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On the reactivity of the *Melanocarpus albomyces* laccase and formation of coniferyl alcohol dehydropolymer (DHP) in the presence of ionic liquid 1-allyl-3-methylimidazolium chloride

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ABSTRACT

Some ionic liquids are able to dissolve wood, including lignin and lignocellulose, and thus they provide an efficient reaction media for modification of globally abundant wood-based polymers. Lignin can be modified with laccases (EC 1.10.3.2), multicopper oxidases, which selectively catalyze the oxidation of phenolic hydroxyl to the phenoxy radical in lignin by using oxygen as the co-substrate and an electron acceptor. Many enzymes, including laccases, retain their catalytic activity in the presence of ionic liquids. However, the enzyme activity is usually decreased in the presence of ionic liquids, and the most deactivating ionic liquids have been observed to be those dissolving wood most efficiently. In the present study the activity, pH optimum and catalyzed oxidation of coniferyl alcohol by the laccase from the ascomycete *Melanocarpus albomyces* was investigated in the ionic liquid 1-allyl-3-methyl-imidazolium chloride ([Amim]Cl), known to dissolve wood and expected to affect the laccase activity. Indeed, with an increasing concentration of [Amim]Cl, the activity of *M. albomyces* laccase decreased, and the pH range of the enzyme activity was narrowed. The pH optimum, using 2,6-dimethoxyphenol as the substrate, was shifted from 6.5 to 6.0 when the amount of [Amim]Cl was increased to 60% (m-%). It was also found that the inhibition of laccase with NaN_3 was not as severe in the ionic liquid as in water. The insoluble fraction of the dehydropolymer (DHP) formed in the presence of [Amim]Cl had clearly higher molecular weight compared to the one formed in water. DHPs formed in the absence and presence of [Amim]Cl both contained β-5, β–β, β-*O*-4, α-C=O/β-*O*-4 and α-*O*-4/β-*O*-4 structures. However, in the presence of [Amim]Cl, less β-*O*-4, slightly less β-5 and more β–β structures were formed.

1. Introduction

Lignin is a heterogenous polymer consisting of phenylpropane units, which are coupled to form an irregular polymer network. Wood contains approximately 30% of lignin, which is, after cellulose, the second most abundant biopolymer [1,2]. Today, lignin is mainly used for production of energy in pulp and paper processes by burning it. Lignin, extracted in the production of pulp (cellulose), is also used as dispersing agents, in concrete, textile dyes, pesticides, batteries and ceramic products or as binding agents in animal foods and briquettes.

For any chemical modification, it is advantageous for the reagents to be soluble in the reaction media, because this increases the probability of molecules to react with each other. Wood and lignocellulose, and their main constituents (cellulose and lignin) are highly insoluble in common solvents because of their large molecular size, the hydrophobic nature of lignin and the crystallinity of cellulose. However, recent studies show that wood and other lignocellulosic material are soluble in some ionic liquids (ILs) and thus they provide potential media for modification of these materials [3–7].

Swatloski et al. reported first the dissolution of cellulose to an imidazolium-based IL, 1-butyl-3-methylimidazolium chloride ([Bmim]Cl) [8]. More recently, 1-allyl-3-methylimidazolium chloride ([Amim]Cl) and 1-ethyl-3-methylimidazolium acetate ([Emim]Ac) have also turned out to be able to dissolve cellulose [7,9,10]. The two most powerful solvents for lignin are 1-butyl-3-methylimidazolium trifluoromethanesulfonate [Bmim]CF_3SO_3 and 1,3-dimethylimidazolium methylsulfate [Mmim]$MeSO_4$, which on the other hand are not able to dissolve wood flour [10,11]. Other good ILs for dissolving lignin are [Amim]Cl and [Emim]Ac, while [Bmim]Cl is less efficient [5,11]. According to comparative analysis

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[12], [Emim]Ac is the most efficient IL for dissolving cellulose and [Amim]Cl for dissolving wood chips.

The dissolution mechanism of wood and its constituents, cellulose and lignin, by ILs is not yet completely understood. Both the cation and the anion, and apparently also their combination, play a role in the dissolution [10,13]. Dissolution of cellulose is proposed to be based on the interruption of inter- and intramolecular hydrogen-bond-network, responsible for crystalline nature of the polymer. Thus, the cations of ILs that are able to dissolve cellulose are mainly imidazolium-based and relatively small (e.g. $[Amim]^+$, $[Emim]^+$ and $[Bmim]^+$, and the anions are small hydrogen-bond acceptors (e.g. Cl^-, AcO^-). However, even this generalization seems to be somewhat inadequate, as a recent article shows that also tetramethylguanidine-based ILs are able to dissolve cellulose efficiently [14]. Generalizations are also difficult in the case of lignin, because all of the existing results are not comparable [10]. It has, however, been shown that the nature of the anion is decisive and large, non-coordinating anions, such as PF_4^- and BF_6^-, are not favorable. The allyl chain in [Amim]Cl could also interact with lignin, since [Amim]Cl is a better solvent for lignin than [Bmim]Cl [10].

Lignin can be selectively modified with oxidative enzymes, such as laccases [15,16]. Laccases (EC 1.10.3.2) are multicopper oxidases that are found from plants, fungi, insects and bacteria [17,18]. In nature, plant laccases take part in lignin biosynthesis and those of the white-rot fungi in lignin biodegradation. Laccases use oxygen as their co-substrate, which is reduced to water during the catalytic cycle [19]. Laccase oxidizes the phenolic group of lignin by removing one electron to form a phenoxy radical, which then reacts further [20]. Based on electron-spin resonance spectrometric studies of laccase-oxidized beech wood fibers, it seems that lignin-derived phenoxy radicals in the solution can introduce radicals further to the matrix lignin in wood fibers [21]. Studies with syringylic β-1 and β-*O*-4 lignin model compounds have revealed that in laccase-catalyzed oxidation, several degradation products are formed as a result of side chain cleavages from various positions, as well as oxidation of the benzylic hydroxyl [22]. Oxidation of benzylic hydroxyl can also take place in laccase-catalyzed oxidation of guaiacylic compounds, which however prefer more oxidative coupling to form biphenylic- and dibenzodioxepin-type products [23–25]. It has been suggested that the differences of product distributions observed in laccase-catalyzed oxidation of lignin model compounds could be affected by the enzyme dosage used and the pH of the reaction media [24,25].

