PERGAMON

Phytochemistry 62 (2003) 139–146

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Initial steps of the peroxidase-catalyzed polymerization of coniferyl alcohol and/or sinapyl aldehyde: capillary zone electrophoresis study of pH effect

David Fournand[a], Bernard Cathala[b], Catherine Lapierre[a,*]

[a]*Laboratoire de Chimie Biologique, INRA/INA-PG, Institut National Agronomique, F-78850 Thiverval-Grignon, France*
[b]*INRA/URCA, Équipe Paroi et Biomatériaux Fibreux, CRA 2 Esp R. Garros, F-51686 Reims Cedex, France*

Received 26 July 2002; received in revised form 23 October 2002

Abstract

Capillary zone electrophoresis has been used to monitor the first steps of the dehydrogenative polymerization of coniferyl alcohol, sinapyl aldehyde, or a mixture of both, catalyzed by the horseradish peroxidase (HRP)–H_2O_2 system. When coniferyl alcohol was the unique HRP substrate, three major dimers were observed (β-5, β-β, and β-*O*-4 interunit linkages) and their initial formation velocity as well as their relative abundance varied with pH. The β-*O*-4 interunit linkage was thus slightly favored at lower pH values. In contrast, sinapyl aldehyde turned out to be a very poor substrate for HRP except in basic conditions (pH 8). The major dimer observed was the β,β′-di-sinapyl aldehyde, a red-brown exhibiting compound which might partly participate in the red coloration usually observed in cinnamyl alcohol dehydrogenase-deficient angiosperms. Finally, when a mixture of coniferyl alcohol and sinapyl aldehyde was used, it looked as if sinapyl aldehyde became a very good substrate for HRP. Indeed, coniferyl alcohol turned out to serve as a redox mediator (i.e. "shuttle oxidant") for the sinapyl aldehyde incorporation in the lignin-like polymer. This means that in particular conditions the specificity of oxidative enzymes might not hinder the incorporation of poor substrates into the growing lignin polymer.

Keywords: Horseradish peroxidase; Coniferyl alcohol; Sinapyl aldehyde; Dehydrogenative polymerization; Dehydrodimers; Hydrogen peroxide; Lignin; Lignification; Radical coupling reaction

1. Introduction

Lignin is conventionally defined as a complex hydrophobic network of phenylpropanoid units derived from the oxidative polymerization of one or more of three types of hydroxycinnamyl alcohol precursors, thus giving rise to *p*-hydroxyphenyl, guaiacyl and syringyl subunits in lignin (Boudet et al., 1995; Whetten et al., 1998). These alcohols can react with oxidative enzymes (peroxidases and laccases) and then radically couple at several sites with each other or with the growing oligomer to produce a complex polymer with a variety of intermolecular linkages (Adler, 1977). Unexpected variation in lignin subunit composition has been recently evidenced in mutants and in genetically engineered variants of a diverse range of plant species (Ralph et al., 2001a,b; Pinçon et al., 2001; Jouanin et al., 2000; Lapierre et al., 2000; Sederoff et al., 1999; Piquemal et al., 1998; Yahiaoui et al., 1998). These results highlight the need for a better understanding of the factors controlling the incorporation of phenolic compounds into lignins during the enzyme-initiated oxidative coupling reactions.

The in vitro dehydrogenative polymerization of monolignols has been widely used in plant phenolic research as a model to study lignification. However, the chemical structure and molecular weight of dehydrogenation polymers (DHP) are different from those of native lignin. Among the different intermolecular linkages observed in vivo for a guaiacyl-type lignin (Fig. 1), the β-*O*-4 interunit linkage is predominant and accounts for about half of the total (Adler, 1977). In contrast, in typical dehydrogenative polymerization performed in

* Corresponding author. Tel.: +33-130-815-470; fax: +33-130-815-373.
E-mail address: lapierre@grignon.inra.fr (C. Lapierre).

