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Effect of reaction media concentration on the solubility and the chemical structure of lignin model compounds

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Abstract

In plant cell walls, lignin polymerization occurs in concentrated polysaccharide gel. The effect of high polysaccharide concentrations on the structure of lignin-like polymers (DHPs = dehydrogenation polymers), were investigated by running lignification-like polymerization under three reaction conditions in which the concentrations of all reactants (xylan/coniferyl alcohol (CA)/oxidising system) were gradually increased. Control experiments were also run in similar conditions but without polysaccharides. DHPs showed increased solubility with increased concentrations of reactants in the presence of xylans but were mostly insoluble in buffer control experiments. The structures of DHPs were characterized by thioacidolysis and size exclusion chromatography (SEC). Results indicated that the frequency of β-alkyl aryl ether bonds and DHP molecular weight increased with increasing concentration of the reaction mixture in the presence of xylans whereas those of control DHPs decreased slightly under the same conditions. This emphasizes the role of the pre-existing polysaccharide gel and high concentrations existing in the cell wall during construction of the lignin polymer.

Keywords: Arabinoxylan; Aggregation; Dehydrogenation polymers; β-*O*-4 linkages; Lignification; Molar mass

1. Introduction

Lignified cell walls are composite materials resulting from the assembly of different biopolymers. Cell wall formation is a complex process in which polymers are deposited in a successive and highly controlled manner. The initial deposits are of polysaccharides (cellulose and hemicelluloses) whereas lignin polymerization occurs at the end of the process in the pre-existing polysaccharide environment. Lignin is formed through the dehydrogenative polymerization of three aromatic monomers (*p*-coumaryl, coniferyl-CA- and sinapyl alcohol). Polymerization occurs by an enzyme-initiated but chemically controlled process that is influenced by the physicochemical characteristics of the environment. Information on the relationship between the reaction environment and the chemical structures of lignin has been obtained by model approaches. The dehydrogenation process can be reproduced *in vitro* by the synthesis of dehydrogenation polymers (DHPs) generated by oxidative polymerization of monolignols using either peroxidase or laccase (Freudenberg and Neish, 1968). This versatile system has allowed a wide range of modifications of polymerization conditions to be studied. Thus, the effect of many physicochemical parameters, such as addition mode of the reactants (Sarkanen, 1971), type of

Abbreviations: CA, coniferyl alcohol; AX, arabinoxylan; DHPs, dehydrogenation polymers; SEC, size exclusion chromatography; CAC, critical aggregation concentration; M_w, weight average molecular weight; M_n, number average molecular weight; THF, tetrahydrofuran.

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monomer (Sarkanen, 1971), oxidizing enzyme (Wallace and Fry, 1999), pH (Terashima et al., 1995, 1996; Fournand et al., 2003), presence of polysaccharide (Higuchi et al., 1971) and many others have been investigated. Despite all these efforts, the synthesis of DHPs structurally similar to lignin has never been achieved implying that the laws governing lignin polymerization are far from being fully understood.

Among all the various parameters previously studied, the concentrations of the reactants have received little attention. Indeed, model experiments were mostly run in dilute solutions whereas the cell wall environment contains high concentrations of polysaccharides. A few attempts have been made to investigate this effect. In the early eighties, Higuchi's team prepared DHP by diffusion of coniferyl alcohol through a dialysis tube containing the oxidizing system and 2% polysaccharide solutions (pectin, mannan and xylan) (Tanahashi et al., 1981). The molecular weights of the DHPs were found to increase in the presence of mannan and pectin but remained unchanged in the case of xylans, compared to reference DHPs obtained by classic dropwise addition of the reactants. Later on, Terashima et al. prepared DHPs in the presence of pectin solution. The structures of the resulting DHPs were more condensed than that of the control (Terashima and Seguchi, 1988). The reverse was found when DHPs were synthesized in the presence of concentrated pectin solutions (Terashima et al., 1995, 1996). Indeed, dioxan–water extracted DHPs from a pectin reaction mixture were found to be structurally closer to lignin than DHPs prepared in dilute solution (Terashima et al., 1995, 1996). Similar trends were also reported by our group using bacterial cellulose/pectin composites as polymerization medium (Touzel et al., 2003) whereas the Fukushima's group reports recently that polymerization of coniferyl alcohol in the presence of cyclodextrin led to the same conclusion (Nakamura et al., 2006). However, these structural variations cannot be unambiguously attributed to the high concentration of polysaccharide. In Terashima's work (Terashima et al., 1995, 1996), DHPs were obtained by dioxane–water extraction and represented only a minor proportion of the total DHPs. Thus, an effect of DHP fractionation cannot be ruled out. In the case of a diffusion cell (Touzel et al., 2003) or dialysis tube (Tanahashi et al., 1981), very slow addition rates of the monomer may also be partly responsible for the observed structural modifications.

