

Effect of Salts on Laccase-Catalyzed Polymerization of Lignosulfonate

Sebastian A. Mayr,^[a] Harald Rennhofer,^[b] Lars Gille,^[c] Nikolaus Schwaiger,^[d] Gibson S. Nyanhongo,^[e] Renate Weiss,^{*,[a]} and Georg M. Guebitz^[a, f]

Enzymatic polymerization of lignosulfonate (LS) has a high potential for various applications ranging from coatings to adhesives. Here, the effect of different ions in low concentrations on enzymatic polymerization of LS was investigated, including salt solutions consisting of mono- and dicarboxylic acids, sulfate, phosphate and chloride with sodium as counter ion. LS polymerization was followed by viscometry and size exclusion (SEC) chromatography. Interestingly, there was only a small effect of ions on the activity of the laccase on standard substrate ABTS, while the effect on polymerization of LS was substantially different. The presence of acetate led to a 39% higher degree of polymerization (DP) for LS. Small angle X-ray

scattering (SAXS) revealed that the structure of the enzyme was largely unaffected by the ions, while the determination of the zeta potential showed that those ions conveying higher negative surface charges onto LS particles showed lower DPs, than those not affecting the surface charge. Further, electron paramagnetic resonance (EPR) spectroscopy showed 5-times higher intensity in phenoxyl radicals for the monovalent ions compared to the divalent ones. It was concluded that the DPs of LS could be tuned in the presence of certain ions, by facilitating the interaction between the laccase substrate-binding site and the LS molecules.

Introduction

The concept of an integrated biorefinery uses renewable resources, represented by lignocellulosic biomass, and is thought to hold the potential to replace products of today's petrochemistry, which is based on the use of environmentally

harmful and ceasing fossil resources.^[1–4] As it is well known by now, usage of the latter causes severe damage to the climate and the environment in contrast to lignocellulosic biomass, consisting of cellulose, hemicellulose and lignin.^[5–7] While cellulose and hemicellulose are used for high-value applications, such as fine chemicals, high-quality paper or biofuels, the inhomogeneous and recalcitrant lignin is mostly burned to regenerate some of the energy needed during the isolation process. Only about 2% of the annually produced lignin are used for mainly low-value applications.^[8–10] However, in the sense of a circular bioeconomy also these side streams should be used to generate valuable products.^[11]

Especially for lignin, the value lies buried within its structure. It presents the second most abundant biopolymer comprising aromatic moieties which can be used for the synthesis of fine chemicals.^[12] Generally, lignin consists of so-called monolignol units, which structurally are cinnamyl alcohols differing only in the number of methoxy substituents. There is no methoxy group present in *p*-coumaryl alcohol while coniferyl alcohol carries one methoxy group at position C3 of the aromatic ring and sinapyl alcohol has two at positions C3 and C5. These monolignols form phenylpropanoid units, known as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) upon incorporation into the growing lignin polymer. The proportion of these units differs with the type of wood. In softwoods, mainly G- and low amounts of H-units are found, while in hardwoods G- and S-units are predominant. Grasses also contain higher amounts of H-units besides G- and S-units. The distribution of the monolignols affects the reactivity of lignin.^[13]

Generally, three main approaches are followed today to obtain valuable products from lignin. The first one is depolymerization, where lignin is degraded into its simple aromatic compounds, which can be further used for the synthesis of fine

[a] S. A. Mayr, R. Weiss, G. M. Guebitz
University of Natural Resources and Life Sciences, Vienna, Department of Agrobiotechnology, IFA-Tulln, Institute of Environmental Biotechnology, Konrad Lorenz-Strasse 20, 3430 Tulln an der Donau, Austria
E-mail: renate.weiss@boku.ac.at

[b] H. Rennhofer
University of Natural Resources and Life Sciences, Vienna, Department of Material Science and Process Engineering, Institute of Physics and Material Science, Peter-Jordan-Strasse 82, 1190 Vienna, Austria

[c] L. Gille
University of Veterinary Medicine, Vienna, Department of Biomedical Sciences, Institute of Pharmacology and Toxicology, Veterinärplatz 1, 1210, Vienna, Austria

[d] N. Schwaiger
Sappi paper holding GmbH,
Brucker Strasse 21, 8101 Gratkorn, Austria

[e] G. S. Nyanhongo
University of Johannesburg, Department of Biotechnology and Food Technology, Faculty of Science, Corner Siemert and Louisa, Doornfontein 2028, John Orr Building, Johannesburg, South Africa

[f] G. M. Guebitz
Austrian Centre for Industrial Biotechnology (ACIB),
Konrad Lorenz Strasse 20, 3430 Tulln, Austria