The number of reports concerning reactions catalyzed by different enzymes in ILs is still limited, but based on the knowledge acquired some generalizations can be made [26–28]. Many enzymes, such as lipases, proteases, glycosidases and oxidoreductases retain some of their activity in the presence of ILs, even in very low water contents. It has been observed that the anion of an IL has a significant influence on the enzyme activity. Enzymes are most active in ILs that have large, hydrophobic and non-coordinating anions such as BF_4^- and PF_6^-. The most deactivating ILs contain anions (like Cl^-), which are capable of breaking hydrogen bonds. For example, cellulases from *Trichoderma reesei* are inactive in [Bmim]Cl [29]. On the other hand, these types of anions are required for efficient dissolution of wood components.

The enzyme activities of laccases are usually decreased with increasing IL concentration [30–33]. The decrease of enzyme activity is caused by decreasing maximum reaction velocity (V_{max}) and usually with decreased substrate affinity (increased K_M). When the anion of the IL is of alkylsulfate or alkylsulfonate type, K_M is slightly decreased, but nevertheless the observed enzyme activity is decreased because the presence of the IL affects more to the V_{max} [32]. When the IL cation is imidazolium-based, laccase deactivation increases with the length of the alkyl chain of the ring [33].

The aim of the present study was to investigate reactivity of the laccase from the ascomycete *Melanocarpus albomyces* in the presence of the ionic liquid, 1-allyl-3-methylimidazolium chloride ([Amim]Cl). This IL was expected to affect the laccase activity, because it dissolves wood by breaking the hydrogen-bonding network of cellulose. The *M. albomyces* laccase was chosen for this study, as it is thermostable (optimum temperature 60–70 °C), which is expected to improve the general stability of the enzyme. This enzyme also has rather a broad pH optimum (e.g. pH 5.0–7.5, determined with guaiacol, and pH 6–7 with syringaldazine) [34]. The *M. albomyces* laccase has also been shown to remain active in the presence of various organic solvents [35]. Activity of the enzyme was determined in different pH-values and different [Amim]Cl concentrations by following the product formation of 2,6-dimethoxyphenol. Coniferyl alcohol was oxidized to dehydropolymers (DHPs), and possible structural differences caused by the IL were investigated with GPC and NMR. Formation of the oligomeric coniferyl alcohol oxidation products were followed with HPLC to detect possible changes compared to aqueous solvent.

2. Experimental

2.1. Materials

All commercial reagents and solvents were used as received unless otherwise mentioned.

The *M. albomyces* laccase was overproduced in *T. reesei* and purified as described earlier [36].

[Amim]Cl was synthesized according to Zhang et al. [9] with slight modification to the procedure; both allyl chloride (Aldrich) and 3-methylimidazole (Aldrich) were distilled prior to use. To remove traces of color, [Amim]Cl was further purified by dissolving the crude [Amim]Cl in water and refluxing with activated charcoal for 18 h. The solution was filtered through silica plug and water was removed by distillation and drying for 2 days in vacuum to yield [Amim]Cl as a pale yellow crystalline solid, with a melting point of 52 °C.

From the used substrates 2,6-dimethoxyphenol (Aldrich) was commercially available and coniferyl alcohol was synthesized according to a well-known method [37].

2.2. Determination of laccase activity

The laccase activity was determined using 2,6-dimethoxyphenol (1 mmol kg^{-1}) as substrate in different buffers at room temperature. The buffers used were 50 mmol kg^{-1} sodium citrate, pH 5.0–7.0; McIlvaine buffer (0.2 mol kg^{-1} disodium hydrogen phosphate, 0.1 mol kg^{-1} citric acid), pH 3.0–8.0 and 25 mmol kg^{-1} sodium succinate, pH 4.5. The measured solution contained also 0.5 μl g^{-1} ethanol from the substrate stock solution.

Oxidation of the substrate was monitored for 5 min by absorbance measurements, and the activity was determined from the slope of the linear curve. The determined absorbance maxima of the 2,6-dimethoxyphenol oxidation product at different [Amim]Cl concentrations were ([Amim]Cl m-% – λ_{max}): 0% – 469 nm, 20% – 474 nm, 40% – 479 nm, 60% – 480 nm, 80% – 475 nm. The filtered oxidation product was not soluble in water and thus, the same molar absorption coefficient (ε = 49 600 kg mol^{-1} cm^{-1}) was used for activity calculations in all concentrations [38], different wavelengths.

2.3. Determination of pH optima in different [Amim]Cl concentrations

The optimum pH for *M. albomyces* was determined in solutions containing 0%, 20% or 60% (m-%) [Amim]Cl by activity

determinations in buffers with different pH values. The buffer pH was determined before [Amim]Cl was added, because addition of the [Amim]Cl salt did not significantly change the pH. McIlvaine buffer was used at a pH range 3.0–8.0 with the 20% [Amim]Cl. Because of the low solubility of phosphate at pH values above 6.0 in 60% [Amim]Cl, Na-citrate buffer was used at the pH range 5.0–7.0. Activity determinations were done in both buffers without [Amim]Cl. To produce linear absorbance curves, enzyme stock solution was added to reaction mixtures in different amounts: 0% [Amim]Cl 0.33 μl g^{-1}, 20% [Amim]Cl 0.67 μl g^{-1}, 60% [Amim]Cl 1.33 μl g^{-1}. Thus, the highest activity of each solvent system (buffer – [Amim]Cl concentration pair) was set to 100% and other activities in the same solvent system were calculated as percentages related to this highest activity.