Our aim in this study was to investigate the effect of increasing the concentration of the polymerization medium during DHPs synthesis in order to mimic cell polymerization conditions. Xylans are the major hemicellulose present in hardwood and graminaceous cell wall. We chose to focus of one type of xylans, the arabinoxylans (AX) that are typical xylans of grass cell walls. As model of lignin, we polymerized coniferyl alcohol (CA) since it is the first monolignol polymerized in the presence of arabinoxylans. We run the polymerization at three concentrations of the reactants. The concentrations of all the reactants (CA, peroxidase and hydrogen peroxide) were calculated to keep the ratios between them identical for all three concentration values studied (0.1%, 0.5% and 1%). These concentrations were higher than the critical concentration for xylan aggregation, determined by fluorescent probe, to ensure that reactions did not occur in a dilute solution system. The solubility and chemical structure of the AX-DHPs (DHPs prepared in presence of AX) and control DHPs (DHPs prepared without AX) were evaluated.

2. Results and discussion

2.1. Characterisation of AX aggregation

At high concentrations, polymer chains can form interchain aggregates (Winnik and Regismond, 1996). Aggregation induces the formation of hydrophobic domains that can be detected by fluorescence probe studies using, for example, pyrene as probe molecule. Pyrene is a hydrophobic molecule and would thus be located predominantly in the hydrophobic region. The fluorescence spectrum of pyrene is highly sensitive to the polarity of the medium. The emission spectrum of pyrene exhibits several fine vibronic peaks. The relative intensity of peaks 1 (372 nm, I_1) and 3 (382 nm, I_3) (Fig. 2) is sensitive to solvent polarity and is usually considered indicative of the polarity of the microenvironment surrounding the pyrene moiety (Kalyanasundaram and Thomas, 1997). For example, I_1/I_3 is equal to 0.6 in hexane and to 1.7 in water. Hence, the I_1/I_3 value can be used to probe the micelle or aggregate formation.

In aqueous solution, depending on the concentration range and ramification levels, the arabinoxylan chains can be associated, leading to the formation of aggregates or gel networks (Roubroeks et al., 2000; Dervilly-Pinel et al., 2004; Warrand et al., 2005). In Fig. 2, the intensities of peaks 1 and 3 of the fluorescence spectra of pyrene in 0.25% and 0.5% AX solution are different. This clearly indicates the formation of hydrophobic domains in the 0.5% solution that can be related to aggregation phenomena. The I_1/I_3 ratio was plotted as a function of the AX concentrations (Fig. 3). The curve shows a sigmoidal decrease as the AX concentration increases. In the low AX concentration range, the I_1/I_3 ratio is constant and roughly equal to 1.65, indicating that pyrene acts in a polar environment. The I_1/I_3 value then shows a sharp decrease that evidences the formation of AX aggregates. The CAC value (critical aggregation concentration) is determined from the point of intersection between linear extensions of the rapidly decreasing part and the horizontal part of the curve (Aguiar et al., 2003) (see Fig. 3). The estimated CAC value is 0.1% (v/w). These results suggest the formation of inter-chain aggregates by hydrophobic interaction in agreement with previous results (Shigematsu et al., 1994a). This clearly indicates that xylans do not exist as free soluble individual chains at concentrations higher than