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chemicals, such as vanillin or syringaldehyde. The second approach is further polymerization of lignin, presenting a more direct usage. With this approach, new lignin-based biomaterials and biopolymers can be generated. The third approach is modification of lignin with foreign molecules, thus altering its properties resulting in new materials, such as lignin-based resins able to replace phenol-formaldehyde formulations. The reactions for these applications can be conducted either thermally, chemically or enzymatically.^[14–16] Although the resources are sustainable, the reaction conditions mostly are not. Especially chemical and thermal treatments of lignin often require the use of harmful chemicals (often in the form of organic solvents or chemical catalysts), harsh reaction conditions (strongly acidic or alkaline pH) and have high energy demands (high temperatures and pressures). One way to make these processes more sustainable and ecologically feasible would be the use of biocatalysts, which mostly require slightly acidic to neutral milieus, mild solvents and ambient temperatures and have the advantage of high selectivity leading to less waste accumulation.^[17,18] Recently, enzymatic polymerization of water soluble lignosulfonates (LS), resulting as side stream from the sulfite pulping process, has been described for various applications, such as adhesives,^[19,20] coatings^[21] or hydrogels.^[22,23] Further, tailoring of LS properties by enzymatic modification with functional molecules^[24] and the applicability of enzymatic polymerization to other types of technical lignins, such as the water insoluble kraft lignin (KL), was shown.^[25] Although the enzymatic modification of technical lignins has been investigated in the past, the main focus was put on KL.^[26,27] Whereas this process focuses mainly on the use of LS as the reactions can be carried out at moderate conditions, without requiring organic solvents or chemical catalysts.

In nature, microorganisms use a mix of different enzymes which need to work synergistically to break down lignocellulosic biomass. One of these enzymes is laccase (EC 1.10.3.2). The first laccase was isolated from the lacquer tree *Rhus vernicifera* and was later also found in fungi. By now, laccases and laccase-like enzymes are known to be widely distributed as they have also been detected in bacteria and insects.^[28] Laccases belong to the copper-containing oxidoreductases and can oxidize phenolic substrates, generating phenoxyl radicals, while simultaneously reducing molecular oxygen to water.^[29–31] In nature, this reaction mechanism can assist in both the formation and the degradation of lignin. By adjusting the reaction conditions accordingly, the laccase can be influenced to tend toward one activity or the other. The phenoxyl radicals that are generated during laccase-assisted oxidation of phenols can react further, either by undergoing another oxidation catalyzed by enzymes, or by non-enzymatic reactions, such as polymerization.^[32]

Based on this reaction mechanism a process was developed, allowing for extensive polymerization of LS resulting in polymers with altered properties, such as insolubility in water.^[33] For the present process, standard conditions, directing the laccase activity towards polymerization, are already defined.^[21] These conditions fit perfectly well for the fungal laccase *Myceliophthora thermophila* (MtL), which is known to show high activities in the acidic (pH 3) and neutral (pH 7) milieu and

further can stand elevated temperatures since it originates from a thermophilic fungus.^[34] Although this process for laccase, or more precisely, MtL-catalyzed polymerization of LS is frequently used, important aspects influencing the reaction, such as the effect of ions, are still not fully understood. Yet, higher reaction rates could dramatically reduce process costs. Thus, to date, several studies on the effect of various ionic species (cations and anions of varying valences, mono-, di- and tricarboxylic acids but also short-chain fatty acids) on laccase activity were conducted. However, it turned out that the effect of exogenous ions on the enzyme catalyzed reaction strongly depended on both, the individual laccase itself and on the substrate, while furthermore it turned out that lignins as substrate have not been studied yet.^[35–39]

Therefore, in this study, for the first time the effect of different mono- and divalent anions on enzymatic polymerization of LS was mechanistically investigated. A variety of analytical tools were used to elucidate their influence on the enzyme itself (e.g., small angle X-ray scattering (SAXS)), on the substrate properties (zeta potential measurement) and on the interaction between enzyme and substrate (electron paramagnetic resonance spectroscopy (EPR)).

Results and Discussion

The potential of enzyme-catalyzed polymerization of lignosulfonates (LS) for the production of various value-added products has been demonstrated recently.^[19–23] However, the enzymes (i.e., laccase) represent a considerable cost factor and hence attempts to optimize the polymerization efficiency are essential to allow industrial implementation of this process. The presence of ions can strongly affect the activity of enzymes by inducing structural changes. For example, ions can interact with the substrate binding site, altering its affinity to the substrate or by directly binding to the active center thereby blocking it.^[40,41] Hence, the effect of different ions on the activity of a fungal laccase (*Myceliophthora thermophila* laccase; MtL)^[34] and their effect on the degree of polymerization (DP) of LS were investigated herein.

