Journal of Molecular Catalysis B: Enzymatic 72 (2011) 122–129

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Oxidation of lignans and lignin model compounds by laccase in aqueous solvent systems

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ARTICLE INFO

Article history:
Received 16 March 2011
Received in revised form 23 May 2011
Accepted 25 May 2011
Available online 1 June 2011

Keywords:
Melanocarpus albomyces
Laccase
Solvent
Reactivity
Lignin
Lignans
Dehydrogenation polymers

ABSTRACT

The stability and activity of the low redox potential *Melanocarpus albomyces* laccase (MaL) in various aqueous organic (acetone, ethanol, propylene glycol, diethylene glycol monomethyl ether) solvent systems was studied spectrophotometrically using 2,6-dimethoxyphenol (2,6-DMP) as substrate. In addition, reactivity of the enzyme with two lignans; matairesinol (MR) and 7-hydroxymatairesinol (HMR), was examined by oxygen consumption measurements in the most potential aqueous organic solvent systems. Polymerization of the lignans by MaL was verified by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and size exclusion chromatography (SEC). Polymerization of the higher molecular weight lignin model compound, dehydrogenation polymers (DHPs), was studied by SEC. The solubilities of industrial softwood and hardwood kraft lignins were evaluated as parameters for investigation of enzymatic modification in aqueous organic solvent systems. The functioning of MaL in different aqueous organic media was excellent. Propylene glycol and diethylene glycol monomethyl ether were better solvents than ethanol or acetone in enzymatic oxidations. Even though they were the best solvents for enzyme oxidation, ethanol and propylene glycol were selected for further tests because of their different physicochemical properties. The results obtained in this study for the use of laccase-catalysed reactions in organic solvents to improve the efficiency of lignin oxidation may be exploited in several applications and areas in which the solubility of the reactants or products is a limiting factor.

1. Introduction

Lignin is regarded as a by-product from the pulp and paper industries and is still today mainly burned and exploited as an energy source. Currently, only a low number of applications producing value-added polymers and materials from lignin exist. Hence, various biorefinery concepts propose novel uses for this remarkably aromatic biopolymer, and several studies on its catalytic modification have recently been published [1–3]. Oxidoreductases, such as laccases in particular, are promising for enzymatic modifications of lignin, as currently they are the only lignin activating enzymes that are produced in industrial scale [4,5]. Laccases are polyphenol oxidases which are able to oxidize a wide array of substrates *via* catalysing the single electron oxidation of phenols and amines to reactive radicals, which thereafter undergo further chemical reactions [6].

Degradation of industrial lignins, either by enzymatic or chemical means, is still an extremely challenging task due mainly to the heterogeneity, sulphur content, strong smell and dark colour of the existing lignins [7,8]. In addition, lignins as well as several lignans [9] are not soluble in buffered aqueous solutions, a characteristic that restricts their exploitation in laccase catalysed reactions [10]. It is unlikely that significant enzymatic transformations of the bulky, solid lignin substrate could be effected in aqueous media [11] and therefore several attempts have been made to carry out and analyse enzymatic transformations in organic aqueous solvent systems [12–14]. However, laccase-catalysed oxidative radical reactions in aqueous organic solvent mixtures are still not well understood.

Abbreviations: MaL, *Melanocarpus albomyces* laccase; 2,6-DMP, 2,6-dimethoxyphenol; MR, matairesinol; HMR, 7-hydroxymatairesinol; MALDI-TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; SEC, size exclusion chromatography; DHPs, dehydrogenation polymers; GC, gas chromatographic; HRP, horseradish peroxidize; THF, tetrahydrofuran; HPLC, high-performance liquid chromatography; ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid; NaN_3, sodium azide; ACN, acetonitrile; ThL, *Trametes hirsuta* laccase; TMP, thermomechanical pulp.

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doi:10.1016/j.molcatb.2011.05.009

Table 1
Physicochemical properties of organic solvents used in the enzymatic reactions.

Solvent	Property				
	Log *P*	Acidity (pK_a)	Density ($g\,cm^{-3}$)	Viscosity (cPa)	Boiling point (°C)
Acetone	−0.23	24.2	0.793	0.3075	57
Ethanol	−0.24	15.9	0.789	1.200	78
Diethylene glycol monomethyl ether	−0.53	14.36	1.021	3.9	190
Propylene glycol	−0.92	–	1.036	2.9	188

Organic water-miscible solvents have a great influence on the catalytic activity of enzymes [15,16], and the activity is strongly dependent on the amount of water available to maintain the active conformation of the enzyme [17]. By selection of the appropriate solvent and the amount of the catalyst, enzymatic reactions can be optimized and yields may be shifted in the desired direction, as exemplified by lipase- and esterase-catalysed reactions [18–20]. In these hydrolytic reactions polar solvents have shown negative effects due fact that the solvents inactivated the enzyme, whereas in the case of oxidative reactions involving hydrophobic substrates, polar solvents have had a positive impact [21,22]. Suitable solvent systems must be tested separately based on the substrate and the mode of action of the enzyme.