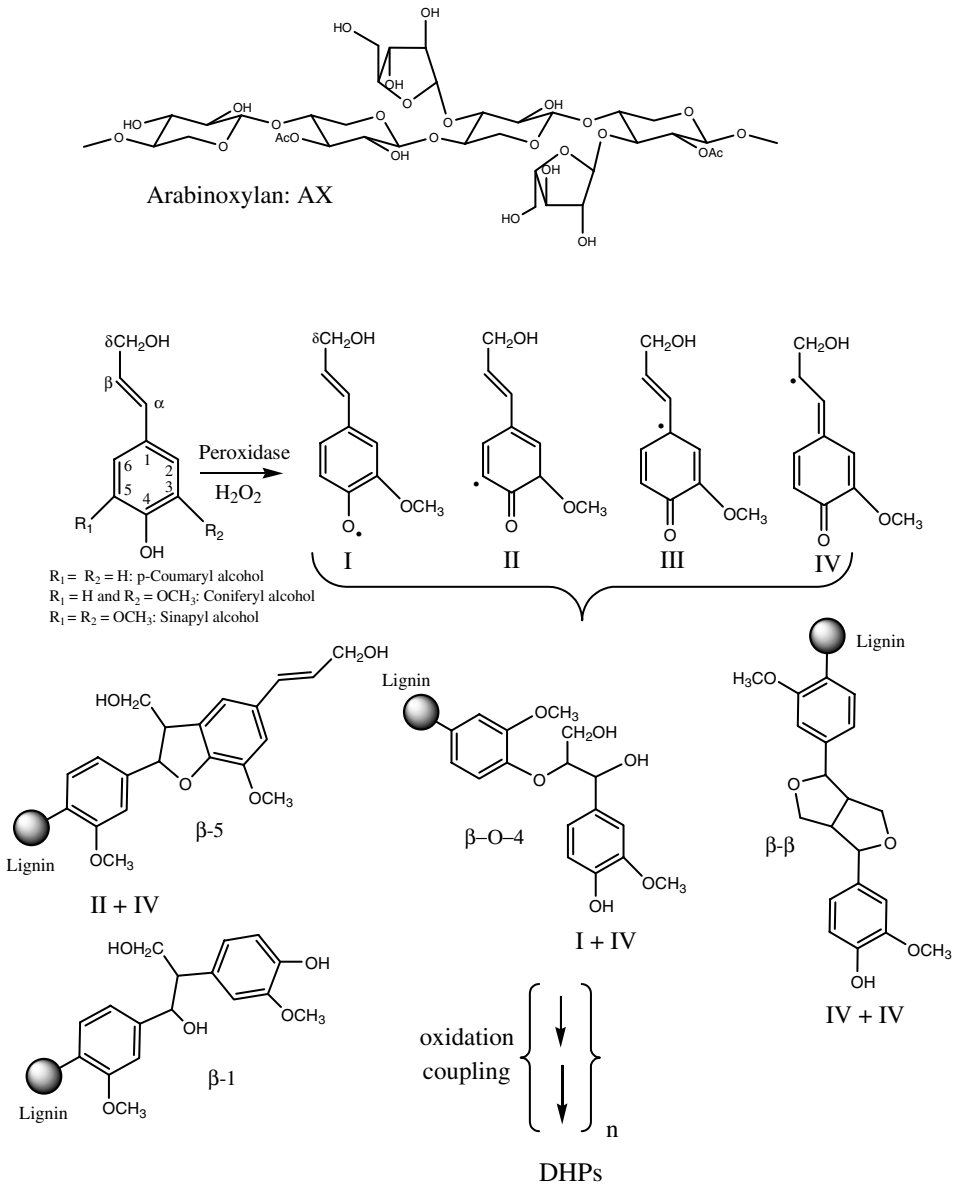

Fig. 1. Structure of arabinoxylan (AX) and mechanism of polymerization of coniferyl alcohol.

0.1% w/v but in an associated form. The polymerizations were therefore carried out at the same or higher CAC concentrations, namely 0.1%, 0.5% and 1.0% w/v.

2.2. Polymerization of coniferyl alcohol and DHPs solubility in buffer and in AX solutions

The end-wise polymerization (Zutropfverfahren; ZT) mode was used to prepare DHPs without AX and in AX solutions (AX-DHP). The AX/CA ratio was kept equal to 1, while the amounts of added peroxidase and hydrogen peroxide were adapted to be similar between the three conditions. The main difference between all polymerizations is the final concentration in AX-DHP. However the only parameter that differed here was the rate of addition of monomers. Addition time was also kept constant (240 min) in order to have an equal ratio between radicals and polysaccharides in all experiments. However, this has the disadvantage of increasing the addition rate with increasing xylans concentration so that the experiment with 1% w/v exhibits more pronounced bulk polymerization (Zulaufverfahren; ZL, all reactants added simultaneously) than that with 0.1% w/v.