Initially, sodium acetate (NaAc) was investigated for its known inhibitory effect on laccases.^[38] Surprisingly, it turned out that the polymerization was accelerated when carried out in a 0.1 M NaAc solution. To investigate this observation in more detail, the effect of different concentrations of NaAc was tested. Thereby, it turned out that the best results were achieved at a concentration of 0.1 M. Based on these findings, sodium salts of mono- and divalent anions (NaCl and Na₂SO₄), monocarboxylic acids (Na-formate, Na-acetate and Na-propionate) and a dicarboxylic acid (Na₂-oxalate) were investigated, regarding the effect of chain length and valency. The results were compared to the reaction in water (as the standard solution for the polymerization process and therefore taken as the benchmark) and in a Na₂HPO₄/NaH₂PO₄ buffer (NaP), which is used as the standard buffer for MtL activity determination.

First, the activity of MtL in the respective solution was investigated. Generally, when the standard assay using ABTS as

substrate was used, only moderate changes in the specific activity were observed when compared to the standard buffer used for the activity assay (NaP). At pH 7, phosphoric acid is present as a mixture of its first (H_2PO_4^-) and second (HPO_4^{2-}) dissociation stages, with the latter being predominant.^[42] Thus, when talking of phosphoric acid in its ion state, HPO_4^{2-} will be used hereinafter. In the presence of HPO_4^{2-} ions (NaP), the overall lowest activity was found, followed by SO_4^{2-} (Na_2SO_4) showing only minor increases in activity. Sodium chloride (NaCl), sodium-oxalate (Na_2Oxal), sodium-acetate (NaAc) and the control (H_2O) showed slightly increased activities when related to NaP. The highest activities were found for sodium-propionate (NaProp) and sodium-formate (NaForm). However, also the highest activity reached in NaForm solution, was only 0.4-fold higher compared to NaP (Figure 1). The reason why some of the tested solutions showed high variations may be, that some of the solutions are out of their buffering range e.g., the buffering region of acetate salts would be in the range from pH 4 to 6.

However, when one relates the activity results to water set to pH 7 (control, as the standard solution for the enzymatic polymerization reaction) the anions of the sodium salts of the monocarboxylic acids (formate⁻, acetate⁻, propionate⁻) activate MtL, while the other tested anions (Cl^- , SO_4^{2-} , HPO_4^{2-} and oxalate²⁻) inhibit MtL activity (Figure 1).

In their active form, laccases contain four copper ions distributed in three reactive centers. These centers are designated according to their spectroscopic and electronic features. It was found that there is one type 1 (T1), one type 2 (T2) and two type 3 (T3) copper centers. These four copper centers together form the active center of the laccase enzyme. The T1 is located nearby the substrate-binding site at the surface of the protein, where the one-electron oxidation of substrate occurs. The subtracted electron is then transferred via a conserved His-Cys-His electron transfer route to the trinuclear copper cluster formed out of the T2 and two T3 copper centers. At this site, molecular oxygen is reduced to water. For the reduction of one molecule of oxygen to two molecules of water, four electrons are required, which are generated through the sequential oxidation of four phenolic substrates.^[32,43]

The effects of different ions on laccase activity have been investigated in several studies. Therein, it was found that

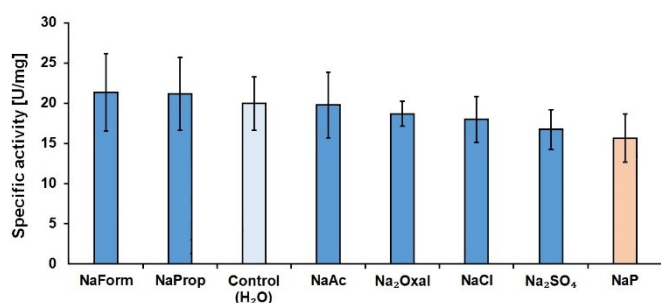


Figure 1. Specific activity of MtL in the presence of different ions (0.1 M) and water (control) at pH 7 on 10 mM ABTS as substrate. Standard buffer for the activity assay (NaP, highlighted in light red) and standard solution for the LS polymerization reaction (Control (H_2O), highlighted in light blue).