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PII: S0031-9422(02)00573-3

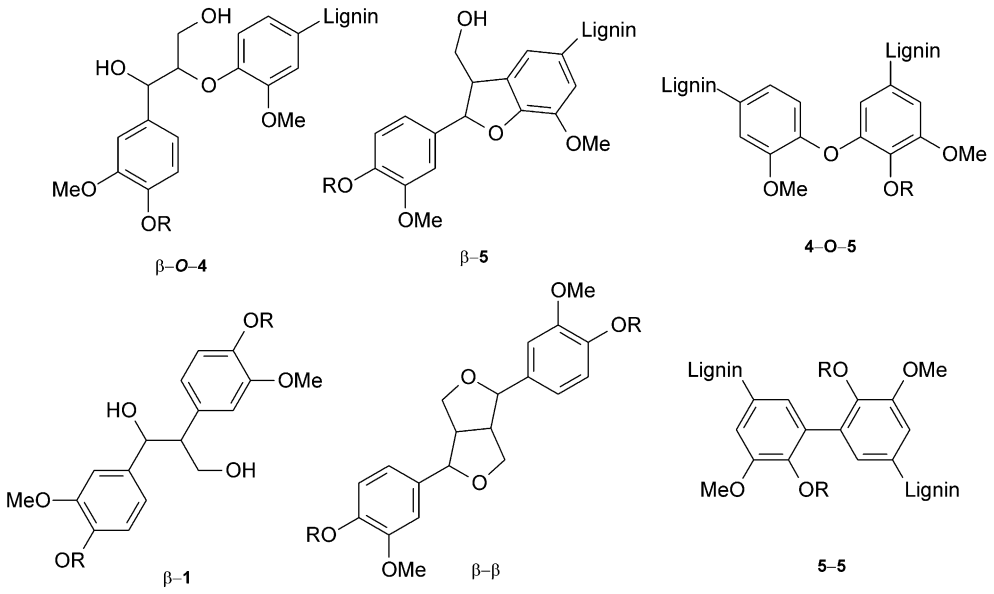

Fig. 1. Main interunit linkages in a guaiacyl-type lignin. R = H or lignin.

vitro with coniferyl alcohol as the unique monomer, the main interunit linkages observed are the β-5, β-β and β-*O*-4 linkages (Fig. 1) (Sarkanen, 1971). Despite this discrepancy between natural and synthetic lignins, the in vitro dehydrogenative polymerization remains a unique tool to study the oxidative coupling reactions. However, because of the time-consuming analytical HPLC or gas chromatography techniques, the chemical structure of DHP are conventionally determined after a given reaction time, without any kinetic consideration. To overcome this analytical limitation, we recently developed a capillary zone electrophoresis technique for the rapid determination of coniferyl alcohol oxidation products (Fournand and Lapierre, 2001).

In this study, we have investigated, with a kinetic point of view, the initial steps of the dehydrogenative polymerization of coniferyl alcohol and/or sinapyl aldehyde as a function of pH. Objectives of this investigation were two-fold: (i) to test the effect of pH on the enzyme-initiated radical coupling reactions and (ii) to elucidate the incorporation mode of sinapyl aldehyde, an unusual monolignol, into lignins.

2. Results and discussion

2.1. Dehydrogenative dimerization of coniferyl alcohol and further oligomerization

When coniferyl alcohol was used as the unique monolignol substrate for the HRP–H_2O_2 system, very high apparent turnover numbers were observed (Table 1). Coniferyl alcohol thus turned out to be rapidly oxidized by HRP. The highest turnover number was observed at pH 6. Whatever the pH, three major dehydrodimers were observed (Fig. 2, **4**, **5**, **6**). They were representative of β-5, β-*O*-4, and β-β interunit linkages. Coupling modes producing 4-*O*-5 or 5-5 bonds

Table 1
Apparent turnover numbers (k) of HRP towards different starting compounds as a function of pH. Initial concentration in the reaction medium was 0.67 mM for each compound

Starting compound	k[a] (s^{-1})		
	pH 4.5	pH 6	pH 8
Coniferyl alcohol	2936	4307	3465
Sinapyl aldehyde	89	123	660
Coniferyl alcohol/sinapyl aldehyde mixture	2182/2112	3053/3863	1628/2526
β-5 Dehydrodimer **4**	Nd	510	nd
β-*O*-4 Dehydrodimer **5**	Nd	132	nd
β-β Dehydrodimer **6**	Nd	1197	nd

[a] The HRP molecular mass used for calculation is 44,000 Da. Each k value results from 2 or 3 experiments. nd, Not determined. k is defined as the number of substrate molecules that can be oxidized by one enzyme molecule per second.

