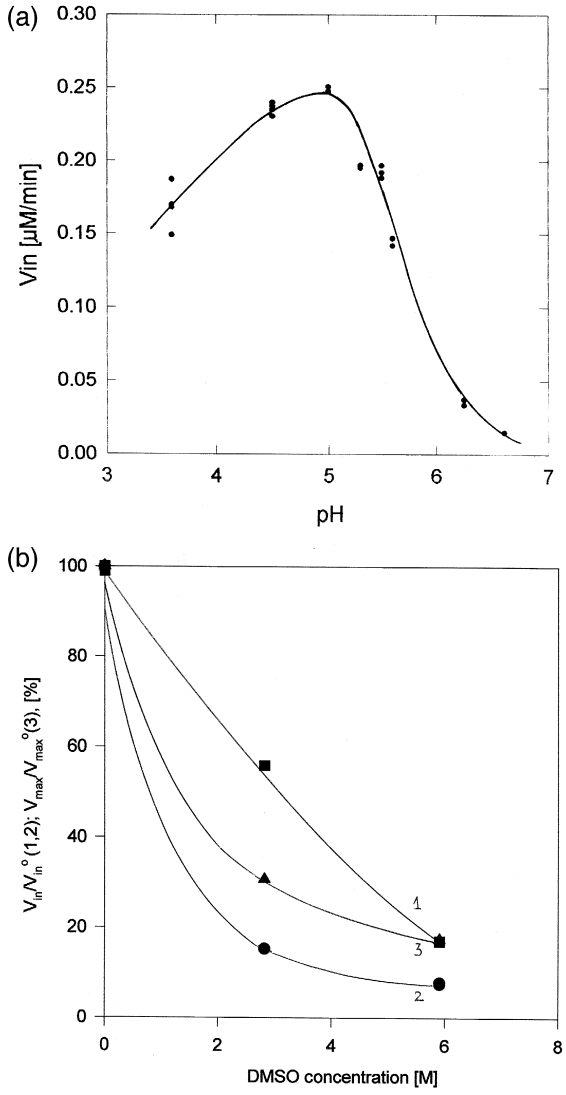

Fig. 5. (a) Effect of pH on activity of PRL in acetate/phosphate buffer solutions in the absence of DMSO; 111 μM 2,6-DMOP. (b) Variation of the experimental (1) and predicted from the pH change (2) initial rate values of 2,6-DMOP oxidation in solutions of various DMSO contents. (3) Variation of the respective V_{max} in solutions of various DMSO contents.

acetate buffer and in phosphate buffer solutions. Rather strong pH effects may be found. Fig. 5b shows the initial rate of 2,6-DMOP oxidation in function of DMSO concentration. Curve 1 is a theoretical line, drawn based on the results presented in Figs. 4a and 5a. It shows the predicted effect of DMSO, resulting purely from the pH variation caused by DMSO addition. Curve 2 represents the experimental data. Curve 3 illustrates the corresponding reduction of V_{max} value caused by DMSO addition. It may be seen that the contribution to the rate decrease that might be ascribed solely to pH changes, under the chosen experimental conditions, is significant, and varies with DMSO concentration.

3.2.5. Effect of the substrate hydrophobicity on inhibitory effects

SYR and 2,6-DMOP, the substrates considerably differing in hydrophobicity ($\log P = 2.78$ and 1.15, respectively), were oxidized in the presence of various laccases (PRL, CBL and PBL), in aqueous and aqueous–ethanol solutions. The data summarized in Table 3 indicate that the change in the $\log(K_I^{2,6\text{-DMOP}}/K_I^{SYR})$ values for ethanol is more or less parallel to that of $\log(K_m^{2,6\text{-DMOP}}/K_m^{SYR})$ values. The higher the binding constant of the particular substrate, K_m, the stronger is the observed ethanol competitive inhibition.