anions, such as SO_4^{2-} do not affect laccase activity, while HPO_4^{2-} decreased and NO_3^- increased activity at higher concentrations.^[35] Halides (F^- , Cl^- , Br^- , I^-) were shown to have an inhibitory effect on laccase activity.^[44] The inhibitory effect of halides originates in their ability to form stable complexes with the central copper ions in laccases. With increasing size of the halide ions, access of the substrate and oxygen to the active center is hampered, which is the reason why F^- has a stronger inhibiting effect than Br^- .^[37,44] Monovalent cations (Na^+ , K^+ , NH_4^+) did not show a significant effect on laccase activity, while di- and trivalent cations (Mg^{2+} , Ca^{2+} , Cu^{2+} , Zn^{2+} , Mn^{2+} and Al^{3+}) showed variable impacts.^[35] Monocarboxylic acids (formate, acetate, propionate, butyrate) were found to have an inhibitory effect on *Trametes villosa* laccase, while di- and tricarboxylic acids (oxalate, succinate, citrate) were found to be beneficial.^[38] The deviating results found in this study may be explained by the fact that most of these previous studies were conducted at acidic pH, while in this study, activity was determined at neutral pH (to allow comparison with LS polymerization), where e.g., the inhibitory effect of Cl^- becomes detrimental since it was found to weaken with increasing pH.^[41,45] This may also be true for the other ions used, such as carboxylic acids. In addition, the salts used herein were added in low concentrations (0.1 M), hence the ionic effects may be less pronounced. Further, previous studies have demonstrated that the effect of ions depended on individual laccases. *Trametes*, *Phlebia*, *Lentinus* and *Dichomitus* laccases were inhibited by acetate while *Pleurotus eryngii* laccase was not affected and the activity of *Pycnoporus* laccase was even increased.^[38]

In summary, on ABTS as a substrate and related to NaP as a standard buffer, mono- and dicarboxylic acids (formate, acetate, propionate and oxalate), chloride and sulfate activated the herein used MtL while the lowest activity was found in the presence of phosphate. On the other hand, when relating MtL activity to water, monocarboxylic acids were found to show the same (acetate) or an increased (formate and propionate) activity, the dicarboxylic acid (oxalate) and chloride slightly inhibited laccase activity while sulfate and phosphate inhibited MtL activity (Figure 1).

The herein used initial and polymerized material was thoroughly characterized in a recently published work.^[46] (An overview of some important numbers on the initial LS material can be found in the Supporting Information). For the enzymatic polymerization reaction of LS, the most pronounced increases in viscosity of about 30% were found for chloride and acetate, for propionate and formate no significant differences were seen when compared to the control, while sulfate, phosphate and oxalate had about 50% lower viscosities. The molecular weight was determined using an HPLC system equipped with a size exclusion chromatography (SEC) column coupled to a multi angle laser light scattering detector (MALLS). This system allows the determination of the weight average molecular weight (M_w ; large molecule fragments), number average molecular weight (M_n ; small molecule fragments), their ratio in the form of the polydispersity (Đ) and the hydrodynamic radius (r_h) of the molecules. The molecular weight (M_w) of LS after the polymerization (pLS) (chromatograms can be found in the Supporting

Information) was found about 55% higher in the presence of NaAc related to the control. Only slight changes in molecular weights were found for pLS in the presence of chloride, propionate and formate, showing the same molecular weights than the control, while in the presence of sulfate, phosphate and oxalate pLS had about 65% lower molecular weights than the control (Figure 2a and b).

An important characteristic when comparing polymerization reactions is the degree of polymerization (DP). The DP is calculated by relating the final M_w to the initial M_w ($DP = M_{w \text{ final}} / M_{w \text{ initial}}$), which was further normalized to the control and multiplied by 100 (e.g., $(DP_{\text{NaAc}} / DP_{\text{Control}}) * 100$), resulting in the degree of polymerization in percent. In the presence of Na_2Oxal , NaP and Na_2SO_4 the degree of polymerization was found 30 to 80% lower than that of the control. Only slight changes between -10 to $+14\%$ in DP were found for NaForm, NaProp and NaCl, while NaAc showed a nearly 40% higher DP (Table 1).