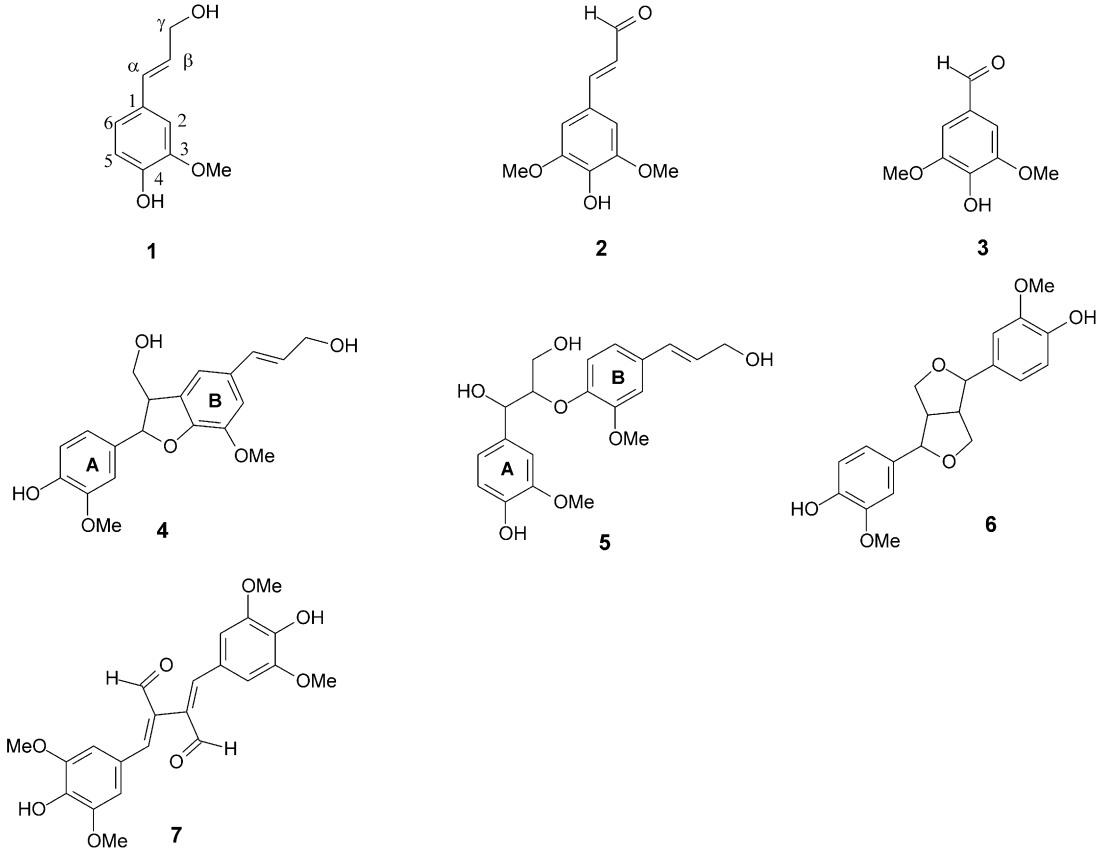

Fig. 2. Chemical structure of coniferyl alcohol, sinapyl aldehyde and their main oxidation products. Coniferyl alcohol **1**, sinapyl aldehyde **2**, syringaldehyde **3**, β-5 dehydrodimer **4**, β-*O*-4 dehydrodimer **5**, β-β dehydrodimer **6**, β-β′-di-sinapyl aldehyde **7**.