Since DHPs are mostly insoluble in aqueous solutions, they are usually collected by centrifugation. All the reaction mixtures (DHP synthesized with and without AX) were subjected to centrifugation. The solid residue was washed with ultra pure water and then freeze dried to obtain the water insoluble fraction (Fig. 4). The yields of DHPs prepared without AX were between 67% and 71% (based on starting coniferyl alcohol) in agreement with previous works (Terashima et al., 1995; Cathala and Monties,

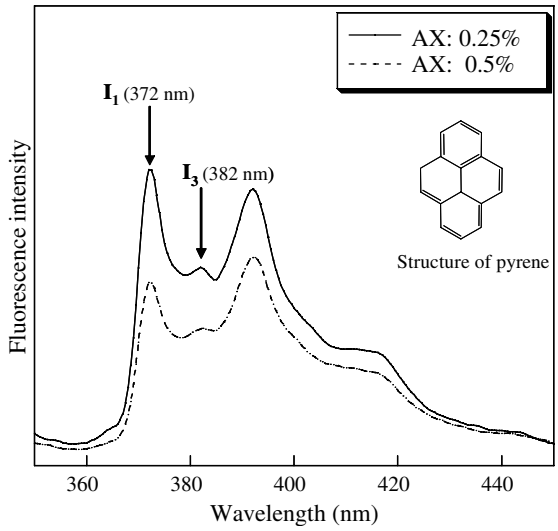

Fig. 2. Fluorescence spectra of pyrene (5×10^{-7} M) in the presence of an increasing concentration of AX: continued line 0.25% and dashed line 0.5% of AX.

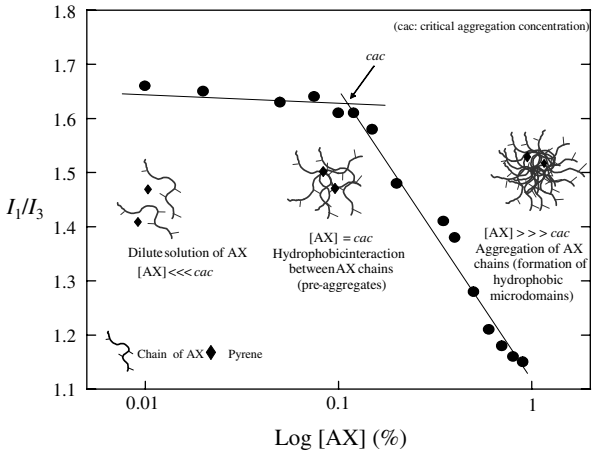

Fig. 3. CAC determined by fluorescence probe study: change in the I_1/I_3 ratio of vibronic band intensities of pyrene (5×10^{-7} M) with AX concentration and schema of the aggregation pattern of AX chain.

2001), whereas those of water-insoluble DHP prepared in AX solutions decreased drastically from 5% to 22% (Fig. 4). We concluded from these results that the majority of DHP molecules remain in the aqueous supernatant. A similar effect had already been noted when DHPs were synthesized in the presence of pectin (Cathala and Monties, 2001). Polysaccharides improve the solubility of DHPs, either by the formation of colloidal aggregates as demonstrated previously (Lairez et al., 2005; Barakat et al., 2007a) or by the formation of covalent bond (Barakat et al., 2007b) and even more so when the two are combined. This effect is notably more efficient at higher polysaccharide concentrations despite the increase in DHP concentration which emphasizes the role of density of the medium.

2.3. Molar mass determination and thioacidolysis analysis of the dioxane water soluble DHP fraction

The yields of DHPs precipitated out of the AX solution were rather low compared to the starting CA, so analyses of this fraction would not be representative of the total DHPs formed. We therefore decided to treat the freeze dried samples with dioxane–water (95:5), which is a specific solvent of DHPs, in order to extract a greater proportion of DHPs (Fig. 5). The yields of dioxane–water soluble DHPs (S-AX-DHP) were 72% for the 0.1% w/v, 64% for the 0.5% v/w and 32% for the 1% v/w synthesis (based on starting CA, Fig. 5). Carbohydrate analyses (see Section 3.5) indicate that dioxane–water soluble DHPs do not contain any polysaccharide contamination whereas the insoluble DHP fraction (R-AX-DHP) contained both arabinoxylans and DHP. The AX recovery yields exceeded 90–95% of the starting AX materials indicating that no significant solubilisation of xylans had occurred during dioxane–water extraction. The carbohydrate contents gradually became lower with the increase in polymerization media, to attain 84% (R-AX-DHP0.1), 76% (R-AX-DHP0.5) and