3.2.6. Solvent effects for various enzymes catalyzing the same reaction

When various enzymes catalyze the same reaction, the difference in behaviour on the presence of a solvent may be ascribed primarily to the influence of the solvent on the enzyme molecules. Such data were collected for oxidation of SYR and 2,6-DMOP, catalyzed by PRL, CBL and PBL in the presence of ethanol, and for oxidation of 2,6-DMOP, catalyzed by PRL and POL, in the presence of DMSO.

The apparent inhibition constants, K_I, for ethanol in 2,6-DMOP oxidation varied in the ratio 1.0:1.1:2.7 and in SYR oxidation in the ratio 1.0:0.8:1.3, for PRL, CBL, and PBL, respectively. The values of K_{IS} were low and close for PRL and CBL, whereas they are higher for PBL. The inhibition constant, K_I, observed for DMSO, was much higher for POL than for PRL laccase (5.5:1). For all studied enzymes DMSO, behaved as a relatively strong inhibitor. The data for PRL are consistent with the observation of Rogalski et al. [13]. The data obtained for the commercial sample of POL are more difficult to interpret. The relatively low values of K_{IS} and relatively high values of K_I may be caused by some contamination of the sample.

An unusually high inhibitor effect of DMSO was also observed in the case of two other commercial enzymes: mushroom tyrosinase (oxidation of *t*-butyl catechol) and horseradish peroxidase (oxidation of ABTS with the use of hydrogen peroxide): $1/K_I = 6.80$ and 1.66 M^{-1}, for tyrosinase and peroxidase, respectively.

Table 3
Oxidation of two substrates in the presence of the same enzyme in one solvent system

Solvent	Enzyme	$\log(K_m^{2,6\text{-DMOP}}/K_m^{SYR})$
Buffer	PRL [14]	1.87
	CBL [16]	0.96
	PBL [16]	0.92
Solvent	Enzyme	$\log(K_I^{2,6\text{-DMOP}}/K_I^{SYR})$
Ethanol	PRL [14]	1.47
	CBL [16]	1.16
	PBL [16]	0.81

$\log P_{SYR} - \log P_{2,6\text{-DMOP}} = 2.78 - 1.15 = 1.63.$

3.2.7. The inhibitory effect and denaturating power of the studied solvents

Studies of any enzymes under denaturating conditions are very difficult, mainly because of the differences in denaturation kinetics and a very strong dependence of enzyme activity on changes in the solvent concentration, over a narrow range of their concentrations. Khmelnitsky et al. [19] showed that the denaturation ability of organic solvents depends more or less linearly on $\log P$ and E_T^N values of the studied solvents. Similar relations were obtained here for the inhibition constants. The question arises whether the data obtained under non-denaturating conditions may be used to predict denaturation of the particular enzymes by the same solvents. To answer this question, we correlated our inhibition constants, K_I, with the predicted values of C_{50} (solvent concentration needed to reduce enzymatic activity to 50%

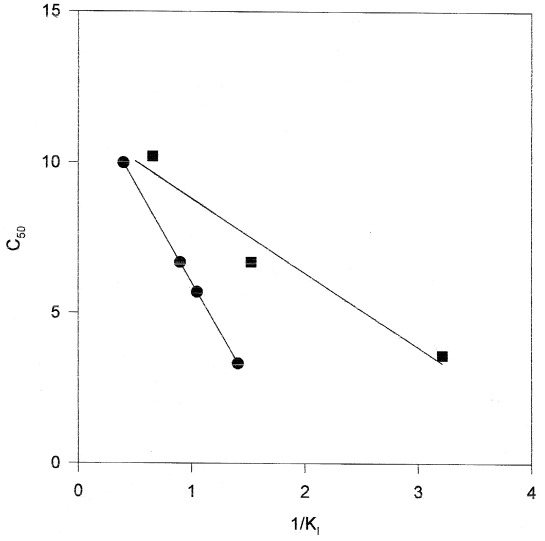

Fig. 6. Correlation between denaturation and inhibition of PRL by various organic solvents; ● oxidation of 2,6-DMOP; ■ oxidation of SYR.

of the initial value in the absence of the solvent), assessed by Khmelnitsky et al. [19] for laccase from *C. versicolor*, in oxidation of pyrocatechol (Fig. 6).

