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SHORT COMMUNICATION

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Enzymatic modification of kraft lignin through oxidative coupling with water-soluble phenols

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Abstract The aromatic polymer lignin can be modified through promotion of oxidative coupling between phenolic groups on lignin and various phenols. The reaction is initiated by an oxidation of both components, e.g., by using the oxidoreductases laccase or peroxidase. Coupling between phenolic monomers and lignin has previously been studied by the use of radio-labeled phenols. In this study, incorporation of water-soluble phenols into kraft lignin, using laccase as catalyst, was investigated. Several phenols with carboxylic or sulfonic acid groups were used as markers for the incorporation. The modified lignin was isolated and the amount of phenol incorporated was characterized by means of titration, quantitative ^{1}H-NMR, and quantitative ^{31}P-NMR after modification with 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane. Only a few of the phenols studied were found to be incorporated into lignin. When the phenol guaiacol sulfonate was incorporated into kraft lignin, the lignin became water-soluble at pH 2.4 and a low ionic strength due to the introduction of sulfonic acid groups. The content of sulfonic acid groups in the product was 0.5–0.6 mmol/g lignin. A lower amount of 4-hydroxyphenylacetic acid was incorporated under similar conditions.

Introduction

The most abundant biopolymer in nature, next to cellulose, is lignin, and breakdown products of this aromatic network polymer are a huge byproduct from the pulp and paper industry. It has been suggested that these materials could serve as replacements for various petroleum-derived chemicals, including resins, plastic, and fillers (Allen et al. 1980). However, to suit these purposes some modification of the recovered lignin is necessary. Lignin can be modified in a variety of ways using classical chemical modification; alternatively, it may also be modified with the oxidoreductase enzymes laccase and peroxidase. Laccase and peroxidase oxidize phenolic substrates, including lignin, to phenoxy radicals with concomitant reduction of oxygen or hydrogen peroxide, respectively. The phenoxy radicals generated in situ are unstable, and reactions involving polymerization as well as depolymerization of lignin can take place (Ishihara and Miyazaki 1972; Leonowicz et al. 1985). Many phenols are enzymatically oxidized into homopolymers, but in the presence of lignin coupling between lignin and the phenols occurs. Thus, Dordick and coworkers showed that it was possible to incorporate a phenol into lignin through a radical-based oxidative coupling mechanism by oxidizing a mixture of lignin and ^{14}C-labeled *para*-cresol in 50% dioxane with a peroxidase. The product was a highly cross-linked lignin-based copolymer containing 20% *p*-cresol (Blinkovsky and Dordick 1993; Popp et al. 1991). Milstein et al. (1994) used the same concept for binding vanillic acid to lignin.

The present study further investigates the structural changes that occur during the incorporation of water-soluble phenols into kraft lignin using oxidoreductases as catalysts. Aqueous dioxane solutions of mixtures of lignin and different water-soluble phenols were oxidized with laccase. From this mixture, the lignin fraction could be separated from remainders of nonincorporated phenols. This was made possible by using phenols that remained soluble upon enzymatic oxidation into their homopolymer and which subsequently could be washed out of the water-insoluble lignin fraction. The phenols had either a carboxylic acid or a sulfonic acid group as substituent, and incorporation of phenols into lignin was characterized by means of potentiometric and conductometric titrations as well as quantitative ^{1}H- and ^{31}P-NMR.

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Materials and methods

Materials

Trametes villosa laccase (E.C. 1.10.3.2) was provided by Novo Nordisk A/S, Bagsvaerd, Denmark. Enzymatic activity of laccase was measured in units (U) with 1 U defined as the amount of enzyme that oxidizes 1 µmol syringaldazine per min in 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer, pH 5.5, at 30 °C.

Indulin AT, which is an effluent lignin isolated from black liquor recovered from the kraft pulping process, was obtained from Sigma (St. Louis, Mo.) and used without further purification. 1,4-Dioxane, obtained from Aldrich (Milwaukee, Wis.), was distilled over sodium borhydride prior to use. All other reagents used were of analytical grade.

Enzymatic oxidation of mixtures of lignin and phenols

Five hundred mg indulin AT was suspended in 300 ml freshly distilled dioxane and mixed with a 200 ml aqueous solution of the phenol. The concentration of the phenol in the final reaction mixture was 2 or 4 mM. Sulfuric acid (0.1 M) or sodium hydroxide was used to adjust the pH to 4.5. Laccase was added at a dosage of 1 U/ml. The solution was stirred overnight at ambient temperature. *p*-Dioxane was removed by evaporation on a rotary evaporator at 40 °C. The subsequent isolation and purification of the modified lignin from the aqueous phase varied among the different phenols used.