were insignificant, if any. This was in accordance with previous results (Sarkanen, 1971; Syrjänen and Brunow, 1998) showing that coupling modes producing a covalent bond at the β-carbon of at least one of the participating radicals were the dominant ones, probably because of a higher reactivity of β-radicals. Relative amount of the three major compounds was also in accordance with literature (Sarkanen, 1971). In all cases, the β-5 dehydrodimer **4** accounted for about half of the total, the β-*O*-4 dehydrodimer **5** and β-β dehydrodimer **6** being formed approximatively in equal amount. Some slight differences were nevertheless observed as a function of pH. As shown in Fig. 3, the relative initial formation rate of the β-*O*-4 dehydrodimer **5** was slightly improved at pH 4.5 (28% instead of 25 and 23% at pH 6 and 8, respectively). As described by Sipilä and Brunow (1991), the formation of the β-*O*-4 dehydrodimer differs from the other two dimers in that it requires the addition of water to an intermediate quinone methide. This water addition proceeds more rapidly at acidic pH (Sipilä and Brunow, 1991). The unidentified products observed in higher amounts at pH 6 and 8 might correspond to quinone methides. These products could be observed by capillary zone electrophoresis (CZE) in large amount during the first five minutes of an oxidative dimerization performed in a concentrated medium containing acetone (data not shown), and their abundance was correlated to a yellow coloration of the reaction medium at the early stage of the oxidation. Quinone methides are known to give such a coloration in solution (Brunow et al., 1989). Moreover, Brunow and co-workers also described a higher stability of quinone methides in alcaline conditions.

The decrease of dehydrodimer concentration as a function of time (Fig. 3, A1–A3) suggests that they are involved in oligomerization reactions. It was nevertheless possible to stop the reaction at a stage where more than 80% of initial coniferyl alcohol appeared in a dimerized form. As for coniferyl alcohol dimerization, the lowest dimer oligomerization was observed at pH 4.5. According to Fig. 3 (A1–A3), it seems that β-β and β-5 dehydrodimers undergo oligomerization more rapidly than β-*O*-4 dehydrodimer. Considering the maximum amount of each compound at the beginning of oligomerization, these results suggest that β-β dehydrodimer **6** is more reactive towards the HRP–H_2O_2 system. To confirm these results, different oxidation reactions were performed using standard dehydrodimers **4**, **5** and **6** as substrate for the HRP–H_2O_2 system. At pH 6, the initial turnover number of β-β dehydrodimer