2.4. Determination of laccase activity in different [Amim]Cl concentrations

M. albomyces laccase activity was measured at pH 4.5 (Na-succinate) and pH 6.0 (Na-citrate) using [Amim]Cl concentrations (m-%) 0%, 20%, 40%, 60% and 80%. To produce a linear absorbance curve, the reaction mixture contained the used enzyme stock solution 0.67 μl g^{-1} at pH 4.5 and 0.33 μl g^{-1} at the optimum pH 6.0. Because the used enzyme dosages were different, the results were expressed as the activities of the enzyme stock solution as nanokatals (nmol of substrate oxidized per second).

2.5. Oxidation of coniferyl alcohol and analysis of the oligomeric products as a function of time

Oxidation of coniferyl alcohol (CA) was followed as a function of time with [Amim]Cl concentrations 0% and 40% by stopping the reactions with NaN_3 (Riedel-de Haën), an inhibitor of laccase [39] after 0.5, 1, 2 and 4 h.

In reference experiments without the IL ([Amim]Cl 0%), CA (10 mg) was dissolved in 100 μl dioxane (synthesis grade, distilled over sodium). To the resulting solution, 125 μl of 400 mM Na-citrate, pH 6.0, 765 μl of water and *M. albomyces* laccase in 10 μl of 50 mM Na-citrate buffer was added. The final laccase activity of the resulting solution was 2 nkat g^{-1}. After the appropriate reaction time, 2 ml of 10 mM NaN_3 was added to stop the reaction, followed by 0.4 g [Amim]Cl and 1 ml acetonitrile (ACN, HPLC grade). The HPLC sample was prepared by adding, for internal HPLC standard, 10 μl of guaiacol (Aldrich) in ACN (100 mg ml^{-1}) to 990 μl of the reaction mixture. The sample was filtered using a syringe filter (PTFE, 0.45 μm pore size) and analyzed immediately.

In experiments with [Amim]Cl concentration of 40%, CA (10 mg), [Amim]Cl (0.4 g), 400 mM Na-citrate, pH 6.0 (125 μl), water (460.5 μl) and *M. albomyces* laccase stock solution (14.5 μl) were added to the reaction mixture. The decrease in laccase activity compared with 0% [Amim]Cl was compensated by increasing the enzyme dosage 4.3 times. The reaction was stopped by adding 2 ml of 10 mM NaN_3. After that, 100 μl of dioxane, 300 μl of water and 1 ml of ACN were added to make the samples identical with 0% [Amim]Cl samples. The HPLC sample with internal HPLC standard was prepared as in the case of 0% [Amim]Cl.

HPLC analyses were performed with Waters HPLC, including 600 Pump, 600 Controller, 996 Photodiode Array Detector and 717plus Autosampler. The used column was Waters Symmetry C_{18} 5 μm (4.6 mm × 150 mm) and a wavelength of 280 nm was used for detection. The DAD (diode array detector) UV spectra of the compounds were acquired. ACN-water was used as the eluent and the flow rate was 0.8 ml min^{-1}. Isocratic flow (ACN-H_2O, 30:70) was continued for 11 min, followed by 9 min gradient phase (ACN-H_2O to 100:0).

Dimers **1**–**3** (*β*-5, *β*–*β* and *β*-*O*-4) were identified with authentic compounds. The dimers used for identification were isolated from oxidation mixture of coniferyl alcohol and structures verified with NMR [40].

The structure of trimer **5** [41] was confirmed by analyzing a sample collected from HPLC using high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). HR-ESI-MS was acquired with Bruker Daltonics microTOF ESI-TOF and Agilent ES Tuning Mix (G2421A) was used for internal calibration. HR-ESI-MS (positive) *m*/*z* 561.2088 $[M+Na]^+$ ($C_{30}H_{34}O_9Na$ requires 561.2095).

The structure of dimer **4** was deduced based on HPLC retention times, characteristic carbonyl absorbance in the UV spectrum and chemical structure of the polymerized DHP (Section 3.4).

HPLC retention times and UV absorption maxima (λ_{max}) for compounds: CA 3.89 min, 221 nm, 265 nm; **1** 5.84 min, 230 nm, 277 nm; **2** 9.65 min, 230 nm, 277 nm; **3** 3.25 min, 221 nm, 268 nm; **4** 4.64 min, 230 nm, 279 nm, 310 nm [1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2′-methoxyphenoxy)-1-ethanone 218 nm, 314 nm] [24]; **5** 8.90 min, 230 nm, 268 nm.

2.6. Oxidation of coniferyl alcohol and analysis of the formed dehydropolymer

Coniferyl alcohol was oxidized to dehydropolymer (DHP) in 0% and 40% [Amim]Cl concentrations. The high-molecular-weight fraction of the polymer (high-fraction) from both experiments was isolated, acetylated and analyzed with GPC and NMR.

For the oxidation of coniferyl alcohol in 0% [Amim]Cl, coniferyl alcohol (100 mg) was first dissolved in dioxane (1 ml, distilled over sodium). To the resulting solution, 400 mM Na-citrate, pH 6.0 (1.25 ml) was added, followed by water (7716.5 μl) and laccase stock solution (33.5 μl). The final laccase activity in the reaction mixture was 2 nkat g^{-1}. The reaction mixture was stirred for 20 h and centrifuged (2500 rpm, 20 min) to separate the high-fraction DHP. The polymer was washed with water, dried under an argon flow and acetylated with pyridine and acetic anhydride (1:1). Yield of the fine structured, oily and light brown acetylated DHP was 80 mg.

In the case of 40% [Amim]Cl, coniferyl alcohol (100 mg), [Amim]Cl (4 g), 400 mM Na-citrate (1.25 ml), water (4605 μl) and laccase stock solution (145 μl) for the reaction were added. The laccase dosage was 4.3 times compared to 0% [Amim]Cl to compensate the decrease in activity due to the ionic liquid. The high-fraction DHP was isolated as with 0% [Amim]Cl, except that water was added prior to centrifugation to dilute the [Amim]Cl. Yield of the dense and dark brown acetylated DHP was 80 mg.