When a mixture of lignin and guaiacol sulfonate was oxidized, the lignin became soluble in water at pH 2.4 after the enzyme treatment, and it was necessary to increase the ionic strength to precipitate it. After evaporation of dioxane and adjustment of the pH to 2.4, sodium chloride was added to a concentration of 0.5 M, which made the modified lignin precipitate. The precipitate was collected after centrifugation followed by washing three times with 0.5 M sodium chloride at pH 2.4 to remove residues of non-incorporated guaiacol sulfonate. Salt was removed from the washed precipitate by dialysis against water. The outer solvent was replaced three times. The dialysis bag was a 500 D Spectrapor CE membrane (Spectrum, Calif.). The theoretical yield was calculated from the estimate of the amount of phenol incorporated into lignin, giving a percentage yield of 80%.

Enzymatically oxidized 4-hydroxybenzoic acid and 4-hydroxyphenylacetic acid were separated from lignin after removal of dioxane by salting the lignin out in 1 M sodium chloride at pH 4.5–5. The precipitate was washed three times with 250 ml of 1 M sodium chloride, pH 4.5–5, followed by three washes with 250 ml water, pH 2.4, to remove the salt and protonate carboxylic groups. 4-Hydroxybenzene sulfonic acid remained soluble after enzymatic oxidation and could be removed from the mixture after evaporation of dioxane by precipitating the lignin at pH 2.4 and washing the precipitate three times with acidified water.

Titration of sulfonic and carboxylic acids

The amount of guaiacol sulfonic acid incorporated into lignin was determined by conductometric titration. The method of Öster et al. (1988) for determining sulfonic acids in sulfonated kraft lignin was used with a few modifications. The desalted, modified lignin was protonated by elution through an Amberlite IR-120 cation exchanger in the acid form and then freeze-dried. The modified and protonated lignin (100 mg) was dissolved in a minimum amount of acetone and water (4:1 v/v), diluted with 400 ml water, and 5 ml 0.1 M sodium chloride was added. This solution was titrated with 0.010 M sodium hydroxide under argon atmosphere while changes in conductivity were measured. The content of carboxylic acids was determined by potentiometric titration with lithium hydroxide according to the method described by Zakis (1994). In brief, 30 mg of dried lignin was dissolved in 5 ml DMSO/water (9:1) followed by addition of 0.5 ml pyridine and water to a total volume of 20 ml. The solution was titrated with 0.010 M lithium hydroxide. The standard deviation was 0.03 mmol/g.

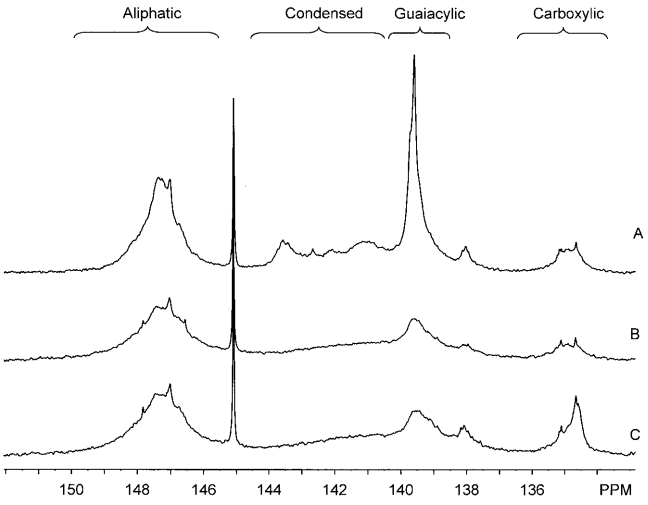

Fig. 1 Quantitative ^{31}P-NMR spectra of Indulin AT derivatized with 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane. *A* Untreated indulin AT, *B* indulin AT oxidized with laccase, *C* indulin AT oxidized in the presence of 4-hydroxyphenylacetic acid. Integration regions for aliphatic hydroxy groups, condensed- and guaiacylic phenols, and carboxylic acids are shown on *top*. The peak at 145.1 ppm is the internal standard, cyclohexanol