In aqueous buffered solvents, precipitation of the laccase-polymerized product has been found to be the limiting step of the oxidative polymerization [23]. Hence, in order to efficiently catalyse enzymatic oxidations, the substrate as well as preferably also the oxidized reaction products should remain dissolved in the selected solvent system. In this study, the stability and activity of MaL in different aqueous mixtures of acetone, ethanol, propylene glycol and diethylene glycol monomethyl ether were studied using 2,6-DMP as substrate. In addition, lignin-type molecules such as the lignans MR and HMR, as well as the higher molecular weight DHP were used as substrates. These molecules have been shown to be useful model compounds for studying the reaction mechanisms of laccases [23–27]. Enzymatic reactions were monitored by different analytical methods. To increase the industrial relevance of the work, the solubility characteristics of industrially relevant polymeric kraft lignins from softwood and hardwood were also investigated. Thus, a generic approach to exploit enzymes in aqueous organic media for the modification and degradation of poorly soluble, high molecular weight substrates was adopted.

2. Experimental

2.1. Chemicals

Organic solvents (acetone, ethanol, propylene glycol, diethylene glycol monomethyl ether) and all other chemicals used in this study were of analytical quality. The physico-chemical properties of the solvents are shown in Table 1.

2.2. Lignans

HMR ($C_{20}H_{22}O_7$, molecular weight: 374.39 g mol^{-1}, exact mass: 375.149038 Da, CAS number: 20268-71-7) was isolated from a Norway spruce knotwood hydrophilic extract by precipitating with potassium acetate, according to a modification of a previously described method [28]. The purity of the white crystalline product was 99% according to gas chromatographic (GC) analysis. MR ($C_{20}H_{22}O_6$, molecular weight: 358.39 g mol^{-1}, exact mass: 358.141638 Da, CAS number: 580723) was prepared from HMR by reduction with $NaBH_4$, and purified by column chromatography with silica. The purity of the white crystalline product was 93%, according to GC analysis. Molecular structures of the lignans are shown in Fig. 1.

2.3. Dehydrogenation polymer

DHPs (molecular weight: ca. 3000 g mol^{-1}) of coniferyl alcohol were prepared according to methods described by [29–31]. Coniferyl alcohol was prepared according to the method described by [32] and used as a substrate for polymerization by horseradish peroxidase (HRP) obtained from Serva (Heidelberg, Germany). The reaction mixture was incubated for 24 h with constant stirring and continuous addition of hydrogen peroxide (Sigma–Aldrich, Seelze, Germany). For the molecular weight determination the acetylated DHPs (2 mg) was dissolved in 1 mL tetrahydrofuran (THF) and filtered through a 0.45 μm Acrodisc® GHP Membrane high-performance liquid chromatography (HPLC) filter (Waters, Milford, MA, USA). An Agilent 1100 HPLC system equipped with an Agilent 1050 diode array detector operating at 280 nm and an autosampler (Santa Clara, CA, USA), and two Styragel columns (HR5E and HR1) from Waters were used for the analyses. Injection volume was 30 μL and flow rate of THF eluent was 0.5 mL min^{-1}. The temperature was 23 °C. Calibration of the columns was performed using polystyrene standards (Mw: 434, 177, 42, 9 and 1.24 kg mol^{-1}) from Polymer Standards Service – USA Inc.

2.4. Lignins

The industrial kraft lignins, Indulin AT from soft wood and PC-1369 from hard wood, were obtained from MeadWestvaco (USA). The selected lignins represent relevant lignins with potential industrial interest in enzymatic modification.

2.5. Preparation of aqueous organic solvent mixtures

The presence of ethanol and propylene glycol in varying concentrations of 10–70% (V:V) in 25 mM Na–citrate buffer affected the pH, especially at the lower pH range (<4.5) but less at pH 5.0. For the activity and stability measurements of the MaL enzyme, as well as for reactions with lignin and lignin model compounds, the pH of the solvent mixtures was controlled by use of the buffer, resulting in final pH of 5.5.

2.6. Solubility of lignins and lignans

Solubilities of the selected hardwood and softwood kraft lignins in 25–100% (V:V) ethanol and propylene glycol were determined according to the standard procedure of the International Lignin Institute (ILI, Italy) [33]. The solubilities of MR and HMR in 20%, 30%, 40%, and 50% (V:V) ethanol at concentrations of 1, 5, or 10 mg mL^{-1} were determined by filtering an aliquot of the solution through a syringe filter with 0.45 μm nylon membrane after 1 h and 2 h of magnetic stirring at room temperature. A known amount of cholesterol was added as an internal standard for quantitative GC determinations. The solubility was calculated as the amount of the lignans determined by GC of the total theoretical amount of the lig-