Overall, these results are substantially different from those obtained for the activity measured on ABTS. Many substrates used for the determination of laccase activity are small molecules or lignin model substrates and thus do not represent the interaction between the enzyme and the real lignin, which is known to be different due to electrostatic and steric variations present in the real lignin molecules.^[47] The results obtained, suggest that the ionic effect is not so much based on their influence on the laccase but more likely on the LS molecules and the interaction between enzyme and substrate. In an aqueous solution, the shape of LS molecules depends on the surrounding milieu in terms of pH, temperature and the presence of ions. When suspended in water LS is expected to be a branched polymer with hydrophilic side groups (sulfate, carboxylate and phenolate) located on the outside of the molecule and a hydrophobic backbone (aromatic rings) tending

to be in the interior of the molecule. In salt-free aqueous solutions, the LS molecule is present as a loose, randomly branched anionic polyelectrolyte. The loose conformation is due to electrostatic repulsion caused by the negatively charged residues present in the side groups. While in saline solutions, the added ions will lower the charges on the LS molecule leading to less electrostatic repulsion resulting in the formation of denser-packed oblate ellipsoid spheres. Depending on the pH, the presence of ions and on their concentrations, these spheres are more or less densely packed.^[48–53] At low pH, LS is present as a dense, tightly packed spheroid molecule tending to precipitate. In this state, the interaction between laccase and LS is sterically hindered because the enzyme cannot access the reactive groups. While at high pH, the LS molecule is present in a highly loose state, with dissociated phenyl groups and thus highly reactive.^[54,55] However, many fungal laccases are not active at such high pH values.^[13] Aside from pH, it is well known that salts can either promote (salting-out) or prevent (salting-in) precipitation of proteins or polymers in aqueous solutions. Salting-out increases protein stability leading to dense and compact structures resulting in precipitation, while salting-in destabilizes the protein leading to loose structures promoting solubility. The tendency of different ions to promote either the first or the latter effect is shown in the Hofmeister series.^[56,57] When adding salts into an aqueous solution of LS its shape will start to transform from the open polyelectrolyte state into the closed spheroid shape becoming denser with increasing salt concentrations. Ludovitskaya and Kolmachikhina stated in their study that, at low salt concentrations (around 0.1 M) the LS molecule is in a transient state between aggregation and solubilization, which is highly susceptible to minor environmental changes.^[58] Although, only KCl salt was used, it may be also true for other salts at this concentration. By drawing a conformation plot based on the SEC-MALLS data, where the rms radius is plotted against the molecular weight, a good estimation of the molecular shape can be done by comparing the respective slopes. Thereby, it was found that the initial LS molecules are present in a spheroid shape tending to become random coils upon polymerization (the conformation plots are shown in the Supporting Information). Therefore, at the conditions used for the reaction herein, the LS molecules are thought to be present at highly reactive (transient state due to the presence of ions e.g., acetate) but at the same time stable (neutral pH) conformations. This state might facilitate the interaction between LS and the laccase by lowering the steric restrictions of LS.

A general investigation on the solution and storage stability of the pLS was performed in earlier works.^[46,59] While the

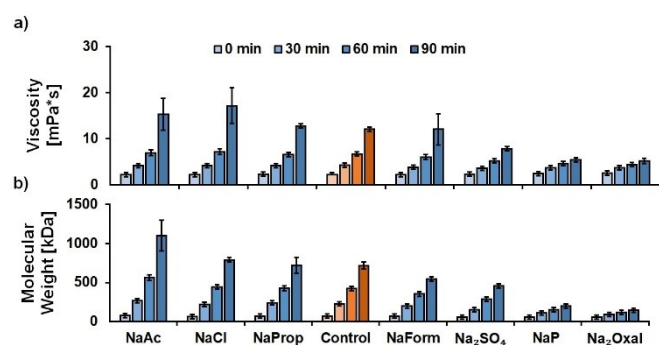


Figure 2. Changes in viscosity (a) and molecular weight (M_w) (b) during the enzymatic polymerization of LS by MtL in the presence of various ions (0.1 M) at pH 7. The control is highlighted in orange.

Table 1. Initial and final M_w of LS in the respective ion solutions with the calculated degree of polymerization and the relative degree of polymerization, respectively.

	NaAc	NaCl	Control	NaProp	NaForm	Na_2SO_4	NaP	Na_2Oxal
Initial M_w [kDa]	73.85	64.68	66.55	72.62	65.90	56.40	58.25	57.98
Final M_w [kDa]	1102.80	792.70	716.25	721.55	565.60	427.70	199.35	142.95
Degree of polymerization [%]	138.75	113.88	100.00	92.32	79.75	70.46	31.80	22.91