Quantitative ^{31}P- and ^{1}H-NMR

Quantitative ^{1}H and ^{31}P NMR spectra data were acquired with a 400 MHz Bruker DMX spectrometer. Prior to acquiring the ^{31}P NMR data, the lignin was phosphitylated with 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane, which reacts with all labile protons in lignin, i.e., alcohols, phenols, and carboxylic acids. The ^{31}P chemical shift of the derivative formed depends on the specific functional groups, thus allowing for a quantification of functional groups in lignin containing a labile proton. The quantitative ^{31}P-NMR measurements were carried out following methods described in the literature (Argyropoulos and Ying 1998; Granata and Argyropoulos 1995; Jiang et al. 1995). In brief, a lignin sample (approximately 30 mg) was dissolved in 200 µl anhydrous DMF and 400 µl of a mixture of anhydrous pyridine and deuterated chloroform in the ratio 1.6:1 (v/v) was added. The solution was added a mixture of 100 µl pyridine and deuterated chloroform (1.6:1 v/v) containing cyclohexanol (4.0 mg/ml) and chromium(III) acetylacetonate (3.6 mg/ml), which served as internal standard and relaxation reagent, respectively. Acquisition of the data was started within 10 min after addition of the derivaterization agent; 150 scans were collected with a 30° pulse and a 25-s delay between pulses. A local baseline correction was done for the internal standard peak. Integration regions shown in Fig. 1 are consistent with those in the literature (Argyropoulos and Ying 1998; Granata and Argyropoulos 1995; Jiang et al. 1995).

Quantitative ^{1}H-NMR was performed according to the method described by Runge and Ragauskas (1999). Approximately 30 mg was dissolved in 500 µl DMSO-d_6 and quantified relative to the sodium salt of 3-trimethyl-propionate-2,2,3,3-d_4 (0.75 mg/ml). Two hundred transients were acquired at a 90° pulse, with 14 s between pulses. The method was only used for quantifying carboxylic acids, which were integrated from 13.5 – 10.5 ppm. The standard deviation on the integration of the carboxylic acid peak was 0.03 for ^{31}P- and 0.11 mmol/g lignin for ^{1}H-NMR.

Results

Enzymatic oxidation of mixtures of lignin and phenols

Enzymatic oxidation of indulin AT, alone, darkened the color of the solution. Upon removal of dioxane from the solution and adjustment of the pH to 2.4, the lignin precipitated. The isolated and dried indulin AT, oxidized with laccase, appeared as a dense black powder that required prolonged stirring to dissolve in DMF or DMSO.

Several different phenols (4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, vanillic acid, coumaric acid, ferulic acid, guaiacol sulfonate, and 4-hydroxybenzene sulfonic acid) were investigated for their binding to lignin. When a mixture of lignin and a phenol was oxidized with laccase, lignin could be isolated from the solution after evaporation of dioxane and changing the pH and ionic strength such that lignin precipitated without precipitating oxidation products of the phenol. An aqueous suspension of lignin has its lowest stability when its carboxylic acids are protonated, but can also be precipitated at higher pH by increasing the ionic strength. Lignin was separated from oxidized 4-hydroxyphenylacetic acid and 4-hydroxybenzoic acid at pH 4.5–5 and a high ionic strength because oxidation products of these phenols showed a minor precipitate at pH 2.4, but not at pH 5. Upon enzymatic oxidation and removal of dioxane, phenols like vanillic acid, coumaric acid, and ferulic acid were found to precipitate over a wide pH range and could not be separated from lignin.

When indulin AT was enzymatically oxidized in the presence of guaiacol sulfonate and the dioxane was subsequently removed, the product appeared as one clear phase without any turbidity at pH 2 and low ionic strength. This suggests that guaiacol sulfonate had been incorporated into the lignin, making it water-soluble under acid conditions. Addition of salt to a solution of the lignin-guaiacol-sulfonate derivative that was formed made it precipitate. Upon removal of salt by dialysis against water, the solution again appeared as one clear phase. Enzymatic oxidation of guaiacol sulfonate alone gave a yellow product that did not precipitate in 1 M sodium chloride at pH 2.4. If the ratio of guaiacol sulfonate to indulin AT in the reaction mixture was too low, none or only a part of the lignin stayed in solution after removal of dioxane and adjustment of the pH to 2.4. To determine whether the solubilization of lignin was due to noncovalent association between lignin and an oxidation product of guaiacol sulfonate, lignin was added to a solution of 2 mM guaiacol sulfonate in 60% dioxane that was oxidized overnight with laccase. The enzyme was inactivated with sodium azide prior to addition of lignin to stop any further oxidation. When dioxane was removed from this solution and the pH adjusted to 2.4, the lignin precipitated. Thus, it was concluded that the solubilization of lignin is due to a covalent binding of guaiacol sulfonate to lignin.