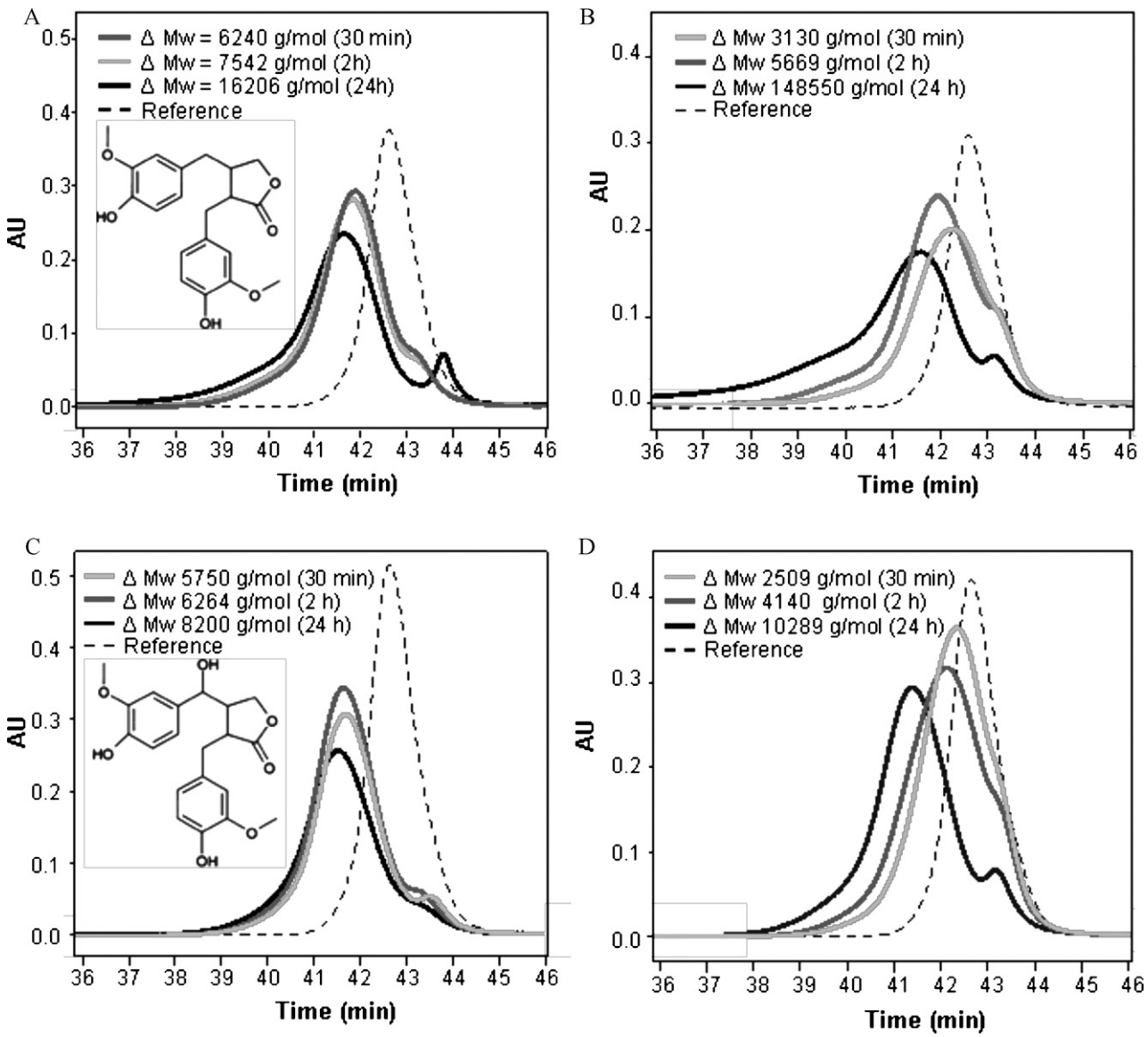

Fig. 1. Polymerization of lignans by MaL in different solvent systems as analysed by SEC. **A**. MR solubilized in 20% ethanol and **B**. in 50% propylene glycol. **C**. HMR solubilized in 20% ethanol and **D**. in 50% propylene glycol. Chromatograms of the untreated lignans are shown with black dotted lines and chromatograms of the MaL-treated lignans are shown with black to grey solid lines after different incubation times. ΔMw values show the increase of the molecular weight after MaL treatment. Molecular structures of MR and HMR are shown as inserts in **A** and **C**, respectively.

nans in the sample. The solubility of MR and HMR lignans in 50% (V:V) propylene glycol was evaluated by weighing.

2.7. Activity and stability of laccase

The recombinant low redox potential MaL was expressed in *Trichoderma reesei* and purified as described by [34]. Enzyme activity was determined using 2,2′-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid (ABTS) as substrate in 25 mM Na–succinate buffer at pH 5 and room temperature [35]. The oxidation was monitored at 436 nm (ε = 29,300 M^{-1} cm^{-1}) using a Perkin Elmer Lambda 45 spectrophotometer (Waltham, MA, USA). The activity of MaL was also determined using 1 mM 2,6-DMP as a substrate in 25 mM Na–citrate buffer at pH 5.5 at 22 °C. The oxidation of 2,6-DMP was monitored with a Shimadzu UVmini-1240 spectrophotometer (Kyoto, Japan) at 476 nm (ε = 11,300 M^{-1} cm^{-1}).

The effect of the organic solvent, *i.e.* 10–70% (V:V) of acetone, ethanol, propylene glycol or diethylene glycol monomethyl ether, on the activity of MaL was analysed by adding the laccase to a mixture of the organic solvent and 25 mM Na–citrate buffer and measuring the activity against 2,6-DMP as described above. In addition, the stability of MaL in the above solvent systems was investigated by incubating the enzyme at 25 °C for 1, 2 and 20 h. After incubations the residual activity of the enzyme was determined against 2,6-DMP as described above.

