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Peroxidase activity can dictate the *in vitro* lignin dehydrogenative polymer structure

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Abstract

The objective of this study was to assess the influence of the peroxidase/coniferyl alcohol (CA) ratio on the dehydrogenation polymer (DHP) synthesis. The soluble and unsoluble fractions of horseradish peroxidase (HRP)-catalyzed CA dehydrogenation mixtures were recovered in various proportions, depending on the polymerization mode (Zutropf ZT/Zulauf ZL) and HRP/CA ratio (1.6–1100 purpurogallin U $mmol^{-1}$). The ZL mode yielded 0–57%/initial CA of unsoluble condensed DHPs (thioacidolysis yields <200 μmol g^{-1}) with a proportion of uncondensed CA end groups increasing with the HRP/CA ratio (7.2–55.5%/total uncondensed CA). Systematically lower polymer yields (0–49%/initial CA) were obtained for the ZT mode. In that mode, a negative correlation was established between the β-O-4 content (thioacidolysis yields: 222–660 μmol g^{-1}) and the HRP/CA ratio. In both modes, decreasing the HRP/CA ratio below 18 U $mmol^{-1}$ favoured an end-wise polymerization process evidenced by the occurrence of tri-, tetra- and pentamers involving at least one β-O-4 bond. At low ratio, the unsoluble ZT DHP was found to better approximate natural lignins than DHPs previously synthesized with traditional methods. Besides its possible implication in lignin biosynthesis, peroxidase activity is a crucial parameter accounting for the structural variations of *in vitro* DHPs.
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Keywords: Dehydrogenation polymers; Coniferyl alcohol; Horseradish peroxidase; Lignin structure; Glycerol β-aryl ether bonds

1. Introduction

Lignin plays an essential role in plant growth, development and uses. It improves water conduction through tracheary elements, limits pathogen attacks but also restricts the degradation of cell wall polysaccharides by enzymes, thus decreasing feeding value. Although studies on lignin biosynthesis have started in the early-50s (Higuchi, 1990), important findings occurred within the last few years. Facilitated by the combination of molecular biology, genetics, bioinformatics, biochemistry and physiology, these findings underlie the successive updated schemes proposed for the monolignol biosynthetic pathways (Boerjan et al., 2003; Barriere et al., 2004; Ralph, 2005; Sibout et al., 2005; Chiang, 2006). After their synthesis, the monolignols are thought to be transported under a glycosylated form to the cell wall where oxidative polymerization takes place (Steeves et al., 2001). This last step in lignin synthesis still raises questions but the involvement of peroxidases (among others proteins) to catalyze the production of monolignol radicals is quite widely admitted (Onnerud et al., 2002; Boerjan et al., 2003; Ros Barcelo et al., 2004). Formation of the polymer would result from radical couplings between two dehydrogenated compounds, the oxidized monomer and the growing polymer phenoxy radical (endwise polymerization) or two oligomer phenoxy radicals (bulk polymerization) (Brunow, 1998).

Given the colossal complexity of lignin in terms of biosynthesis, structure and interactions with the cell wall polysaccharidic network, the investigation of normal, mutants or transformant plants altered in their lignification profile is not enough for plainly deciphering lignin structure and properties. A variety of model systems have consequently

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been developed, including the *in vitro* oxidase-catalyzed polymerization of lignin precursors into dehydrogenation polymer (DHP) initiated by Freudenberg 50 years ago. As previously reviewed (Boerjan et al., 2003), much of what is known today about the radical coupling process and the parameters governing lignin structure, and more particularly the frequency of the different types of interunit bonds, is due to this system. It offers the main advantage that monolignol types, matrix components and polymerization conditions can be accurately adjusted, which enables a high level of control over the composition, structure and lignin–matrix interactions. As recently reviewed (Grabber, 2005), DHP lignins are supposed to not fully mimic the structure of plant lignins. DHPs are reported as highly condensed polymers (low proportion of β-O-4 linkages) and abnormally rich in coniferyl alcohol (CA) end-groups (15–40%), regardless of whether they are formed under bulk (Zulauf mode) or endwise (Zutropf mode) polymerization conditions (Terashima et al., 1996).