Molar masses of the obtained DHP samples were determined by using organic gel permeation chromatography (GPC). Samples were dissolved (1 mg ml^{-1}) in THF (HPLC grade, without stabilator) and filtered using syringe filter (PTFE, 0.45 μm pore size). After filtration samples were injected to GPC system. GPC system included degasser, pump, autosampler and diode array UV detector (Waters 1050 series). Mobile phase was THF, flow 0.5 ml min^{-1}. Columns were Waters Styragel guard, HR-5E and HR-1 (7.8 mm × 300 mm) connected in series. GPC system was calibrated using polystyrene standards (500, 890, 1000, 4000, 9000, 42,300, 177,000, 434,000, 1,270,000 Da) using UV detection at multiple wavelengths (220, 254, 280, 350 nm). Molar masses of the samples were calculated using calibration. GPC system was run under Agilent Chemstation, which had GPC add-on software.

Varian Inova 500 MHz spectrometer was used for NMR measurements, which were performed at 27 °C. Deuterated dimethyl sulfoxide (d_6-DMSO) was used as a solvent and internal reference in analysis of the acetylated high-fraction DHPs. The selected NMR (500 MHz) data for structures **1**–**5** from acetylated DHPs are

Table 1
Selected NMR data (500 MHz) from analysis of acetylated high-fraction DHPs (in d_6-DMSO).

Structure	^{1}H assignments			^{13}C assignments		
	Hα	Hβ	Hγ	Cα	Cβ	Cγ
1	5.60 (5.46–5.63)	3.74 (3.71–3.77)	4.30, 4.36 (4.25–4.41)	86.9 (86.6–87.2)	49.3 (49.1–49.5)	64.7
2	4.70 (4.64–4.76)	3.06 (3.04–3.08)	3.97, 4.04 (3.79–4.21)	84.7	53.6 (53.5–53.7)	71.2 (71.0–71.3)
3	5.96 (5.92–6.00)	[a]	[a]	73.2 (71.6–74.8)	[a]	[a]
4	–	5.52 (5.45–5.58)	4.64, 4.86	–	78.7	~64.7[b]
5	5.62	4.87	4.07, 4.22	79.5	79.5	62.5

[a] Could not be assigned because of overlapping correlations.
[b] Estimated value, because of overlapping correlations.

presented in Table 1. The quantitative Q-HSQC spectra were measured according to Heikkinen et al. [42].

3. Results and discussion

The activity and reactivity of laccase *M. albomyces* in the presence of the ionic liquid, 1-allyl-3-methylimidazolium chloride ([Amim]Cl) was investigated with two monomeric lignin model compounds. First, 2,6-dimethoxyphenol was used as a substrate to investigate the effect of [Amim]Cl to the optimum pH and enzyme activity in different [Amim]Cl concentrations. Coniferyl alcohol was then used to study polymerization reactions in the presence and absence of [Amim]Cl.

3.1. Effect of [Amim]Cl concentration on the pH optimum of the laccase

The optimum pH of laccase *M. albomyces* was determined in the absence and in the presence of 20% and 60% [Amim]Cl with 2,6-dimethoxyphenol as the substrate (Fig. 1). Full, 100% activity refers to the highest activity in that particular solvent system (buffer – [Amim]Cl concentration pair). Measurements were done in two different buffering systems, McIlvaine and Na-citrate buffers, because phosphate was insoluble at higher [Amim]Cl concentrations above pH 6.0.

In the absence of [Amim]Cl, the optimum pH was 6.5 for 2,6-dimethoxyphenol. The obtained value is very similar to the pH optima of *M. albomyces* laccase determined for other phenolic substrates, guaiacol (5–7.5) and syringaldazine (6–7) [34].

When the concentration of [Amim]Cl was increased to 20%, the pH optimum remained the same. However, the activity of the enzyme at the acidic and the basic ends of the pH scale was markedly reduced, as compared to 0% [Amim]Cl. At pH values 4.0 and 8.0 there were only 7% and 17% left of the maximum activity.

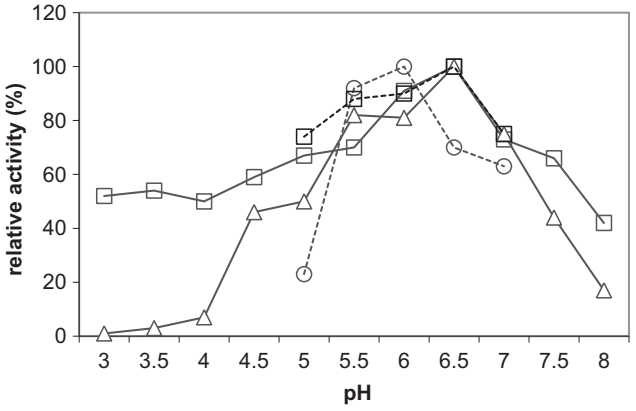

Fig. 1. Relative activity of the laccase *M. albomyces* at different pH values and different [Amim]Cl concentrations. Straight line: McIlvaine buffer, dotted line: Na-citrate buffer, [Amim]Cl concentrations: square 0%, triangle 20% and circle 60%.

At 60% [Amim]Cl concentration the pH optimum was shifted to 6.0. Also, the pH scale at which the laccase remained active was further narrowed compared to 20% [Amim]Cl. The activity decreased more at the acidic end of the scale, even though the pH optimum shifted to a more acidic value.

3.2. Effect of [Amim]Cl concentration on the laccase activity

The activity of *M. albomyces* laccase was determined at five different [Amim]Cl concentrations (0–80%) with 2,6-dimethoxyphenol as substrate. Activity measurements were carried out in two different buffers, Na-citrate (pH 6.0) and Na-succinate (pH 4.5), i.e. at optimal and suboptimal pH values.