The data collected in Fig. 6 show a very good correlation of the inhibition constants with C_{50} values for all studied solvents, except DMSO. pK_I value predicted by Khmelnitsky et al. [19] for DMSO is about 0.24 units lower than the value presented in this paper.

The fact that the data compared were obtained in two different laboratories for different laccases and various substrates gives some support to the idea of using the inhibition data as a marker of denaturation ability (although the data are too limited for generalization).

3.3. The solvent effects and thermodynamic activity of the species present in reaction media

The reduction of enzymatic activity by the presence of organic solvents may also be discussed in terms of their effect on thermodynamic activity of the species participating in the reaction. The thermodynamic measure of the variations in enzyme activity is the ratio of its specificity coefficients k_s/k_s^0, where $k_s = V_{max}/K_m$ in the presence of organic solvent and $k_s^0 = V_{max}/K_m$ in its absence. Both the models (CI and MI) form a family of equations for which the k_s/k_s^0 values depend only on K_I, according to the formula: $k_s/k_s^0 = 1/(1 + [I]/K_I)$. This fact considerably facilitates the comparison of the results.

As organic solvents affect thermodynamic activity of water in the studied systems, we have chosen for analysis a simple model derived by Lee and Kim [20], taking into account variations of thermodynamic activity of all reactants. The model is based on two assumptions only: that "hydration of the enzyme is a prerequisite step for its catalytic action and the hydrated enzyme molecule follows the Michaelis–Menten kinetics." These assumptions lead to the following formula (Eq. (2)) :

$$\ln\left(k_s/k_s^0\right) = c + \ln\left(\gamma_s/\gamma_s^0\right) + \ln\left(\gamma_{ES}^0/\gamma_{ES}\right) + \ln\left(\gamma_E/\gamma_E^0\right) + n\ln\left(a_w\right), \quad (2)$$

where γ_s, γ_{ES} and γ_E denote the respective activity coefficients of the substrate, the hydrated enzyme–substrate complex and the hydrated enzyme, a_w is water thermodynamic activity, n is the number of water molecules that hydrate the enzyme molecule and c is the constant dependent on the water binding constant and the ratio of enzyme activity coefficients in the absence and in the presence of the solvent. The values of $\ln(\gamma_s/\gamma_s^0)$ vary with the solvent concentration similarly as substrate solubilities in the respective solutions. For SYR in aqueous buffer–ethanol solutions, we obtained the following relationship: $(\delta \ln(S/S_0)/\delta[I] = 0.22 \pm 10\%)$. Assuming that the values of the sum $\ln(\gamma_{ES}^0/\gamma_{ES}) + \ln(\gamma_E/\gamma_E^0)$ are constant or vary linearly with the solvent concentration, we obtain the following simplified equation:

$$\ln\left(k_s/k_s^0\right) = \ln c + b^*([I]) + n\ln\left(a_w\right), \quad (3)$$

where $b^*([I])$ replaces the sum: $\ln(\gamma_s/\gamma_s^0) + \ln(\gamma_{ES}^0/\gamma_{ES}) + \ln(\gamma_E/\gamma_E^0)$.

The analysis of the data obtained in the region 3 by van Erp et al. [11] leads to the equation of a very similar form:

$$\ln\left(k_s/k_s^0\right) = \ln(\exp(S) + 1) - n^*\ln(x_w) - b^*[I], \quad (4)$$

where x_w is the molar ratio of water in the system and S is the constant dependent on enzyme hydra-

tion and solvent properties, linearly correlated with $\log P$. Contrary to Eq. (3), in Eq. (4), the terms: $n * \ln(x_w)$ and $b^*[I]$ have negative signs. Water is one of the products of the studied reactions. At very low water concentrations, its content should vary during the process studied, shifting the reaction equilibrium. At moderately low water concentrations in region 3, water seems to behave like a competitive inhibitor of the enzyme.