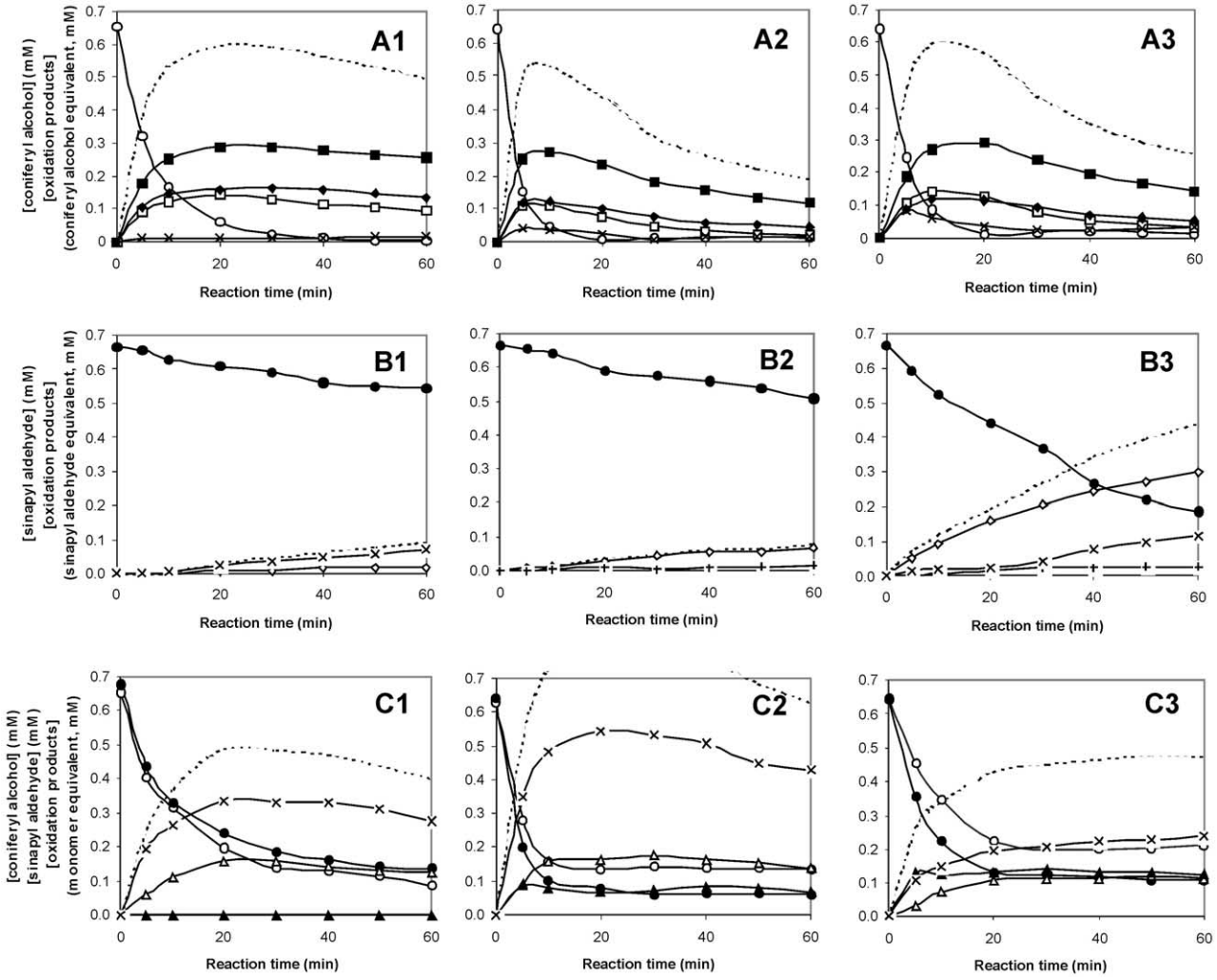

Fig. 3. Formation of dehydrodimers with the HRP–H_2O_2 system as a function of pH. Substrate: (**A**), coniferyl alcohol; (**B**), sinapyl aldehyde; (**C**), equimolar mixture of coniferyl alcohol and sinapyl aldehyde. (**1**), pH 4.5; (**2**), pH 6; (**3**), pH 8. (○), Coniferyl alcohol; (●), sinapyl aldehyde; (■), β-5 dehydrodimer **4**; (◆), β-*O*-4 dehydrodimer **5**; (□), β-β dehydrodimer **6**; (×), unidentified products; (- - -), sum of oxidation products; (◇), β-β′-di-sinapyl aldehyde **7**; (+), syringaldehyde **3**; (△), sum of β-5, β-*O*-4, and β-β dehydrodimers (**4**, **5**, **6**); (▲), sum of syringaldehyde and β-β′-di-sinapyl aldehyde (**3**, **7**).

6 was effectively two-fold and ten-fold higher than that of β-5 dehydrodimer **4** and β-*O*-4 dehydrodimer **5**, respectively (Table 1). This was probably due to the two phenolic sites present in compound **6**, which increased the possibilities of phenoxy radical formation. The turnover number of β-*O*-4 dehydrodimer **5** was surprisingly low and did not correspond to the relative decrease of dimer concentration during oligomerization (Fig. 3, A2). In spite of its lower reactivity towards the HRP-driven oxidation, the β-*O*-4 dehydrodimer **5** might be oxidized by phenoxy radicals from other dehydrodimers, thus acting as "shuttle oxidants".

2.2. Dehydrogenative dimerization of sinapyl aldehyde

When sinapyl aldehyde was used as the unique monomer substrate for the HRP–H_2O_2 system, the apparent turnover numbers were about 30–40-fold lower than those obtained with coniferyl alcohol, except in slightly alcaline conditions (Fig. 3, B1–B3, and Table 1). At pH 8, the apparent turnover number surprisingly increased and was only 6-fold lower than that obtained with coniferyl alcohol. Sinapyl aldehyde thus turned out to be a poor HRP substrate in acidic and neutral conditions. These results highlighted a strict relationship between the HRP substrate specificity and the pH of the reaction medium. Candeias and co-workers recently showed that substrate specificity of peroxidases combined two aspects. The first one is a Michaelis-Menten kinetic control, which is related to dissociation constants of the enzyme-substrate complexes. The second one is a thermodynamic driving force, which corresponds to the reorganization energies of electron-transfer within those complexes and which is related to the difference of

oxidation potentials between the peroxidase and the substrates (Candeias et al., 1997). Both the dissociation constants of the enzyme-substrate complexes and the thermodynamic driving force are dependent on pH and could explain the higher turnover number observed at pH 8 with sinapyl aldehyde as substrate.