Quantification of incorporated phenols

The amount of sulfonic acid incorporated into indulin AT by oxidation with laccase, as measured by conductometric titration, was between 0.5 and 0.6 mmol/g lignin when the concentration of reactants in the reaction mixture were 1 mg/ml lignin and 2 mM guaiacol sulfonate. In one experiment, increasing the concentration of guaiacol sulfonate from 2 mM to 4 mM changed the content of sulfonic acids incorporated into indulin AT from 0.60 to 0.7 mmol/g. Oxidation of indulin AT with laccase in the presence of the analogous sulfonated phenol 4-hydroxybenzene sulfonate did not change the solubility of the indulin AT. Since the enzyme-treated indulin AT remained insoluble at pH 2.4, it was not possible to titrate the sample conductometrically to measure if any incorporation had taken place. Incorporation of 4-hydroxybenzoic acid or 4-hydroxyphenylacetic acid into indulin AT was quantified by measuring changes in content of carboxylic acids by potentiometric titration and by quantitative ^{31}P- and ^{1}H-NMR (vide infra). Based on potentiometric titration, the content of carboxylic acids in untreated indulin AT was found to be 0.85–0.90 mmol/g. When a mixture of indulin AT (1 mg/ml) and 4-hydroxyphenylacetic acid (4 mM) was oxidized with laccase, this value increased to 1.0 mmol/g. No significant changes in carboxylic acid in indulin AT oxidized in the presence of 4-hydroxybenzoic acid could be found by any of the techniques used, and it was concluded that this phenol was not incorporated.

Changes in lignin structure

Figure 1 shows the ^{31}P-NMR spectra of untreated indulin AT (spectrum A), indulin AT oxidized with laccase alone (spectrum B), and indulin AT oxidized with laccase in the presence of 4-hydroxyphenylacetic acid (spectrum C). Functional groups were quantified relative to the internal standard, cyclohexanol. From Fig. 1 it can be seen that the content of condensed and guaiacylic phenolic groups decreases upon oxidation with laccase alone. When the same lignin sample was enzymatically oxidized in the presence of 4-hydroxyphenylacetic acid, the decrease in phenolic groups remained the same as when the sample was treated with laccase alone, and there was an increase in carboxylic acids as seen from the broad increase in the peak around 135 ppm. The increase in content of carboxylic acids from spectrum A to spectrum C was from 0.5 mmol/g to 0.7 mmol/g.

Oxidation of 4-hydroxyphenylacetic acid in 60% dioxane with laccase was accompanied by a change from colorless to reddish/brownish. The ^{31}P spectrum of this compound was identical to the ^{31}P spectrum of pure 4-hydroxyphenylacetic acid and appeared with a sharp peak at 138.2 ppm, representing the phenolic group, and one at 134.6 ppm, representing the carboxylic acid (not shown). Addition of oxidized 4-hydroxyphenylacetic acid to oxidized indulin AT gave a spectrum with two

sharp peaks at these positions on top of the indulin AT spectrum (not shown).

^{1}H-NMR showed an increase in carboxylic acids for laccase-oxidized mixtures of indulin AT and 4-hydroxyphenylacetic acid, from 0.8–0.9 mmol/g to 1.0–1.2 mmol/g (not shown), which is in good accordance with the potentiometric titrations.

Surprisingly, it was found that the ^{31}P-NMR method could not be used to monitor sulfonic acids. Benzene sulfonic acid added 2-chloro-4,4,5,5-tetramethyl-1,2,3-dioxaphospholane did not give rise to any new peaks between 0 and 400 ppm, which suggests that either the sulfonic acid does not react with this compound or the derivative is unstable and decomposes before the measurement can be done.

Discussion

In this study, the oxidative coupling between lignin and a phenol was most strongly illustrated by the incorporation of guaiacol sulfonate into lignin, which made the lignin water-soluble at pH 2.4. The fact that mixing lignin with oxidized guaiacol sulfonate in 60% dioxane did not change the solubility of the lignin suggests that the water solubilization is not due to an association between molecules. Rather, it supports the hypothesis that the phenol is actually incorporated into lignin through a radical-based coupling mechanism. The covalent binding of guaiacol sulfonate to the lignin macromolecule resembles sulfonation of lignin in terms of water solubilization (Öster et al. 1988). 4-Hydroxybenzene sulfonate was found not to alter the water solubility of lignin, probably because no incorporation had taken place.