2.8. Oxidation of substrates by laccase

2.8.1. Oxygen consumption in laccase catalysis

Oxidation of 0.4 mM MR and HMR, solubilized in 25–70% ethanol and 25–70% propylene glycol in 40 mM Na–citrate buffer pH 5.0, by MaL was analysed by monitoring the consumption of dissolved oxygen during the enzymatic reaction. Before addition of the enzyme, the substrate solutions were stabilized at room temperature. After initiation of the reaction by addition of MaL, oxygen consumption was followed by an oxygen electrode FIBOX 3 fiber-optic oxygen meter (PreSens, Regensburg, Germany). The measurements were carried out under constant mixing in tightly sealed flasks in order

to avoid entry of oxygen into the reaction mixture during the measurement.

2.8.2. Oxidation of lignans

The polymerization of 2 mM MR and 3 mM HMR by MaL (in 20–70% ethanol and 25–70% propylene glycol) was studied by MALDI-TOF MS and SEC. The overnight enzyme incubations were performed in the presence of varying amounts of organic solvents in 40 mM Na–citrate buffer at pH 5. The enzyme dosage was 70 nkat μmol^{-1} (per reactive group). The enzymatic reactions were terminated by addition of 0.05% (V:V) NaN_3 prior to freeze drying of the samples. Dried samples were solubilized in 50 mM NaOH in 1 mg mL^{-1} concentration for chromatographic analyses. The oxidation product of HMR from the 50% propylene glycol solvent mixture was mixed with saturated α-cyano-4-hydroxycinnamic acid matrix dissolved in 50% acetonitrile (ACN) containing 0.1% TFA 1:2 (V:V) prior to analysis. The MR precipitate from the 20% ethanol solvent mixture was solubilized in 100% ethanol before mixing with the matrix solution.

2.8.3. Oxidation of DHPs by laccase

The oxidation of DHPs by MaL was performed in 20 mL test tubes at room temperature. The reaction volume was 1 mL containing 10 mg of DHP solubilized in 25, 50 or 70% organic solvent (ethanol or propylene glycol) in 25 mM Na–citrate buffer at pH 5.5. Two enzyme dosages (1 and 10 nkat mL^{-1}) were used and the effect of organic solvent on the enzyme activity was taken into account. However, the effect of the organic solvent on the stability of the enzyme was negligible. The reactions were stirred for 24 h and terminated by addition of 1 mL of 5 mg mL^{-1} NaN_3 solution under vigorous stirring. After addition of 3 mL Milli-Q water the reaction mixtures were transferred into tubes and centrifuged for 1 h at 2500 rpm. Finally, the solids were separated and washed twice with 3 mL of Milli-Q water for molecular weight determination of the polymerized product.

2.9. Analytical methods

2.9.1. Size exclusion chromatography

The polymerization products of MR, HMR and DPH by MaL were studied by Waters (Milford, MA, USA) SEC, in 2000, 250 and 120 Å μHydrogel (Milford, MA, USA) columns using the alkaline elution method, as described by [27].

2.9.2. Mass spectrometry

MALDI-TOF mass spectra of MaL-treated lignans were analysed after 24 h incubation with a Bruker Autoflex II instrument equipped with an N_2-laser (337 nm, 100 μJ) (Bremen, Germany). For the MALDI-TOF MS measurement, 1 μL of the lignan sample mixtures containing the matrix for the ionization was pipetted onto the stainless steel MALDI target plate and dried at room temperature. The positive ion mass spectra were measured from the dried sample spots in a reflector mode. The number of scans was typically 2000. Peptide standard solution purchased from Bruker (Bremen, Germany) was used for the molecular mass calibration of the spectrometer.

3. Results and discussion

3.1. Solubilization of lignins and lignans

The most hydrophilic solvent propylene glycol (Table 1) showed excellent solubilization characteristics for industrial softwood and hardwood kraft lignins in contrast to ethanol, as shown in Table 5. When 100% ethanol was used, only a moderate solubilization of technical lignins (ca. 50%) was obtained.

Table 2
Relative rate of oxidation of 2,6-DMP by MaL in aqueous organic media is given in percentages. Values were calculated relative to the activity determined without the solvent in 25 mM Na–citrate buffer at pH 5.5.

Solvent content (%)	Acetone	Ethanol	Diethylene glycol monomethyl ether	Propylene glycol
0	100	100	100	100
20	53	93	122	92
30	29	78	105	73
40	14	ND	ND	ND
50	10	38	43	58
60	ND	24	21	45
70	ND	ND	10	37

ND = not determined.

HMR was soluble in water at a concentration of ≤6.4 g L^{-1}, whereas MR was insoluble in water. However, MR was rather easily solubilized in the presence of small amounts of ethanol: in 20% ethanol (<1 g L^{-1}), in 30% ethanol (1 g L^{-1}), and in 40% and 50% ethanol (10 g L^{-1}) after 1 h incubation under magnetic stirring at room temperature. HMR was soluble in 20% ethanol (10 g L^{-1}). In propylene glycol the solubility of both lignans was higher.

3.2. Initial laccase activity and stability in aqueous organic solvents

The laccase used was a low redox potential MaL expressed in *T. reesei*. It was expected that the use of organic solvents would affect the oxidation of phenolic substrates by MaL in different ways. Thus, first the tolerance of MaL against organic solvents was studied using a low molecular weight water-soluble phenolic compound, *i.e.* 2,6-DMP as substrate. The initial oxidation rate of MaL in the presence of the four selected solvents, acetone, ethanol, diethylene glycol monomethyl ether and propylene glycol was examined spectrophotometrically. The oxidation of 2,6-DMP by laccase results in the formation of a dimeric quinone structure which shows strong absorptivity at 476 nm. Hence, this method is commonly used as a quantitative laccase assay to monitor enzymatic reactions. As postulated, the increasing content of organic solvent in the reaction mixture diminished the initial activity of the MaL when compared to the oxidation rate in 25 mM Na–citrate buffer (Table 2). The measurements of the initial activity indicated highest oxidation rates in the presence of glycol-type solvents, *i.e.* diethylene glycol monomethyl ether and propylene glycol. The tolerance against ethanol was only moderate. In the case of acetone, relatively poor oxidation of 2,6-DMP was observed.