Russell et al. (2000) recently showed that the coupling rate of sinapyl aldehyde was lower than that of coniferyl aldehyde (monomethoxylated aldehyde) in spite of a higher oxidation rate (phenoxy radical formation). These authors proposed that sinapyl aldehyde formed a more stable radical responsible for the decreased rate of coupling in the presence of the HRP–H_2O_2 system (Russell et al., 2000). In addition, we could also suggest that pH had an influence on the stability of the sinapyl aldehyde radical which could undergo dimerization more rapidly at high pH values.

After radical coupling, the β,β′-di-sinapyl aldehyde (Fig. 2, **7**) was the main dehydrodimer observed at pH 6 and pH 8 (about 80% of total oxidation products; Fig. 3, B2–B3). This compound exhibited a brown-red coloration. Such interunit linkages might thus partly participate in the red coloration usually observed in cinnamyl alcohol dehydrogenase (CAD) deficient angiosperms (Halpin et al., 1998; Yahiaoui et al., 1998; Baucher et al., 1999). In contrast, this dehydrodimer represented only about 20% of total oxidation products at pH 4.5, the main dehydrodimer being a still unidentified product (Fig. 3, B1).

Syringaldehyde observed at pH 6 and 8 probably resulted from the heterolytic cleavage of the C_α–C_β bond in sinapaldehyde, as previously reported for the veratryl alcohol-mediated oxidation of isoeugenyl acetate by lignin peroxidase (ten Have et al., 1999).

2.3. Dehydrogenative dimerization of a coniferyl alcohol/sinapyl aldehyde mixture

When an equimolar mixture of coniferyl alcohol and sinapyl aldehyde was submitted to oxidation by the HRP–H_2O_2 system, a wide range of oxidation compounds was formed. The apparent turnover numbers determined for each of the starting phenolic substrates revealed unexpected results (Table 1). Coniferyl alcohol still remained a very good substrate for HRP since the apparent turnover numbers were higher than 1500 s^{-1} whatever the pH. On the other hand, whereas sinapyl aldehyde was previously shown to be a poor substrate for HRP when used as the unique starting material, it appeared as a very good substrate (apparent turnover numbers higher than 2000 s^{-1}) when added in combination with coniferyl alcohol (Fig. 3, C1–C3). In other words, coniferyl alcohol promoted the oxidation of sinapyl aldehyde. Two hypotheses could be proposed to account for this observation: (i), coniferyl alcohol may act as a redox mediator (i.e., "shuttle" oxidant) for the sinapyl aldehyde oxidation (Fig. 4A), as previously described for the isoeugenyl acetate or 4-methoxymandelic acid oxidation by lignin peroxidase in the presence of veratryl alcohol (ten Have et al., 1999; Tien and Ma, 1997); (ii), coniferyl alcohol could efficiently reduce compound II and thereby promote the sinapyl aldehyde oxidation by closing the catalytic cycle (Fig. 4B), as previously described for the *p*-anisyl alcohol oxidation by lignin peroxidase in the presence of veratryl alcohol (Koduri and Tien, 1994).

If coniferyl alcohol only served to close the catalytic cycle, then at the most 1 mol sinapaldehyde would be expected to be oxidized per mol of coniferyl alcohol consumed. As shown in Fig. 3 (C2–C3) and Table 1, we observed that sinapyl aldehyde was oxidized more rapidly than coniferyl alcohol at pH 6 and 8, which supports the first redox mediation hypothesis. The apparent turnover numbers reported in Table 1 thus have to be considered as total oxidation rates and not as actual turnover numbers. This oxidation rate was the highest at pH 6, as it was observed when coniferyl alcohol was the unique substrate, but more than 60% of this activity still remained at pH 4.5 and 8.