It can be clearly seen from Table 2, that increasing the amount of [Amim]Cl decreased the laccase activity, as could be expected. When the [Amim]Cl concentration increased from 40% to 60%, laccase activity at the optimum pH (6.0) decreased most clearly, from 72% to 23%, as compared to the activity in the absence of [Amim]Cl. At 80% [Amim]Cl concentration there was still 13% of the activity left. At pH 4.5, the most remarkable decrease in activity, from 100% to 41%, occurred already when the [Amim]Cl concentration was increased from 0% to 20% and at 80% [Amim]Cl concentration there was only 2% of the activity left.

3.3. Oxidation of coniferyl alcohol as a function of time and analysis of the oligomeric products

Coniferyl alcohol is oxidized by different oxidants first to the dimeric products (β-5, β–β and β-*O*-4, **1**–**3**, Fig. 2) and then further to oligomeric and polymeric products. Laccases from *Rhus vernicifera* and *Pycnoporus coccineus* also catalyze the formation of these dimers from coniferyl alcohol [43]. Coniferyl alcohol was oxidized at [Amim]Cl concentrations of 0% and 40%. Reactions were stopped with the known laccase inhibitor, NaN_3, at 0.5, 1, 2 and 4 h and the products were monitored with HPLC immediately after sample preparation.

First of all, the inhibitory effect of NaN_3 was interestingly gradually decreased when the ionic liquid was present, but in the absence of other efficient ways to inhibit laccase, this inhibitor was, however, used to terminate the reactions, and the samples

Table 2
Activity of the *M. albomyces* laccase, determined as a function of [Amim]Cl concentration at pH 4.5 (Na-succinate buffer) and pH 6 (Na-citrate buffer). Activity was determined using 2,6-dimethoxyphenol as the substrate. The used enzyme dosage was twice as high at pH 4.5 compared to the dosage used at pH 6.0.

[Amim]Cl (m-%)	Activity at pH 4.5		Activity at pH 6.0	
	nkat ml^{-1}	%	nkat ml^{-1}	%
0	700	100	1189	100
20	288	41	987	83
40	93	13	853	72
60	31	4	273	23
80	11	2	160	13

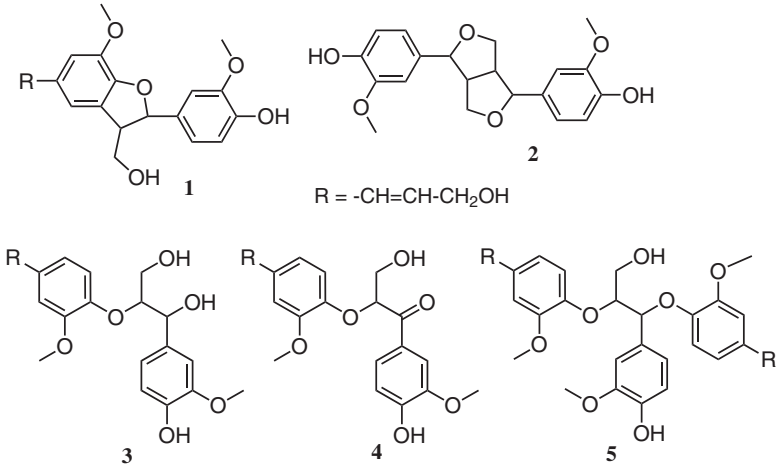

Fig. 2. Dimeric and trimeric products and structures identified from coniferyl alcohol oxidation catalyzed by laccase: β-5 (**1**), β–β (**2**), β-*O*-4 (**3**), α-C=O/β-*O*-4 (**4**), α-*O*-4/β-*O*-4 (**5**).

were analyzed immediately. Although the laccase is relatively thermostable [34], attempts to deactivate the *M. albomyces* laccase by boiling surprisingly also failed. In a previous work, the same HPLC sample preparation method, where the enzyme was first inhibited by adding NaN_3, after which guaiacol was added for internal HPLC standard, was used without any difficulties [24]. As previously shown, after inhibition by NaN_3 the laccase is not able to react with the phenolic guaiacol. Without inhibitor, guaiacol is oxidized by laccase to a red-colored product in a timescale of seconds. Surprisingly it was observed, that in the presence of IL and NaN_3, guaiacol was not oxidized immediately, but the typical red color appeared after a couple of hours. It was thus concluded that NaN_3 first inhibited laccase, but upon prolonged incubation in the presence of IL, the inhibitory effect was gradually decreased. Possibly, the N_3^- anion was first normally bound to laccase, but the presence of ions from the IL gradually weakened the interaction of laccase with the inhibitor.

The HPLC-chromatograms of the samples, in which practically all coniferyl alcohol was oxidized (0% [Amim]Cl at 2 h and 40% at 4 h), are shown in Fig. 3. Authentic compounds were used to identify β-5, β–β and β-*O*-4 dimers (**1**–**3**). The structure of trimer **5** [41] was confirmed with HR-ESI-MS. It is also known, that near neutral pH, addition of phenol to β-*O*-4 type quinone methide to form a α-*O*-4/β-*O*-4 product is dominating, and at more acidic pH, water is added to the quinone methide to form a β-*O*-4 product [44]. The low concentration of α-C=O/β-*O*-4 dimer (**4**) prevented MS analysis of this compound. Thus, the structure was deduced based on the characteristic UV absorbance maximum of ketone and the corresponding structure in the high-polymerized DHP (Section 3.4). In addition, based on previous studies, laccases typically catalyze the oxidation of benzylic hydroxyl groups of lignin model compounds [24].