Two main points can be noticed when considering the literature relative to DHPs. The first one concerns the diversity of the parameters and polymerization conditions tested to better understand and possibly control the DHP synthesis: monolignol structure (Ralph et al., 1992, 1995; Ito et al., 2002; Fournand et al., 2003), pH (Terashima et al., 1996; Fournand et al., 2003; Grabber et al., 2003), solvent environment (Houtman, 1999), monolignol addition mode and rate (Saake et al., 1996; Terashima et al., 1996; Grabber et al., 2003), presence of soluble carbohydrates (Higuchi et al., 1971; Terashima et al., 1996; Cathala and Monties, 2001; Lairez et al., 2005) or of a macromolecular template (Terashima et al., 1996; Guan et al., 1997); polymerization under heterogeneous conditions, under homogeneous conditions (De Angelis et al., 1999) or at the air/water interface (Cathala et al., 2004); use of isolated enzymes, plant tissue culture or cell wall model systems (Whitmore, 1978; Fukuda, 1992; Grabber et al., 1996; Terashima et al., 2004); classical use of horseradish peroxidases (HRP) (Syrjanen and Brunow, 1998, 2000), specific peroxidases isolated from plants (Sasaki et al., 2004), enzymes other than peroxidases suspected to be involved in polymerization (Driouich et al., 1992; Sterjiades et al., 1992, 1993) or to orientate the stereospecificity of the process (Davin et al., 1997). The second point is the severe contrast between this diversity of parameters and conditions tested and the fact that little attention has been paid so far to the peroxidase activity, and more precisely to the peroxidase/monolignol ratio. Peroxidase is generally used in large excess with respect to the monolignols and uncontrolled variations of the peroxidase activity within the same study are not rare. If this parameter is sometimes taken into consideration, however (Tanahashi and Higuchi, 1981), systematic investigations of its influence on the DHP structure are lacking.

Our objective was to assess the influence of the peroxidase/monolignols ratio on the DHP synthesis. We investigated a wide range of ratios covering the values reported in the literature. DHPs were prepared both by the continuous Zutropf (ZT) and discontinuous Zulauf (ZL) methods in order to compare the ratio effect, respectively, on an endwise (EW) and bulk polymerization process. Combined analysis of the soluble oligomers and the insoluble polymer shows that it is possible to increase the proportion of β-O-4 bonds and to decrease that of coniferyl alcohol end-groups, thus to better approximate the structure of normal plant lignins. The influence of the HRP/CA ratio on the polymerization mechanisms is discussed, together with its implications on lignin synthesis *in vivo*.

2. Results

2.1. DHPs recovery and fractionation

Both the traditional Zutropf (ZT) and Zulauf (ZL) methods were implemented to prepare DHPs from CA, at a constant 0.340 mol l^{-1} CA concentration but with HRP/CA ratios ranging from 1.6 to 1100 purpurogallin U $mmol^{-1}$. This range covers not only the conditions mentioned in the literature but also unusually low ratios. Two distinct fractions, respectively, soluble and unsoluble in the reaction medium, could be recovered from the dehydrogenation mixtures in various proportions. The soluble and solvent-extractable fraction was found to consist in oligomers with polymerization degrees (PD) inferior to 10 (size exclusion chromatography, SEC and liquid chromatography–mass spectrometry, LC–MS analyses, Figs. 1 and 2), whereas the precipitate is assumed to correspond to a polymer fraction of higher PD. This polymer was recovered in a yield highly dependent on the polymerization mode (bulk or endwise) and HRP/CA ratio (Fig. 3). Above 9 U $mmol^{-1}$, all the reaction conditions lead to a polymer fraction with a yield ranging between 20 and 60 wt%, determined gravimetrically with respect to the total initial CA. Similar yields are described for ZT and ZL methods (Russell et al., 2000; Tobimatsu et al., 2006) whereas the yields of the DHPs synthesized with dialysis tubes do not exceed 10% (Tanahashi and Higuchi, 1981). Such low yields were also observed in the present study for the unusually low HRP/CA ratio (<4.5 and 1.6 U $mmol^{-1}$, respectively, for ZT and ZL DHPs). Conversely, SEC analysis of the soluble fraction indicates an increased proportion of dimers and oligomers when the HRP/CA ratio decreases (Fig. 1). Good agreement was found between SEC and LC combined with a diode array detector, both enabling the detection of dimers and trimers only below 9 U $mmol^{-1}$ (Figs. 1 and 4). It seems thus possible to adjust this ratio in order to avoid the precipitation of the DHP while favoring the formation of high DP soluble oligomers.

2.2. Structure of the oligomers

The oligomers were analyzed by LC combined with electrospray ionization (ESI) mass spectrometry, a method

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