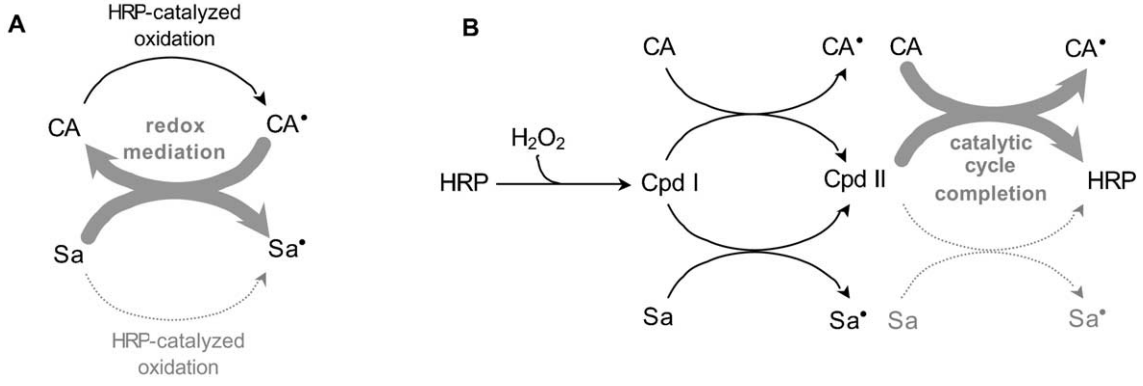

Fig. 4. Proposed mechanism for the coniferyl alcohol-mediated oxidation of sinapyl aldehyde (A) and for the catalytic cycle completion allowed by coniferyl alcohol (B). CA, coniferyl alcohol; Sa, sinapyl aldehyde.

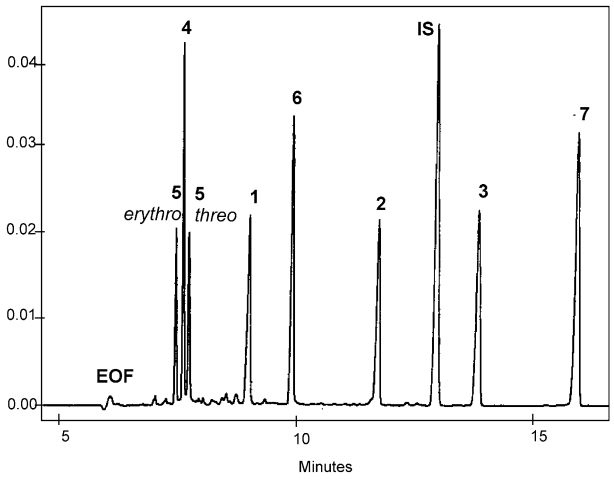

Fig. 5. Electropherogram of a standard mixture. **1**, Coniferyl alcohol; **2**, sinapyl aldehyde; **3**, syringaldehyde; **4**, β-**5** dehydrodimer; **5**, β-*O*-4 dehydrodimer; **6**, β-β dehydrodimer; **7**, β,β′-di-sinapyl aldehyde; IS, internal standard; EOF, electroosmotic flow. See Fig. 2 for compound structures.

Many other oxidation products were observed in addition to the different homodimers previously formed from each of the phenolic compounds as unique substrate. For figure clarity (Fig. 3, C1–C3), the previously identified products were grouped in two classes. Class I was composed of β-5, β-β, and β-*O*-4 dehydrodimers resulting from coniferyl alcohol oxidation and class II was composed of β,β′-di-sinapyl aldehyde and syringaldehyde. Most of the other non identified oxidation products probably resulted from cross-coupling reactions. As shown in Fig. 3 (C1–C3), the ratio between class I and class II compounds was strictly dependent on pH. At pH 4.5, the class II compounds could not be detected whereas they were prominent at pH 8, which confirmed the highest reactivity of sinapyl aldehyde in alcaline conditions.

These results highlighted the importance of pH on the radical coupling modes of a mixture of monolignols. Determination of the actual pH value within plant cell walls is very difficult, but the notion does exist in so far as enzymes need water to be active and monolignols have to diffuse from cytosol to wall. This pH value is probably not constant since cell wall composition (pectin, protein and lignin content) varies in time and space. Thus, pH might be a physico-chemical parameter which participates in the spatio-temporal variability of lignins, in addition with the different expression levels of genes encoding enzymes involved in the lignification pathway.