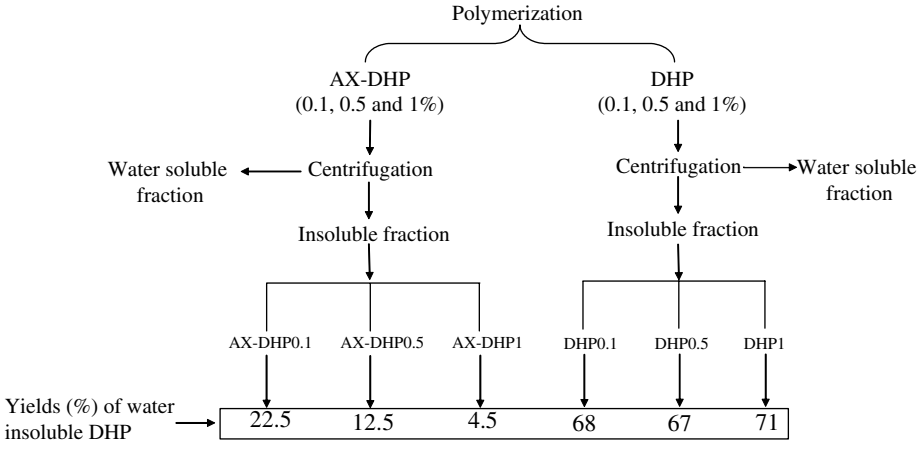

Fig. 4. Purification procedure of AX-DHPs and reference DHPs (without AX) resulting from polymerization of AX and CA at various concentrations. Water-insoluble fractions are collected after centrifugation.

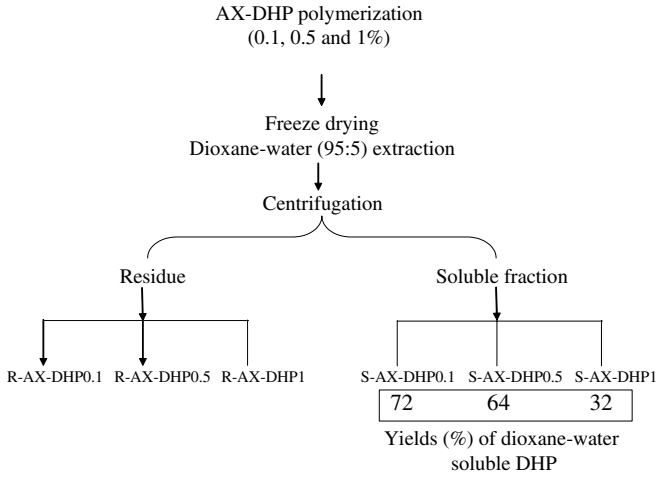

Fig. 5. Fractionation pathway of dioxane–water-soluble and -insoluble DHP recovered from AX-DHP resulting from polymerization of AX and CA at various concentrations.

58% (R-AX-DHP1) since more and more important amount of the DHPs remain trapped (see Fig. 5).

2.3.1. Size exclusion chromatography (SEC) of acetylated dioxane–water soluble DHPs in THF

The control DHPs and S-AX-DHPs were subjected to acetylation and their molecular weights determined by size exclusion chromatography (SEC) in THF. THF-SEC analysis was not possible in the case of acetylated R-AX-DHPs, due to their very poor solubility in THF even after acetylation. This is likely due to the presence of polysaccharide in the samples that may limit DHP solubility either by the formation of non covalent aggregates and/or the formation of covalently bonded complexes. The weight average molecular weight (M_w) and number average molecular weight (M_n) were determined using the relative calibration method based on the elution of 10 polystyrene standards in THF (Faix et al., 1981; Cathala et al., 2003). As shown in Table 2, the molar masses of DHP prepared in the absence of arabinoxylan remain roughly stable or show a slight decrease close to the standard deviation of the measurement. Indeed the values range from 2.6 kDa to 2.3 kDa for 0.1% and 1% v/w polymerization, respectively. This can be attributed to the increased rate of addition of reactant that moves the reaction type nearer to the ZL process that is known to produce DHPs of lower molar mass than the ZT process (Cathala et al., 1998). On the other hand the molar masses of DHPs prepared in the presence of AX (S-AX-DHP) increased with concentration of the medium. Moreover, the latter values were in all cases higher than (Table 2 and Fig. 6) those prepared in the absence of AX.