Organic solvents affect strongly the protein conformation [17] and only a small change in the three dimensional structure of a protein may cause loss of enzyme activity [15,16]. However, in the presence of low concentrations of glycol-type solvents (20% and 30% diethylene glycol monomethyl ether), the substrate oxidation activity of MaL was even slightly increased (Table 2). At high solvent concentrations (>40%) the enzyme activity was clearly decreased and practically lost in the case of acetone. In oxidative enzyme systems, the properties of the substrate and its partitioning between the active site of the enzyme and the solvent has been assumed to be the main factor determining the biocatalytic behaviour of enzymes in organic solvent mixtures [15,36–38]. A similar explanation is assumed to be valid in the case of MaL-catalysed oxidation of 2,6-DMP, as the active site of the laccase is in the middle of the flexible loops, forming a substrate-binding pocket where the T1 copper atom responsible for receiving electrons from the substrate is located [39,40].

Results of the initial laccase activity (Table 2), determined in the presence of acetone, ethanol, diethylene glycol monomethyl ether and propylene glycol, indicated that the tolerance of MaL was high-

Table 3
Initial rate of oxidation of lignans by MaL in aqueous organic solvents as analysed by oxygen consumption measurement.

Substrate	Initial rate ($\mu g\,L^{-1}\,s^{-1}$)					
	Ethanol			Propylene glycol		
	25%	50%	70%	25%	50%	70%
MR	35	3	0	60	15	0
HMR	10	<1	0	25	3	0

est for propylene glycol, followed by ethanol and diethylene glycol monomethyl ether. The listed physico-chemical characteristics of the solvents (Table 1) did not directly correlate with the loss of MaL activity. Thus, based on these results, further experiments were performed using only two organic solvents, propylene glycol and ethanol in the reaction mixtures. As shown in Table 1 both solvents are rather hydrophilic, as the partition coefficients (Log P) are below zero, showing that they are completely water miscible.

To further test the effect of ethanol and propylene glycol on oxidation of the phenolics by MaL, oxygen consumption measurements were performed using MR and HMR lignans as model compounds. The initial rates of lignan oxidation by MaL are shown in Table 3. Although both lignans can be oxidized by MaL, the results clearly show that the initial rate is negligible in the presence of higher concentrations of ethanol (>50%) and propylene glycol (70%). On the other hand, the MaL retains most of its activity even in the highest propylene glycol concentration, whereas in the presence of ethanol the stability is strongly decreased when the concentration of the solvent is >50% (Table 4). It is notable that in both cases of the low organic solvent concentrations, the stability of MaL was fully retained in water-based buffer solution. Low concentrations of ethanol appeared actually to increase the MaL activity. When MaL was incubated in 25 mM Na–citrate buffer alone, some of its oxidation efficiency was lost already after 1 h incubation, although prolonged incubation at 25 °C did not further considerably affect the enzyme activity (data not shown).

On the basis of the three dimensional structure of MaL [39,40], the active site is surrounded by several flexible loops and hence the area interacting with the substrate is relatively large. Interestingly, the effects of propylene glycol and ethanol were quite distinct in the oxidative reactions of MaL, even though both solvents have similar hydrophilic properties. Propylene glycol presumably affected less the conformation of the enzyme, and MaL remained stable even during prolonged incubation, whereas ethanol slowly inactivated the enzyme. The effect of pH change in the aqueous solvent system was ruled out and hence the loss of MaL activity in the tests was due to loss of conformation of the enzyme.

3.3. Polymerization of lignans and DHPs by laccase

The MaL could polymerize both lignans efficiently in the presence of 25–70% ethanol and propylene glycol, although the type of lignan used clearly affected the molecular weight obtained, as shown in Table 6. The increase in the molecular weight of enzymatically polymerized MR was much more significant than that

Table 4
Relative stability of MaL in aqueous ethanol and propylene glycol mixtures in 25 mM Na–citrate buffer, pH 5.5 after 24 h incubations was determined by performing the 2,6-DMP assay.

Enzyme	Relative stability						
	Buffer	Ethanol			Propylene glycol		
		25%	50%	70%	25%	50%	70%
MaL	100	101	50	27	97	86	76

Table 5
Solubility of industrial kraft lignins in aqueous organic media.