stability of the initial LS molecules in the respective aqueous salt solutions was investigated in this work by the determination of the zeta potential. The orientation of the zeta potential, either positive or negative, depends on the kind of ions present on the surface of the particle. If the particle is surrounded by cations, the zeta potential will be in the positive range and if anions are present, it will be in the negative range, but otherwise, they are regarded in the same way. Higher zeta potentials mean higher solution stability and vice versa.^[60] As an anionic polyelectrolyte, the zeta potential of LS was expected to be in the negative range. The herein-used spray-dried LS dissolved in water (control) showed a zeta potential of -25.6 mV which is in accordance with recently published data.^[61] The higher negative charges up to -40.0 mV were found for the salt solutions leading to lower DPs (Na₂Oxal, NaP and Na₂SO₄) while the other tested solutions (NaAc, NaCl, NaProp and NaForm) showed stable but slightly higher negative potentials (around -28 mV) than the control (Figure 3). The solutions showing constant to higher DPs were found to have similar zeta potentials than the control and consist of monovalent anions (acetate⁻, chloride⁻, propionate⁻ and formate⁻). Whereas higher potentials were found for the solutions containing divalent anions (SO₄²⁻, HPO₄²⁻ and oxalate²⁻) showing lower DPs. Thus, it seems that lower negative surface charges of the LS molecules are beneficial for the interaction with laccase.

The formation and decay of the phenoxyl radicals induced by laccase in lignin can be followed by electron paramagnetic resonance (EPR) spectroscopy. EPR allows the measurement of unpaired electrons which can change their spin state in an external magnetic field, such as in radicals.^[47] A study on the investigation of the laccase-catalyzed oxidation of organosolv lignin by EPR found, that after an initial phase of radical formation, a plateau is reached because of substrate depletion and restricted access of the enzyme to some of the phenol groups, followed by radical decay due to non-enzymatic reactions and higher rates of radical decay than formation.^[47,62] Anyhow, the polymerization reaction of LS proceeds further also most likely driven by radical propagation in this state. Herein, the effect of the ions on laccase-induced radical formation in the initial LS was measured by EPR. Those solutions leading to higher (NaAc) and lower (NaP) DPs, respectively,

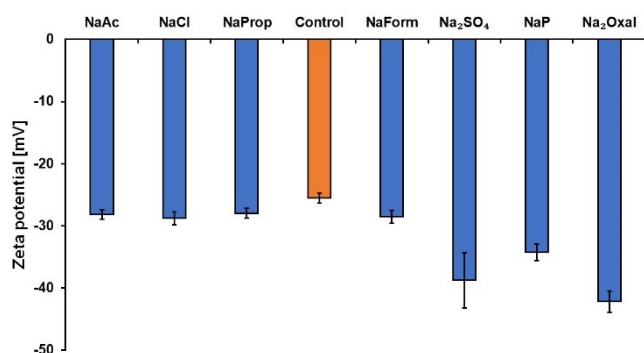


Figure 3. Zeta potentials for LS (1% w/v) dissolved in the respective salt solutions (0.01 M) at pH 7. The control is highlighted in orange.

were investigated. The laccase-induced formation of the radicals started immediately after enzyme addition. The measurement could only be started after a lag time of 2 minutes, needed for sample preparation and measurement set-up. Therefore, at the time the measurement was started, only the radical decay could have been followed. The initial intensities of the radical peaks were about 5-fold higher in NaAc than in NaP. Further, the latter showed a slow but steady decrease, while the former showed a fast decrease in the beginning, which slows down afterwards but anyhow maintains an about 4-fold higher intensity than NaP until the end (Figure 4) (The EPR graphs are shown in the Supporting Information). These findings confirm the higher reactivity of LS in NaAc solution shown by the higher peak intensities. However, as stated before, precipitation of LS in NaP was observed, which is known to lower LS concentration and therefore reactivity.^[52,63] Thus, at least a part of the reactive groups may be bound in the precipitate.

To find out if the ions also affect the structure of MtL, a small angle X-ray scattering (SAXS) measurement was performed with ions leading to low (NaP) and high (NaAc) DPs. Although SAXS has some drawbacks, such as a lower resolution compared to crystallography and elucidation mainly of the flexible regions of the molecules its advantage is that samples can be analyzed directly in the respective solutions.^[64] For data evaluation, the Guinier concept of Radius of Gyration (R_g), a power law approximation of the scattering curve in the low q -range with $I(q) \sim q^{-n}$ and the concept of pair distance distribution function $p(r)$ were applied according to the literature.^[65–68] The Radius of Gyration of MtL was found to be 5.6 nm in NaP and 5.5 nm in NaAc and the power law exponents were found to be 0.52 and 0.51 for the two systems, respectively. These findings indicate that the size and conformation of MtL in the tested solutions are very similar. This can be seen by the pair distance distribution functions, $p(r)$, showing very alike signatures (Figure 5a). The corresponding fit curves from the $p(r)$ -calculation superimposed on the measured scattering curves show a good fit (Figure 5b) (The fitting are shown in the Supporting Information). Thus, the presence of the ions in low