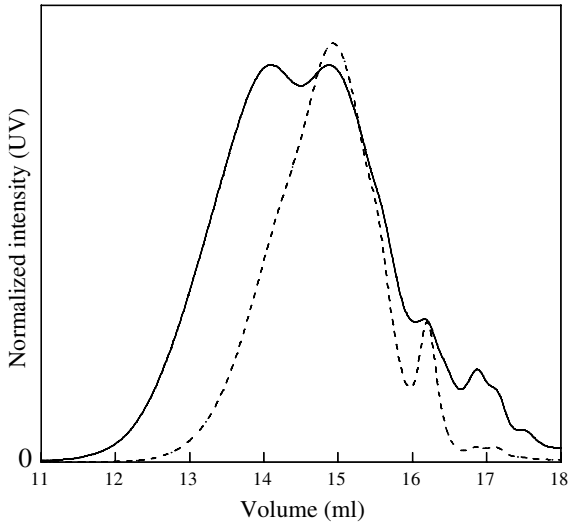

Fig. 6. Steric exclusion chromatography elution profiles of S-AX-DHP1 (continuous line) and control DHP1 (dashed line).

An increased molar mass of DHPs was previously observed with increasing pectin concentrations (Tanahashi and Higuchi, 1981; Cathala and Monties, 2001). As demonstrated above, the presence of polysaccharide induces aggregates formation and avoids the precipitation that otherwise occurs in their absence. As a consequence the DHPs present within the AX-DHPs aggregates can be coupled either with other oligomers or with newly added monomer. Both reaction leads to an increase in molar mass. In the absence of polysaccharide, the DHPs are precipitated as the molar mass increases and are thus no longer reactive. The coupling process is therefore restricted to newly-added monomer and soluble oligomers of lower molar mass yielding lower molar mass compounds.

2.3.2. Evaluation of β-O-4 linkages by thioacidolysis

The contents of β-*O*-4 linkages (alkyl aryl ether, the most abundant structure in native lignin) in the control DHPs experiment (without AX), the S-AX-DHP and R-AX-DHP samples were determined by thioacidolysis. Thioacidolysis is a highly specific method which cleaves the alkyl aryl ether bonds (β-*O*-4). The results for the degradation products of DHP, S-AX-DHP and R-AX-DHP samples at different concentrations reported in Table 3 indicate that the relative content of the β-*O*-4 structure is substantially lower in DHP synthesised without AX than in DHP synthesized in AX solution (AX-DHP), in agreement with previous works (Higuchi et al., 1971; Terashima et al., 1995, 1996). Moreover the β-*O*-4 content increases with the AX concentration whereas in DHP synthesised without AX it remains stable or shows a slight decrease. Table 3 shows that the thioacidolysis yield of DHP (1092 μmol/g) synthesised in concentrated AX solution (1%) is very similar to that obtained from pine lignin (Lapierre, 1993). This finding is also in agreement with the results of Terashima et al. (1995, 1996), when DHPs were synthesized in concentrated pectin solution.

As previously discussed by Sarkanen (1971), the slow diffusion of the monomer radical in the Zutropfverfahren (ZT) reaction type would favour the formation of an "end-wise" polymer containing more β-*O*-4 linkages. On

Table 1
Parameters of coniferyl alcohol polymerization with and without AX

Samples	[AX] (%) (SolnA)	wt_{CA} (%) (SolnB)	$V_{H_2O_2}$ (ml) (SolnC)	Unit of peroxidase[a]	[DHP]/[AX] ratio
DHP0.1	0	0.1	0.1	625–750	–
DHP0.5	0	0.5	0.5	3125–3750	–
DHP1	0	1	1	6250–7500	–
AX-DHP0.1	0.1	0.1	0.1	625–750	1
AX-DHP0.5	0.5	0.5	0.5	3125–3750	1
AX-DHP1	1	1	1	6250–7500	1

V, volume; wt, weight; CA, coniferyl alcohol; AX, arabinoxylan.
[a] One unit will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C.

Table 2
SEC-THF analysis of a series of DHPs synthesised in buffer (without AX) and in AX (AX-DHP) at different concentrations (0.1%, 0.5%, and 1%)

Samples	M_w (g/mol)	P
DHP0.1	2660	1.38
DHP0.5	2480	1.66
DHP1	2322	1.63
S-AX-DHP0.1	3020	2.21
S-AX-DHP0.5	3440	2.54
S-AX-DHP1	4020	1.99

M_w: weight average molecular weight (g/mol); $P = M_w/M_n$: polydispersity (M_n: number average molecular weight).