Oxidation of a mixture of 4-hydroxyphenylacetic acid and lignin did not change the solubility of lignin. The nature of the binding of 4-hydroxyphenylacetic acid to lignin can be revealed from the spectrum shown in Fig. 1, spectrum C, which indicates that 4-hydroxyphenylacetic acid has been covalently bonded to lignin. If the increase in acid groups were due to a contamination, the spectrum would be similar to that of laccase-oxidized lignin with oxidized 4-hydroxyphenylacetic acid added, which would give two sharp peaks, at 138 and 134 ppm, with equal intensity. There is no sharp peak around 138 ppm on the spectrum in Fig. 1, spectrum C, and the broad increase in the peak area at 134 ppm is consistent with a carboxylic acid attached to a macromolecule.

The increase from 0.9 to 1.0 mmol carboxylic acids/g lignin upon oxidation of a mixture of indulin AT and 4-hydroxyphenylacetic acid, as found by potentiometric titration, was in good accordance with the ^{1}H-NMR. Quantitative ^{31}P-NMR of the lignin-4-hydroxyphenylacetic acid derivative suggested an increase in carboxylic acid in the same order of magnitude, but the absolute values were lower as found by potentiometric titration and ^{1}H-NMR. The factors contributing to this discrepancy may be due to side reactions from the phosphitylation procedure (Archipov et al. 1991). The marked decrease in guaiacylic phenolic groups upon treatment with laccase seen from spectrum A to spectrum B in Fig. 1 could be due to the formation of structures in which the phenol is eliminated, e.g., semiquinones. Surprisingly, there was a decrease in aliphatic hydroxy groups upon enzymatic oxidation of indulin AT, which is seen in spectrum B of Fig. 1. Laccase and oxygen oxidize phenols to phenoxy radical, which subsequently rearranges to various condensation products, semiquinones, and quinones (Brown 1967), but oxidation of aliphatic hydroxy groups with laccase has only been reported to take place in the presence of a mediator (Bourbonnais et al. 1997; Potthast et al. 1996). Further investigations are needed to explain what causes the observed decrease in aliphatic hydroxy groups in spectrum B (Fig. 1).

The degree of incorporation into lignin varied among the different phenols studied. The amount of guaiacol sulfonate incorporation into lignin was 0.4–0.6 mmol/g or approximately 10% w/w, whereas its non-methoxy-substituted analogue, 4-hydroxybenzene sulfonate, did not show detectable incorporation at all. Only 0.1–0.2 mmol 4-hydroxyphenylacetic acid/g lignin was incorporated (2% w/w), whereas 4-hydroxybenzoic acid was not incorporated at all. Blinkovsky and Dordick (1993) found that up to 20% (w/w) of *p*-cresol could be incorporated into indulin AT upon oxidation with horseradish peroxidase.

Although the number of phenols tested was limited to four, there seems to be a correlation between their substitution pattern and the degree of incorporation, with only phenols having an electron-donating group being incorporated, i.e., the methoxy on guaiacol sulfonate and the methylene group on 4-hydroxyphenylacetic acid. The presence of an electron-donating substituent decreases the reduction potential of the phenol, making it more oxidizable (Musso 1967), and stabilizes the generated phenoxy radical through polar effects and spin delocalization in the aromatic ring (Wu and Lai 1996). The catalytic rate of laccase oxidation of phenols with substituents such as methoxy and methyl groups is thus increased, whereas an electron-withdrawing substituent such as a nitro group decreases the catalytic rate of oxidation (D'annibale et al. 1997). Both the catalytic rate of oxidation and the stability of the generated phenoxy radical could influence the degree of incorporation. A minimum conversion of the phenol to its phenoxy radical is the requirement for any coupling reaction to take place, whereas the stability of the generated radical could affect the product formed. During oxidation of a mixture of lignin and a phenol, several competing coupling reactions are assumed to take place: polymerization of the phenol to its homopolymer, polymerization of lignin, and incorporation of the phenol into lignin. A lower radical stability of the phenol would be expected to favor formation of dimers and oligomers of the phenols rather than an incorporation of the phenol into the lignin macromolecule. The exact mechanisms and chemical factors contributing to the derivatization of lignin with phenoxy compounds will require further research. Nonetheless, this work

clearly documents some of the changes in lignin structure that occur when it is derivatized with phenoxy substrates by employing an oxidoreductase system.

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