Solvent content (%)	Solubility of kraft lignins (%)			
	Propylene glycol		Ethanol	
	Softwood	Hardwood	Softwood	Hardwood
100	95	80	49	55
75	99	99	92	97
50	40	95	78	98
25	18	54	28	71

of HMR in both organic solvents. This result is in accordance with the estimates of the initial oxidation rates of these model compounds (Table 3). Thus, it was assumed that the third hydroxyl group present in the carbon 7 in HMR forms an electron withdrawal couple that hinders the oxidation of HMR. However, polymerization of both lignans by MaL was obtained even in the highest organic solvent concentrations, in which the initial enzyme activity was low. Increasing the amount of ethanol in the polymerization reaction did not increase the degree of polymerization of the end product of either lignan. Use of propylene glycol, however, altered the polymerization reactions of MR, and the molecular weight was doubled when compared to the molecular weight obtained in the presence of ethanol. On the other hand, HMR in propylene glycol was polymerized to the same low degree as in ethanol. Reaction in 70% propylene glycol resulted in decreased polymerization, and a low molecular weight fraction could still be detected after 24 h incubation (data not shown).

In general, the lignan polymerization reactions catalysed by MaL in 20% ethanol and 50% propylene glycol were rapid. The molecular weight of the oxidized product increased notably after 30 min incubation as evidenced by the SEC profiles shown in Fig. 1. The polymerizations were especially rapid in reactions in which 20% ethanol was used (Fig. 1A and C). A prolonged reaction time yielded a higher molecular weight, although the reaction rate levelled off significantly. The molecular weight in the presence of 50% propylene glycol after 24 h incubation was clearly the highest (Fig. 1B and D). The molecular weight of MR by MaL in 50% propylene glycol was almost 15-fold higher than that of HMR.

MALDI-TOF MS analyses of the MaL-treated HMR in 20% ethanol and in 50% propylene glycol were performed for structural elucidation of the polymerized reaction products after 24 h incubations. The mass spectra of the selected mass range are presented in Fig. 2A and C and the magnification of the trimers in 3 B and D. The detected and predicted masses as well as their differences are listed in Table 7. In both solvents, the polymerization of HMR by MaL was clear and oligomers up to 6 units could be detected from the mass spectra. The polymerization was most probably due to elimination of two hydrogen atoms according to the equation: $[n\mathrm{Mw} - (n-1)2\mathrm{H} + \mathrm{Na}]^+$, where n is the number of monomers and Mw is the exact mass of HMR. Furthermore, on the basis of the detected m/z, HMR oligomers were ionized as sodium salts.

Table 6
Effect of organic solvent on the polymerization of lignans by MaL. ΔMw represents molecular weight increase after polymerization.

	MR (ΔMw)	HMR (ΔMw)
Ethanol (%)		
25	12,600	7400
50	12,300	9200
70	11,200	6900
Propylene glycol (%)		
25	28,500	7700
50	39,200	9100
70	19,900	10,100

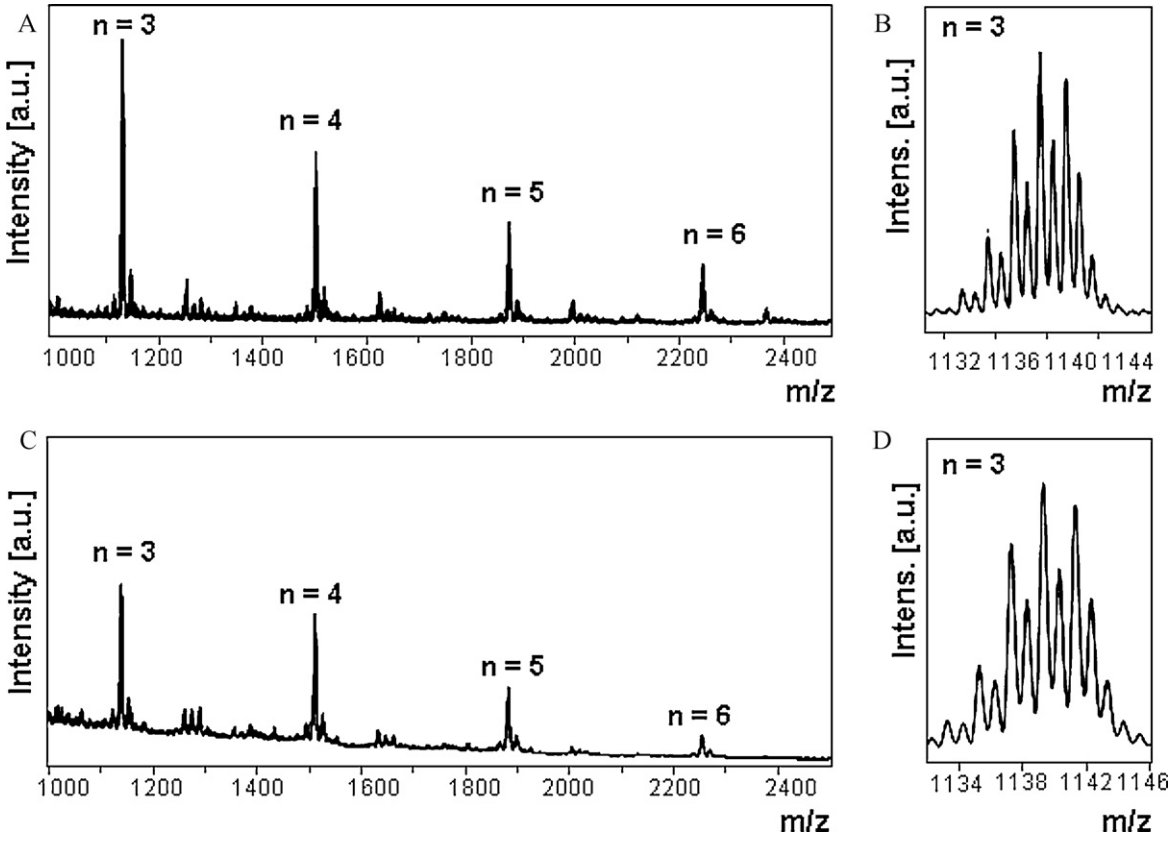

Fig. 2. MALDI-TOF MS spectra of MaL-polymerized HMR in the presence of **A**. 50% propylene glycol and **C**. 20% ethanol. Enlargements of the pattern of trimeric HMR ($n = 3$) are shown in the inserts **B**. and **D**.