The amounts of coniferyl alcohol and oxidation products, relative to the internal standard guaiacol, are presented in Table 3. The amounts are not quantitative, but the results based on HPLC peak areas can be examined to compare the consumption of coniferyl alcohol and formation of each product in the two different solvent systems. The oxidation rate of coniferyl alcohol was clearly slower in 40% [Amim]Cl compared to 0% [Amim]Cl, even though the reduced laccase activity in the presence of [Amim]Cl was compensated by increasing the enzyme dosage accordingly. Obviously, the enzyme was inactivated more in the IL reaction than estimated in the short term activity determination. Possibly, prolonged incubation of laccase in the presence of the IL, caused some additional inactivating effect. However, without understanding how and with which structures of the enzyme the ions of the IL interact on a molecular level, and what the mechanism of the inactivation is, it is impossible to explain these observations at this stage.

Formation of β-5, β–β and β-*O*-4 dimers (**1**–**3**) was very similar in both solvent systems in the beginning (0.5 h) of the reaction. The formation rate of α-*O*-4/β-*O*-4 trimer (**5**) was, however, faster in 0% [Amim]Cl already during the first 0.5 h of the reaction. The α-C=O/β-*O*-4 dimer (**4**) was first detected after 2 h in 0% and 4 h in 40% [Amim]Cl. As the reaction proceeded, all products were formed faster in the absence of [Amim]Cl, until their amount was reduced, as they started to react further. There were also differences in the starting point of the further reactions of the products: the amounts of β-5 and β-*O*-4 dimers (**1**, **3**) started to decrease first in 0% [Amim]Cl, while the amounts of β–β dimer (**2**) and α-*O*-4/β-*O*-4 trimer (**5**) started to decrease at the same time in both solvent systems. There may be several reasons for these differences in the reactivities. The IL may have changed the properties of different substrates due to increased solubility and viscosity. The IL may also have affected to which nucleophiles the quinone methides, formed by the oxidation, would have reacted with. In addition, it is possible that the structure and/or activity of the laccase were affected by the presence of IL, having an impact on the substrate preference or specificity. These different reactivities of dimeric and trimeric products in further reactions, between the two solvent systems, most probably explain also the differences observed in structures of the analyzed DHPs (Section 3.4).

3.4. Oxidation of coniferyl alchohol and analysis of the formed dehydropolymer (DHP)

Coniferyl alcohol was oxidized for a longer period (20 h) in [Amim]Cl concentrations of 0% and 40% in order to investigate the possible structural differences in the formed DHPs. The reduced laccase activity due to the presence of [Amim]Cl was compensated by increasing the enzyme dosage based on the activity determinations in the presence of the IL. Polymers (DHP high-fraction) from both solvent systems were analyzed with GPC and NMR.

The visual appearance of the DHPs was different, as the DHP material formed in 40% [Amim]Cl was more dense and had a darker color, compared to the one formed in without [Amim]Cl. The average molar masses of both DHPs, formed in the absence and in the presence of [Amim]Cl, are shown in Table 4. The DHP formed in the presence of [Amim]Cl, had clearly a higher average molar mass, compared to the DHP formed in water. Apparently, the increased