Table 3
Amounts of β-*O*-4 (μmol/g of DHP) linkage recovered from thioacidolysis from DHPs synthesized with and without AX at different concentrations

Samples	β-*O*-4 (μmol/g of DHP) (thioacidolysis)
DHP0.1	674
DHP0.5	657
DHP1	633
S-AX-DHP0.1	877
S-AX-DHP0.5	924
S-AX-DHP1	1092
R-AX-DHP0.1	755
R-AX-DHP0.5	830
R-AX-DHP1	935

the other hand, a high concentration of the radical in the reaction mixture would result in the formation of a "bulk" polymer containing fewer β-*O*-4 linkages. Concordantly, decreasing proportions of β-*O*-4 linked-structures were recovered with increased concentrations of CA in the case of DHP synthesized without AX.

In contrast, polymerizations in the presence of increasing AX concentrations resulted in a higher frequency of β-*O*-4 linkages in the DHPs. This effect can be attributed to the solubility of lignin monomer in polysaccharides solution (Shigematsu et al., 1994b; Cathala and Monties, 2001), which favors the reaction between monomer and oligomer radicals leading to an "end wise" polymer with an enriched β-*O*-4 content (Sarkanen, 1971). We have also recently shown that DHPs and xylans can interact by non covalent interactions into aggregates in dilute solution (Barakat et al., 2007b) and this association would likely explain the formation of colloidal suspensions of AX-DHP complex. Such a trend would be enhanced by the densification of the AX-DHP system afforded by the increased concentrations of AX and CA. Therefore, preferential location of DHP in these hydrophobic aggregates would permit continuous water removal during the polymerization (Lairez et al., 2005). Hence, increased hydrophobicity of the AX-DHP matrix may promote frequent reactions between the phenoxy radical (Fig. 1, I) and the β-radical (Fig. 1, IV) resulting in an increase of β-*O*-4 linkages. The occurrence of β-*O*-4 rich structures were thus reported in DHP synthesized in aqueous solution containing 1,4-dioxane (Tanahashi and Higuchi, 1990). Similarly, Chioccara et al. (1993) reported that the formation of β-*O*-4 dimer was enhanced with increasing methanol content in a (water–methanol) solvent mixture and Nakamura demonstrated that cyclodextrin could also enhance the β-*O*-4 content of DHPs by forming an inclusion complex with the coniferyl alcohol (Nakamura et al., 2006). In addition, local removal of water will favour the formation of a covalent linkage between arabinoxylan and DHP (Barakat et al., 2007a). Besides the impact of the local environment on coniferyl alcohol reactivity, covalent and non covalent associations between DHP and AX would prevent the precipitation of DHP and oligomers, which could then further react giving rise to high molecular weight DHP intimately associated with AX.

Based on these results, we suggest that the structure of DHPs prepared from coniferyl alcohol in a concentrated AX-DHP system would approximate native lignin more closely than that of DHP prepared in buffer and a dilute solution of polysaccharides. The increases in β-*O*-4 linkages and molecular weight in the presence of highly concentrated polymerization media emphasize the fact that the local density of the polysaccharides–lignin system is critical to the pattern of lignin construction and organisation. This information provides new insights into the assembly of lignified cell wall during lignin deposition.

3. Experimental

3.1. Fluorescence probe study of AX aggregation

AX solutions (0.01–1%) were mixed with pyrene (5×10^{-7} M final concentration). Absorption spectra were

recorded on a Perkins Elmer (lambda 5) UV/vis spectrophotometer. Following excitation at 335 nm wavelength, the emission spectra of pyrene showed vibronic peaks at 372 nm (intensity I_1) and 382 nm (intensity I_3) the relative intensity ($I_1/I_{3)}$ being sensitive to solvent polarity.

3.2. Synthesis of DHPs in buffer and in AX solution

We synthesize dehydrogenation polymers (DHPs) of coniferyl alcohol (CA). In this study, DHPs were also synthesized in the presence of arabinoxylan (AX) (Fig. 1) which is the major hetero-polysaccharide present in angiosperms. The AX, purchased from Megazyme had a molar mass of 75 kDa and an arabinose/xylose ratio of 0.35. They contain less than 1% of glucuronic acid (monosaccharide composition was determined by the method described in Section 3.5) and thus can be considered as arabinoxylans (AX). CA was obtained according to the procedure described by Ludley and Ralph (1996).

Three solutions were prepared for the polymerization experiments.

- Solution A: Solutions of AX at 0.1%, 0.5% and 1% were prepared in phosphate buffer (1/30 N; pH 5.0).
- Solution B: Each weight (100, 500 and 1000 mg) of coniferyl alcohol was dissolved in 3 ml of dioxane and 22 ml of each concentration (0.1%, 0.5% and 1%), respectively of SolnA.
- Solution C: 25 ml of SolnA containing hydrogen peroxide H_2O_2 (2 equiv. compared to coniferyl alcohol) (see Table 1).