The measured m/z values of the oligomerization products corresponded to the predicted values within the error of the method (<1 Da). However, below these masses several very intense peaks separated by one mass unit could be detected from the spectra, suggesting the formation of covalent bonds in the polymerized product *via* elimination of hydrogen atoms. It is also possible that dehydropolymers were formed due to laser irradiation in the MALDI-TOF MS experiment [41]. Hence, detailed structural characterization of the oligomers was difficult. In addition to the main series of HMR oligomers, small series of peaks having equal spacing could be detected from the spectra, suggesting the presence of unknown small molecule weight breakdown products in the reaction mixture. It is possible that these molecules were involved in the enzymatic polymerization reactions [27]. Similar oligomerization products were formed in the enzymatic polymerization reactions performed in 20% ethanol and 50% propylene glycol mixtures.

The molecular weight of the DHPs polymerized by MaL increased with the increasing, 25–70% concentration of ethanol and propylene glycol in the reaction mixture (Table 8). In these experiments the MaL activity was kept constant by dosing the laccase taking into account the enzyme activity in the selected organic solvents (Tables 2 and 4). The polymerization of DHPs by MaL, as analysed by SEC, is shown in Fig. 3. The SEC profiles clearly show the marked differences between the polymerization reactions performed in ethanol and propylene glycol. The highest molecular weight was obtained in the highest solvent concentration of both organic solvents. In the presence of 50–70% solvent, polymerization reactions of especially the larger macromolecules of DHPs appeared to occur, as their proportional amount was reduced, whereas polymers having Mw over 10 kg mol^{-1} increased. The oligomeric DHPs fraction was oxidized efficiently by MaL in lower solvent concentrations. Thus it could be postulated that radicals formed in the enzyme catalysis could further radicalize the substrates present in the reaction mixture, and the major determinants for the second-stage radical action would be determined by the physico-chemical characteristics of the solvents. The viscous propylene glycol (Table 1) allows only slow radical diffusion

Table 7
Assignment of masses of the HMR oligomers detected from aqueous organic solvents (the corresponding MALDI-TOF MS spectra are shown in Fig. 3). The differences ($\Delta m/z$) between detected and predicted masses are shown in parentheses after the detected masses.

Oligomer	Predicted m/z (Da)	Detected m/z in 20% ethanol (Da)	Detected m/z in 50% propylene glycol (Da)
$[(HMR)_3]\,Na^+$	1143.45	1143.28 (−0.17)	1143.43 (−0.02)
$[(HMR)_4]\,Na^+$	1516.60	1516.14 (−0.46)	1516.80 (0.2)
$[(HMR)_5]\,Na^+$	1889.75	1889.02 (−0.73)	1889.05 (0.7)
$[(HMR)_6]\,Na^+$	2262.89	2261.90 (0.99)	2262.26 (−0.63)

Table 8
Effect of MaL dosage on the polymerization of DHP as analysed by SEC. ΔMw represents molecular weight increase after 24 h incubation.

	Enzyme dosage	
	1 nkat mg^{-1} (ΔMw)	10 nkat mg^{-1} (ΔMw)
Ethanol (%)		
25	600	1300
50	1800	8400
70	100	17,100
Propylene glycol (%)		
25	800	1700
50	1000	3400
70	500	4900