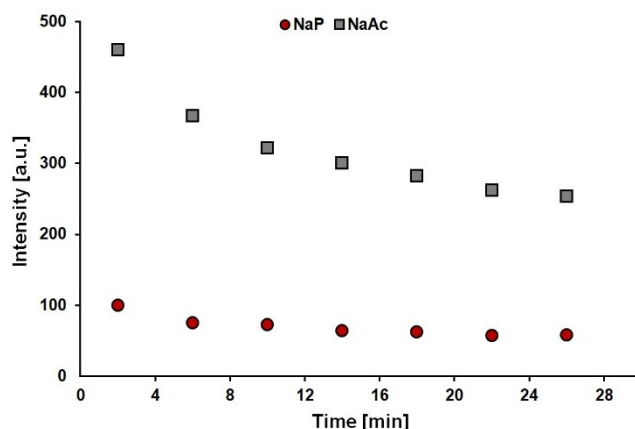


Figure 4. Electron paramagnetic resonance (EPR) spectroscopy results for the laccase-catalyzed polymerization of LS in NaP (red dots) and NaAc (gray squares). Shown is the decay in phenoxyl radical formation, measured every 4 minutes for a period of 26 minutes.

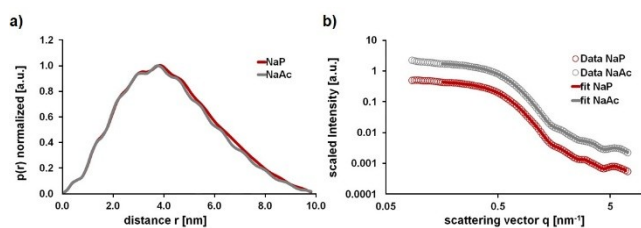


Figure 5. Small angle X-ray Scattering (SAXS) results for enzymatic polymerization of LS in NaP (red) and NaAc (gray) solution, respectively. The pair distance distribution functions, $p(r)$ (a) and the measured SAXS curves (circles) together with the $I(q)$ fit corresponding to the $p(r)$ functions (superimposed lines) (b) are presented.

concentrations does not directly affect the structure of MtL. The results obtained by SAXS analyses confirmed that the effect of the low-concentrated salt solutions on the DPs of enzymatic LS polymerization was not caused by structural changes in the MtL enzyme but rather by changes in the electronic conformation of the LS molecules.

In this study, the DPs for the laccase-catalyzed polymerization of LS, in the presence of the investigated anions, were found to decrease in the following order: acetate⁻ > Cl⁻ > propionate⁻ > formate⁻ > SO₄²⁻ > HPO₄²⁻ > oxalate²⁻ (Figure 6).

These results were found to largely follow the Hofmeister series and are also widely consistent with the reactivity of LS in aqueous salt solutions as found by Myrvold.^[49] Further, it was reported that PO₄³⁻ (HPO₄²⁻ herein) had the strongest salting-out effect on LS followed by SO₄²⁻^[49] which was also found in the present study. The low DP for HPO₄²⁻ and oxalate²⁻ can be explained by the observed precipitation with LS, even at the low concentrations used herein, which would suggest high salting-out effects of these ions. Generally, precipitation is known to lead to lower reactivities, since substrate concentration is lowered and functional groups, needed for the reaction, become inaccessible for binding partners.^[52,63] The low reactivity of LS in sulfate solutions is supposed to be based on the competition between the SO₄²⁻ ions with the sulfonate groups (SO₃⁻) already present in the LS side groups.^[49]

Moreover, the hydrodynamic radii (r_z) of LS in the respective solutions at time point zero (r_{z0}) and at the final time point (r_{zF}), determined by SEC-MALLS, confirmed these findings. The LS molecules dissolved in the solutions with lower DPs (Na₂SO₄, NaP and Na₂Oxal) also showed smaller hydrodynamic radii than in the other solutions (NaAc, NaCl, NaProp and NaForm). This

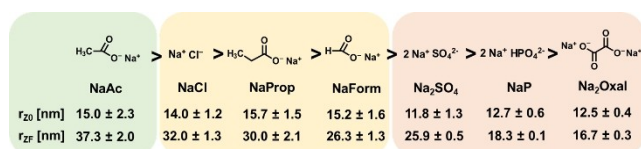


Figure 6. Enzymatic polymerization of LS by MtL in the presence of various ions (0.1 M) at pH 7. Schematic presentation of their effect on DP based on increases in viscosity and molecular weight. The hydrodynamic radii of the LS molecule at time point zero (r_{z0}) and at the final time point (r_{zF}) are given for the respective salts. Green: increased DP; yellow: no effect on DP; red: decreased DP.

would suggest a denser packing of the LS molecules in the former solutions, effectively hindering the interaction between the enzyme and the LS molecules. While in the latter solutions, the hydrodynamic LS radii were the same or bigger than that of the control (r_{z0} = 14.0 nm; r_{zF} = 30.7 nm). Although the hydrodynamic radii did not totally agree with the found DPs, the solutions with higher DPs also showed bigger hydrodynamic radii which facilitates the interaction between laccase and LS due to a looser conformation of the LS molecule (Figure 6) (An overview of all r_z values is given in the Supporting Information).