End-wise polymerization (Zutropfverfahren method, ZT) was achieved as follows: Soln B and Soln C were pumped over a period of 4 h at 25 °C into 50 ml of each concentration of Soln A containing appropriate amounts of the horseradish peroxidase (E1.11.1.7 purchased from Sigma, 250–300 U/mg. One unit will form 1.0 mg of purpurogallin from pyrogallol after 20 s at pH 6.0 at 20 °C). After 4 h of magnetic stirring, three complexes were formed by the reaction: AX-DHP0.1, AX-DHP0.5 and AX-DHP1, corresponding to AX and CA concentrations of 0.1%, 0.5% and 1%, respectively. Control DHPs (without AX) were prepared in the same conditions at 0.1%, 0.5% and 1% of CA in phosphate buffer (1/30 N; pH 5.0). The control DHPs occurred as precipitates that were collected by centrifugation (10,000 rpm, 10 min), washed with distilled water three times and freeze dried to obtain DHP0.1, DHP0.5 and DHP1 fractions. DHP polymerization in the presence of AX leads to a colloidal suspension with no clear-cut formation of precipitates. The solubilities of the DHPs in AX solutions were further estimated from the concentration of DHPs in the supernatant obtained after centrifugation (10,000 rpm, 10 min) of the reaction mixture. The water-soluble and -insoluble DHPs were separated. The amount of water-soluble DHPs was determined by recording the supernatant absorbance at 280 nm. The proportion of insoluble DHP was calculated from the difference between the calculated amounts of DHP in the reaction mixture based on the starting CA and the amount of soluble DHP after centrifugation.

3.3. Preparation of insoluble and soluble dioxane–water DHPs

After freeze drying, the set of AX-DHP complexes (AX-DHP1, AX-DHP0.5 and AX-DHP0.1) was dissolved in dioxane–water (95:5). Subsequent centrifugation produced two fractions: a dioxane–water soluble fraction (S-AX-DHP) as supernatant and the dioxane–water insoluble fraction (R-AX-DHP) as residue. The S-AX-DHP fractions were filtered through a 0.45 μm filter. After freeze drying, both S-AX-DHP and R-AX-DHP fractions were washed three times with distilled water and again freeze dried.

3.4. Size-exclusion chromatography of acetylated samples in THF

Prior to chromatography, the S-AX-DHP samples (dioxane–water soluble) were subjected to acetylation in an acetic anhydride/pyridine mixture (1:1) for 24 h at 40 °C. The reaction products were then poured into ice water and extracted with dichloromethane. The organic layers were washed with dilute hydrochloric acid, saturated sodium bicarbonate solutions and finally water. The organic fractions were dried over magnesium sulphate and concentrated under reduced pressure to give the acetylated S-AX-DHP. The SEC analyses were performed using a multi-detection system consisting of a pump (model 510, Waters), autosampler (U6K injector, Waters), two Polymer Laboratories (PLgel Mixed D, 5 μm) column and UV-detector (model 920, Waters). Separation in THF was performed by injecting 100 μl of 0.1% of the acetylated samples into thermostatically controlled PLgel columns (40 °C). The flow rate was 1 ml/min. Molar mass evaluation was based on the elution of polystyrene standards.

3.5. Carbohydrate analysis

The quantification of carbohydrate was carried out using HPAEC. 5–10 mg of each sample was hydrolysed by sulphuric acid (H_2SO_4) 12 M for 2 h at room temperature, then diluted to 1 M for 2 h at 100 °C. All samples were filtered (PTFE, 0.45 μm) and 50–100 μl solutions were injected onto a CarboPac PA-1 anion exchange column (4 × 250 mm, Dionex). The monosaccharides were quantified using L-fucose as internal standard.

3.6. Thioacidolysis

AX0-DHPs (DHPs synthesised without AX), S-AX-DHPs (dioxane–water soluble) and R-AX-DHPs (dioxane–water insoluble) were degraded by thioacidolysis to

determine the content of β-*O*-4 linked structures according to Lapierre's procedure (Lapierre et al., 1986). The main degradation products were analysed by GC as TMSi derivatives using a J&W Scientific column (DB 1, 30 m 0.25 mm, 0.25 μm film). Detection was by FID using tetracosane as internal standard.

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