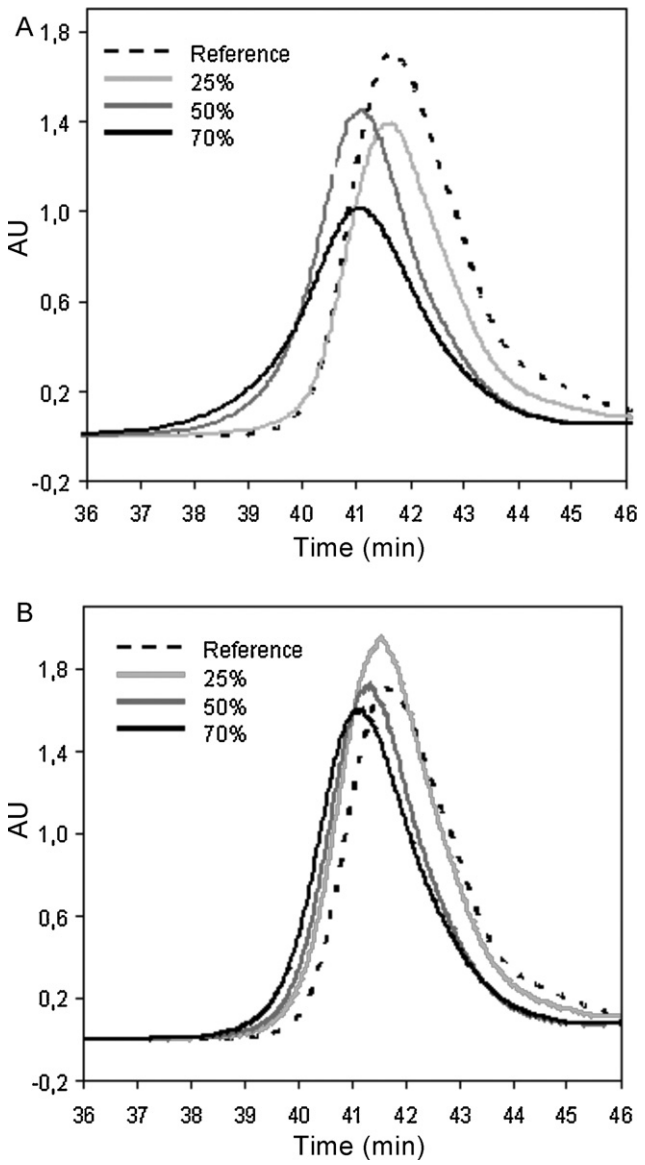

Fig. 3. Polymerization of DHPs by MaL in different solvent systems as analysed by SEC. Solvent reaction mixtures of **A**. ethanol and **B**. propylene glycol.

compared to ethanol, therefore resulting in high molecular weight polymer in prolonged incubations. Based on these results the possibility cannot be ruled out that the substrate molecules may undergo radical intermediate steps or that the stability of intermediates may vary in different solvent systems. It has been shown that reactions may be facilitated in the presence of ethanol in comparison with propylene glycol [36]. Interestingly also observed that ethanol solution supported guaiacol oxidation by non-enzymatic Mn^{3+}, the secondary oxidant in manganese peroxidase-catalysed reactions [37].

The reaction rate of MaL-catalysed oxidation in the presence of organic solvents appears to be controlled mainly by the substrate availability. This is greatly influenced by the substrate solubility characteristics as well as by the solubility of the oxidized reaction product facilitating further enzymatic radical formation, radical coupling and polymerization of the substrate and therefore resulting also in the detection of an increased level of enzyme activity. The results presented in this paper indicate that although the substrate concentration as such influences the rate of laccase catalysis [42], the stability of the enzyme is also an important factor. The polymerization of lignans, when performed in 20% ethanol, resulted in increased reaction rate, but eventually rather low molecular weight products due to loss of enzyme reactivity (Table 6). In 50% propylene glycol the polymerized reaction products remained dissolved, enabling further oxidation by MaL which showed better stability in that solvent system. This resulted in the formation of covalent linkages between the lignans *via* coupling of the radicals, and finally in clearly larger molecular weight reaction products than in ethanol. In addition, the physico-chemical characteristics of the solvent influence the rate of polymerization as the stability of the conformation of the active site of a particular laccase contributes to the final rate of oxidation of phenolic compounds [43].

3.4. Effect of laccase dosage on polymerization efficiency

The dosage of laccase significantly affected the efficiency of the polymerization of DHPs (Table 8). The ability of MaL to oxidize phenolic DHPs was significantly improved when the enzyme activity was increased ten-fold. Interestingly, at low enzyme dosage in 25–70% ethanol and propylene glycol, the degree of polymerization was only little affected by increased concentration of the organic solvent, as also observed by [42]. Thus, it was assumed as observed by [44] that at low laccase dosages demethoxylation of lignan may occur without polymerization. However, no clear evidence for DHPs depolymerization was observed when low laccase dosage was used. At ten-fold higher activity dosage, the molecular weight of the reaction product was significantly increased as a function of organic solvent concentration in both solvents. The highest increase in molecular weight was obtained when 70% ethanol was used. Thus, it was assumed that the higher amount of MaL increased the amount of radicals, as has been previously shown with *Trametes hirsuta* laccase (ThL)-catalysed oxidation reactions of thermomechanical pulp (TMP) [45]. A high amount of radicals in the presence of organic solvent may result in rapid and efficient coupling reactions. Increased concentration of radicals may even have an impact on the course of the reaction route, as has been postulated by [46].

4. Conclusions

It was shown that the reactivity and stability of MaL in various aqueous organic solvent systems was excellent regarding the exploitation of the enzyme in oxidation of selected lignin model compounds. Propylene glycol and diethylene glycol monomethyl ether were better solvents for enzymatic oxidations than ethanol or acetone, as these solvents appeared to have less effect on the activity of MaL. However, even in the presence of solvent concentrations strongly inhibiting the enzyme activity, polymerization reactions still took place, encouraging further studies on laccase reactions in organic solvent systems. The results obtained in this study for the use of laccase-catalysed reactions in organic solvents to improve the efficiency of lignin oxidation may be exploited in several applications and areas where the solubility of the reactants or products is limited. The results also open up possibilities for modification and valorization of technical lignins.

Acknowledgements

The study was part of the LigniVal project with the BioRefine program funded by the Finnish Funding Agency for Technology and Innovation. Funding from Metso Power Oy, Oy Metsä-Botnia Ab, Stora Enso Oyj and Roal Oy is also acknowledged. Financial support was also obtained from the Academy of Finland (research grant no: 1127961). Päivi Matikainen and Tapani Suortti (VTT, Espoo, Finland) and Saskia Araújo (University of Helsinki, Helsinki, Finland) are thanked for skilful technical assistance.

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