We calculated the water thermodynamic activity from the NRTL equation [21] and fitted both Eqs. (3) and (4) for three laccases: PRL, CBL and PBL and two substrates: SYR and 2,6-DMOP in buffer–ethanol solutions, with the molar ratio of ethanol varying between 0 and 0.1. In region 1, the parameter S was close to zero. Fitting Eq. (3) was better than fitting Eq. (4), showing the importance of the water activity coefficient. The results of fitting Eq. (3) are given in Table 4.

It may be seen that within the error of the analysis, hydration of PRL and CBL reaches roughly 200 water molecules per one enzyme molecule and 260 water molecules per one PBL molecule, which may be compared to the crystallographic hydration number of *C. coprineus* laccase [18], equal to 297. A similar order of laccases hydration was also obtained for PRL–SYR in methanol ($n = 188 \pm 50\%$), PRL–SYR in propane ($185 \pm 5\%$), and PRL–2,6-DMOP in acetonitrile ($217 \pm 2\%$). The data on 2,6-DMOP oxidation in the presence of acetone gave a lower value $n = 62 \pm 4\%$. Eq. (3) did not fit into the data obtained for reactions carried out in the presence of DMSO. It is worth noting that the second and third term in r.h.s of Eq. (2) vary in opposite directions with solvent concentration and compensate each other to a great extent. Almost zero values of the parameter c indicate the high hydration of the enzyme. Both the values of c and b change little within the studied systems. The values of b and n are interrelated, especially at very low solvent concentrations, which confirms that Eqs. (3) and (4) oversimplify the reality.

Table 4
Fitting Eq. (3) for various laccases inhibition by ethanol

Enzyme + substrate	n	b [1/M]	c
PRL + SYR [14]	$231 \pm 22\%$	$3.61 \pm 23\%$	$0.916 \pm 7\%$
PRL + 2,6-DMOP [14]	$175 \pm 17\%$	$2.67 \pm 18\%$	$0.951 \pm 4\%$
CBL + SYR [16]	$207 \pm 20\%$	$3.21 \pm 21\%$	$0.933 \pm 5.5\%$
CBL + 2,6-DMOP [16]	$191 \pm 18\%$	$2.94 \pm 18\%$	$0.943 \pm 4.6\%$
PBL + SYR [16]	$255 \pm 24\%$	$4.00 \pm 26\%$	$0.896 \pm 9\%$
PBL + 2,6-DMOP [16]	$273 \pm 26\%$	$4.28 \pm 28\%$	$0.879 \pm 10\%$

4. Conclusions

We have studied the effect of several water miscible organic solvents on activity of several fungal laccases under non-denaturating conditions. The studies were planned so as to compare various factors determining this effect, both theoretically and directly from the experimental data. It was found that:

(i) the studied enzymes are inhibited by the studied solvents without significant enzyme denaturation, within the chosen range of [I],
(ii) in this range of [I], all the studied solvents may be formally treated as the weak competitive or mixed inhibitors of laccases,
(iii) the inhibitory effects are higher for SYR than for 2,6-DMOP,
(iv) under the chosen experimental conditions, the variation in apparent K_m values is predominant,
(v) the primary factor determining the inhibition by the studied solvents is their hydrophobicity,
(vi) a very good correlation between $1/K_I$ values and the Reichardt–Dimroth constant is also observed for most of the solvents,
(vii) the inhibition constant K_I correlates quite reasonably with denaturating ability of the solvents, given by van Erp et al. [11], which might help in predicting the denaturating ability of various organic solvents.

To relate all these findings with the variations in the state of the reactants (enzyme and substrate), we correlated the obtained inhibition constants with the water thermodynamic activity in the systems, using the simple model of Lee and Kim [20]. One of three adjustable parameters of this model is the number of water molecules hydrating one molecule of the catalytically active enzyme. Degree of hydration of laccases resulting from this model is of the order 200–

260 water molecules per one enzyme molecule, which seems to be a quite reasonable value.

One of the investigated solvents, DMSO, turned out to be a relatively strong inhibitor of laccases. The effect is partly caused by variations of the effective pH of the mixed solutions.

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