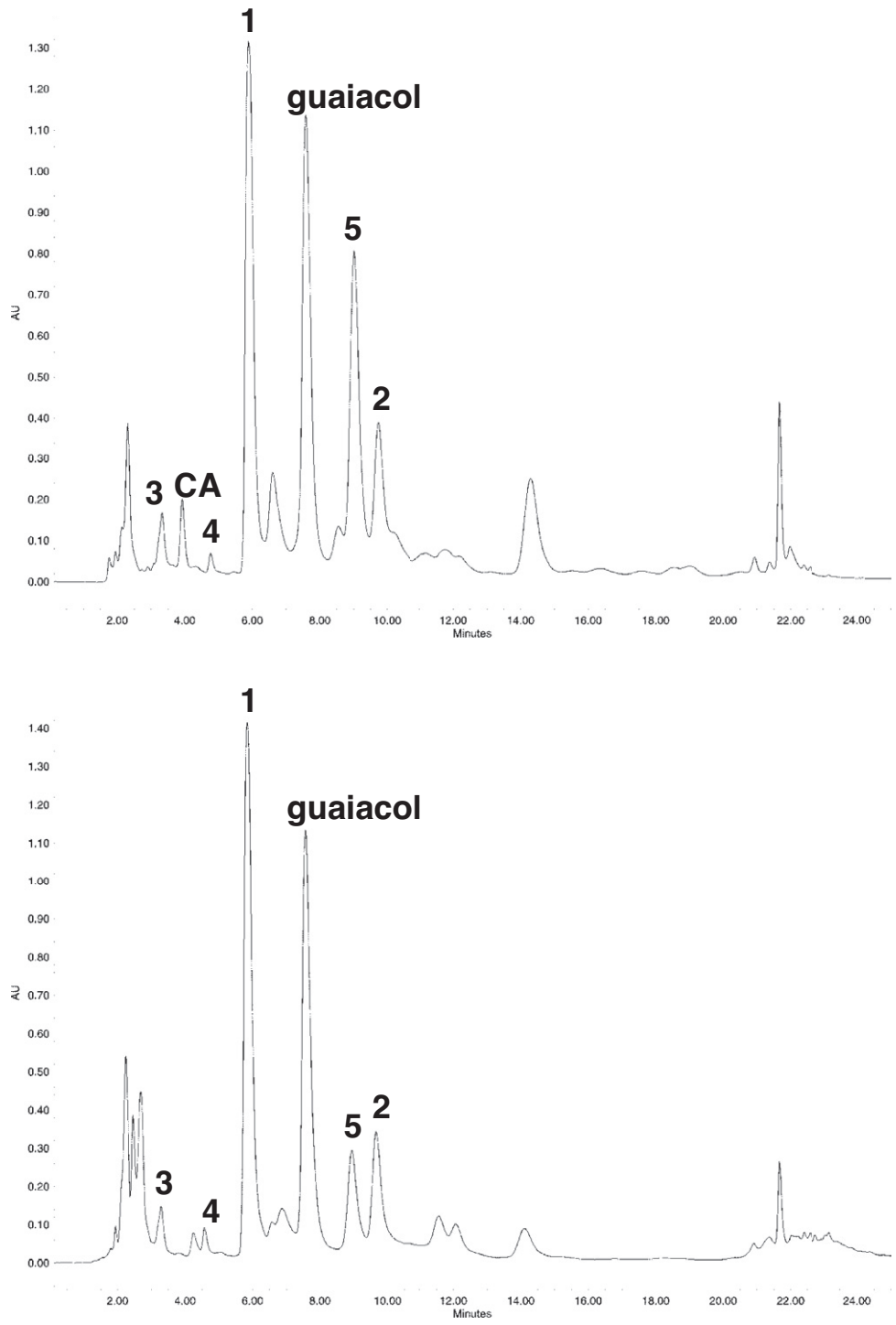

Fig. 3. HPLC chromatograms of products formed from laccase-catalyzed coniferyl alcohol oxidation in 0% [Amim]Cl in 2 h (above) and in 40% [Amim]Cl in 4 h (below).

Table 3
Formation of oligomeric products (**1–5** from Fig. 2) in laccase-catalyzed oxidation of coniferyl alcohol (CA), in 0% and 40% [Amim]Cl as a function of reaction time by HPLC analysis. The ratio of integrated peak areas of coniferyl alcohol and guaiacol (internal reference) was set to 100, and the other values were calculated accordingly.

Coniferyl alcohol (CA) and the oligomeric products												
Time (h)	CA 0%	CA 40%	**1** 0%	**1** 40%	**2** 0%	**2** 40%	**3** 0%	**3** 40%	**4** 0%	**4** 40%	**5** 0%	**5** 40%
0	100.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.5	79.0	85.0	28.7	28.8	12.5	13.1	2.3	2.1	0.0	0.0	33.7	14.7
1	68.1	80.7	45.6	32.7	18.6	15.3	3.3	2.4	0.0	0.0	52.6	15.1
2	4.7	72.7	64.5	50.9	14.0	22.2	6.4	3.2	1.3	0.0	45.8	20.3
4	0.4	0.0	42.6	68.7	10.0	16.4	1.9	4.7	3.4	2.2	48.3	16.6

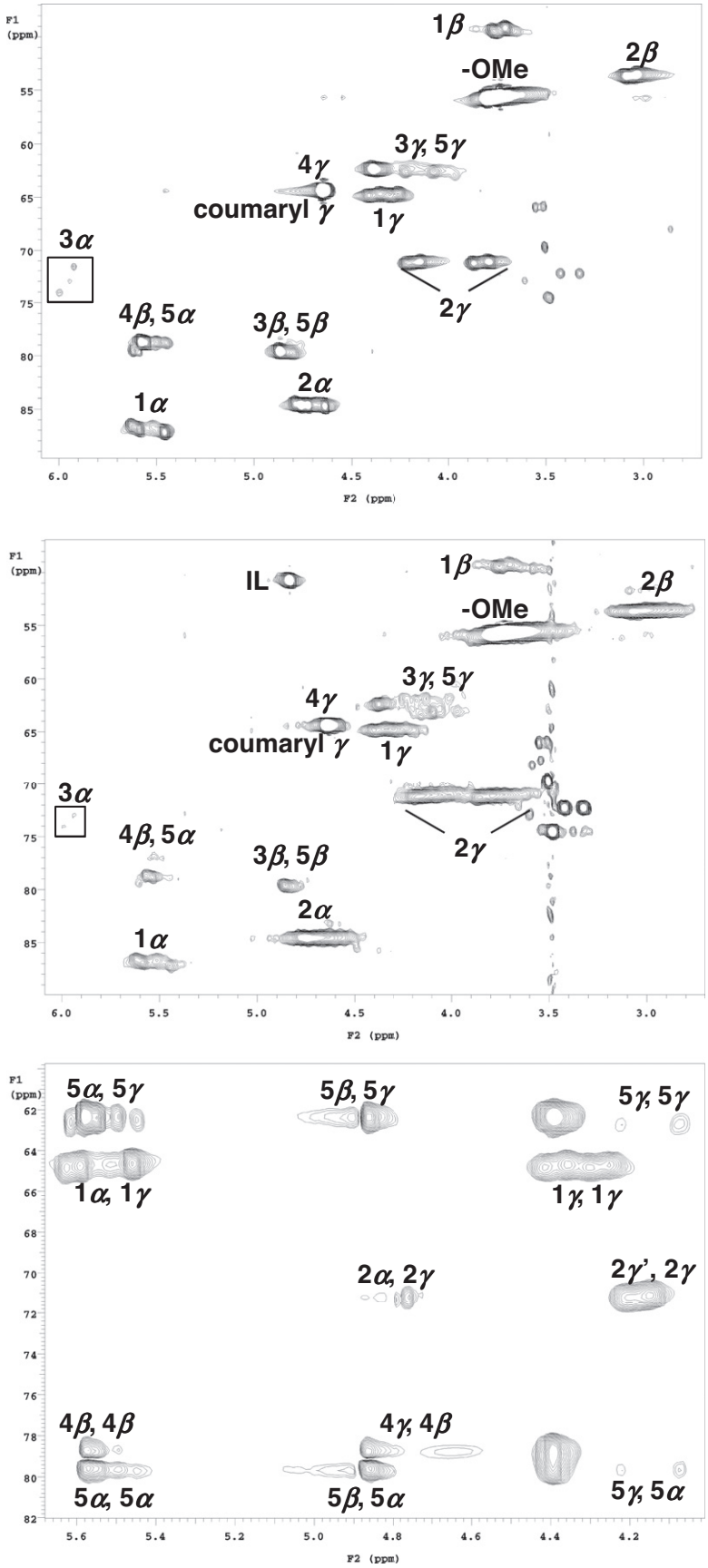

Fig. 4. 2D HSQC spectra (500 MHz, d_6-DMSO) of acetylated DHPs (high-fraction) formed from laccase-catalyzed oxidation of coniferyl alcohol. Top: HSQC spectrum of DHP formed in 0% [Amim]Cl, middle: HSQC spectrum of DHP formed in 40% [Amim]Cl, bottom: HSQC-TOCSY spectrum of DHP formed in 0% [Amim]Cl. Numbering used in Fig. 2 was also used in numbering of the DHP structures.