The affinity of laccase was found to be lower on real lignin molecules than on soluble lignin model compounds, such as hydroxycinnamic acids, which may be caused by lower accessibility of bound lignin-phenols in the LS molecules due to their conformational variations.^[47] Further, the size of the substrate was found to present an important factor for protein-substrate affinity. Lower flexibility of the enzyme is required for smaller substrates, while for larger substrates higher enzyme flexibility is needed. The flexibility of enzymes depends on the pH, where lower pH means lower flexibility and vice versa. Aside from the enzyme, also the substrate needs to fulfil the correct requirements, such as good hydrophobic packing, an optimal shape, optimal water solubility and specific electronic properties, which are not fully defined yet. Further, a distance of below 5 Å between the lignin-phenol to the laccase substrate binding site was found to be necessary to achieve an effective binding and an appropriate electron transfer rate.^[69] When considering these requirements for substrate-laccase interaction, the conditions chosen for the enzymatic polymerization reaction of LS were already close to the optimum. The flexibility of the laccase is given by the neutral pH of the reaction, which facilitates the binding of the large LS molecules to the laccase binding site. At the same time, the LS molecules are present in a moderately loose conformation due to the neutral pH,^[54,55] while simultaneously the presence of the ions in low concentrations keeps them in a transient state increasing their reactivity. On the one hand the presence of monovalent anions (chloride⁻, acetate⁻, formate⁻, and propionate⁻) was found to largely maintain this transient conformation, by mediating slightly higher negative charges to the surface of the LS molecules than water. Thus, leading to less densely packed LS molecules, as was evident by the higher M_w and r_z values found, resulting in the same (chloride⁻, formate⁻ and propionate⁻) or slightly higher (acetate⁻) DPs. While on the other hand the presence of divalent anions (SO₄²⁻, HPO₄²⁻ and oxalate²⁻) clearly increased the already negative charge of the LS molecules, which suggests more stable solutions. Anyhow, since M_w and r_z were found to be lower, they were less reactive resulting in lower DPs. The change in the electronic properties and conformation of the initial LS molecules, induced by the neutral pH and the presence of the salts in low concentrations, leads to conformational changes, which either facilitate or hinder the interaction between the LS molecules and the laccase enzyme. Thus, leading to the observed changes in DP.

Conclusions

The effect of different ions in low concentrations on the enzymatic polymerization of LS by the fungal laccase Mtl was investigated. Overall, the effect of the ions on the DP of the enzyme-catalyzed LS polymerization was substantially different when compared to their effect on enzymatic activity on ABTS. The latter is a model substrate for laccase activity and thus the enzyme activity on the real LS molecule may vary. Further, exogenous ions are known to not only affect the structure of the enzyme but also that of LS. While all investigated salt solutions showed at least slightly higher enzyme activities than NaP, as the standard buffer, SAXS analyses revealed that the presence of these salts in low concentrations does not affect the structure of Mtl directly. Thus, the presence of diverse ions in low concentrations had a more pronounced effect on the conformation and properties of the LS molecules than on the Mtl enzyme. Under the given reaction conditions the LS molecules are supposed to be present in a loose but stable conformation, based on the neutral pH, while at the same time they are kept in a highly reactive state, due to the presence of the low concentrated salts.^[58] These factors were found to influence the DP of the enzyme-catalyzed polymerization reaction. No change in DP was found when the monovalent anions chloride⁻, propionate⁻ and formate⁻ were present. Lower DPs were found in the presence of divalent anions (SO₄²⁻, HPO₄²⁻ and oxalate²⁻). While the presence of acetate⁻ led to an increased DP. It was found that the acetate solution is changing the conformational and electronic properties of LS in a way to facilitate compatibility with the substrate-binding site of the Mtl laccase. In summary, we have clearly demonstrated that the presence of certain ions at low concentrations can tune the DP of the laccase-catalyzed polymerization reaction of LS.

Supporting Information

The authors have cited additional references within the Supporting Information.^[61]

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Conflict of Interests

The authors declare no competing financial interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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