Table 4
Average molar masses of high-fraction DHPs from laccase-catalyzed oxidation of coniferyl alcohol in 0% and 40% [Amim]Cl.

DHP	M_n (10^3 g/mol)	M_w (10^3 g/mol)	M_z (10^3 g/mol)	M_w/M_n
0% [Amim]Cl	1.1	1.4	2.2	1.3
40% [Amim]Cl	2.1	4.1	6.9	2.0

Table 5
Relative amounts of structural units in DHPs formed in 0% and 40% [Amim]Cl from Q-HSQC spectra [42]. The amounts were acquired, assuming each structural unit contains one methoxyl (OMe) group, from HSQC-NMR H/C correlation volumes using the formula: V(Hx/Cx)/[3/V(HOMe/COMe)]. The slight inaccuracy of the integration due to overlapping signals was found insignificant for the final results.

Structural unit	Correlation/ppm (H/C)	0% [Amim]Cl	40% [Amim]Cl
1	5.60/86.9 (Hα/Cα)	0.29	0.20
2	4.70/84.7 (Hα/Cα)	0.53	0.73
3	5.96/73.2 (Hα/Cα)	0.03	0.02
4	5.52/78.7 (Hβ/Cβ)[a]	0.02	0.02
5	4.87/79.5 (Hβ/Cβ)[b]	0.30	0.08

[a] Volume of **5** Hβ/Cβ subtracted from total volume at 5.52/78.7 ppm to acquire volume of **4** Hβ/Cβ.
[b] Volume of **3** Hα/Cα subtracted from total volume at 4.87/79.5 ppm to acquire volume of **5** Hβ/Cβ.

solubility caused by the IL, enhanced the formation of further polymerized material.

Expansions of the HSQC spectra of both DHPs, and expansion of the HSQC-TOCSY spectrum of DHP formed in 0% [Amim]Cl are shown in Fig. 4. The NMR assignments are based on the values found in literature [45]. The Hα/Cα, Hβ/Cβ and Hγ/Cγ correlations of β-5 (**1**) and β–β (**2**) structures separated well in the HSQC spectrum. Also, β-*O*-4 structure (**3**) could be assigned based on correlation at 5.96/73.2 ppm (Hα/Cα) in HSQC. Correlations of α-C=O/β-*O*-4 (**4**) and α-*O*-4/β-*O*-4 (**5**) structures overlapped in the HSQC spectrum (Hβ/Cβ of structure **4** with Hα/Cα of structure **5**, Hβ/Cβ of structure **5** with Hβ/Cβ of structure **3**), and these were assigned based on patterns in the HSQC-TOCSY spectrum. The characteristic Hβ/Cβ correlation at 4.14/82.5 ppm indicating trans-isomer of dibenzodioxocin structure [46] was not observed.

Based on the quantitative Q-HSQC spectra (Fig. 4, and integration results Table 5), there were clear structural differences between the DHPs formed in 0% [Amim]Cl and 40% [Amim]Cl. The DHP formed in 40% [Amim]Cl contained less β-*O*-4 structures (**3**–**5**) and slightly less β-5 structures (**1**) than DHP formed in 0% [Amim]Cl. Also, the DHP formed in 40% [Amim]Cl, contained more β–β structures (**2**) than the one formed in 0% [Amim]Cl. Obviously, based on higher molecular weight, the DHP formed in the presence of [Amim]Cl contained more biphenylic (5–5) cross-linkages. Several different variables are known to affect the frequencies of different bond-types in DHPs: the rate at which monomers are supplied and radicals are formed, the pH, the presence of polysaccharides and the size of the growing polymer [2,44]. The analysis of oligomeric products revealed that the structural differences were formed already at the early stages of oxidation (Section 3.3), but the formation of a larger polymer in the presence of the IL probably also affected the structural differences.

4. Conclusions

The activity of *M. albomyces* laccase decreased with increasing [Amim]Cl concentration. The pH scale, in which *M. albomyces* laccase remained its activity was narrowed and the pH optimum shifted to a more acidic value with increasing [Amim]Cl concentration.

The products formed in laccase-catalyzed oxidation of coniferyl alcohol were identified as β-5, β–β, β-*O*-4, α-C=O/β-*O*-4 and α-*O*-4/β-*O*-4. When reactions were terminated with NaN_3 to examine the oxidation products of coniferyl alcohol as a function of time, this laccase inhibitor was observed not to be as effective in the IL as in water solutions. Consumption of coniferyl alcohol and formation of oxidation products were clearly slower in the presence of [Amim]Cl at later stages of the reaction, although the reduction of laccase activity caused by the [Amim]Cl was at least partially compensated by increasing the enzyme dosage.

The DHPs formed from coniferyl alcohol in 0% or 40% [Amim]Cl differed already in their visual appearance, the one formed in the presence of IL being denser and having a darker color. The 2D NMR analysis revealed that the DHP formed in the presence of [Amim]Cl contained less β-*O*-4, slightly less β-5 structures and more β–β structures. Obviously, the DHP formed in the presence of [Amim]Cl contained more 5–5 bonds, than the DHP formed in the absence of [Amim]Cl.

Clearly, polymerization reactions catalyzed by laccases can be performed in the presence of ionic liquids, such as [Amim]Cl, to enhance the solubility of the substrate and the product, and to form more highly polymerized lignin-based materials. Interestingly, the chemical structure of the formed polymer was also structurally different from that formed in the absence of the ionic liquid.

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