Moreover, the incorporation rate of monolignols into the growing polymer is often related to the reactivity of these monolignols towards oxidative enzymes (peroxidases and laccases). This study revealed that a poorly reactive phenolic species (i.e., sinapyl aldehyde) could be oxidized at a rate higher than a more reactive one (i.e., coniferyl alcohol) provided that the oxidation potentials of the two species enable the redox mediation phenomenon.

3. Experimental

3.1. Chemicals

Sinapyl aldehyde and syringaldehyde were purchased from Aldrich Chemical Co (Milwaukee, WI). Coniferyl alcohol was synthesized according to the method of Ludley and Ralph (1996). Horseradish peroxidase type II (200 purpurogallin units per mg solid) was purchased from Sigma Chemical Co (St. Louis, MO). The internal standard 3,4,5-trimethoxybenzoic acid was obtained from Fluka (Buchs, Switzerland) and hydrogen peroxide (35 wt.% solution) from Acros (New Jersey). Reaction buffers were prepared from sodium dihydrogen phosphate (NaH_2PO_4, $2H_2O$) and di-sodium hydrogen phosphate (Na_2HPO_4, $2H_2O$) (analytical reagents; Prolabo, France).

3.2. Preparation of coniferyl alcohol homodehydrodimer models

Coniferyl alcohol homodehydrodimers were purified by preparative chromatography (from 1 g of coniferyl alcohol), using Et_2O/CH_2Cl_2/MeOH (20/5/1, v/v/v) as an eluent, from a HRP-catalyzed oxidative dimerization of coniferyl alcohol. The reaction was performed at pH 4.5 without internal standard in a 77-fold concentrated medium [containing 18.5% (v/v) acetone] compared to the standard oxidation reaction. ^{13}C NMR spectra were recorded on a Bruker AC250 spectrometer.

β-5 Dehydrodimer (Fig. 2, **4**) (yield 43%): ^{13}C NMR (62.9 MHz, acetone-d_6), δ 54.68 (Aβ), 56.19 ($AOCH_3$), 56.29 ($BOCH_3$), 63.37 (Bγ), 64.53 (Aγ), 88.49 (Aα), 110.39 (A2), 111.53 (B2), 115.65 (A5), 116.02 (B6), 119.53 (A6), 128.24 (Bβ), 130.32 (B5), 130.53 (Bα), 131.85 (B1), 134.27 (A1), 145.10 (B3), 147.22 (A4), 148.33 (A3), 148.86 (B4).

Racemic β-*O*-4 dehydrodimer (Fig. 2, **5**) (yield 7%): ^{13}C NMR (62.9 MHz, acetone-d_6), δ 56.18 ($AOCH_3$), 56.24 ($BOCH_3$), 61.74/61.81 (Aγ, *erythro*/*threo*), 63.24 (Bγ), 73.70/73.74 (Aα, *erythro*/*threo*), 86.47/88.18 (Aβ, *erythro*/*threo*), 110.72/110.86 (B2, *threo*/*erythro*), 111.33 (A2), 115.13/115.21 (A5, *erythro*/*threo*), 119.05/119.34 (B5, *erythro*/*threo*), 120.21/120.30 (B6, *erythro*/*threo*), 120.40/120.48 (A6, *erythro*/*threo*), 129.48/129.52 (Bβ, *erythro*/*threo*), 129.87 (Bα), 132.71/132.82 (B1, *erythro*/*threo*), 133.73/134.16 (A1, *threo*/*erythro*), 146.59/146.77 (A4, *erythro*/*threo*), 147.93/147.99 (A3, *erythro*/*threo*), 148.46/149.05 (B4, *erythro*/*threo*), 151.56/151.74 (B3, *threo*/*erythro*).

β-β Dehydrodimer (Fig. 2, **6**) (yield 9%): ^{13}C NMR (62.9 MHz, acetone-d_6), δ 55.15 (Cβ), 56.17 (OCH_3), 72.14 (Cγ), 86.57 (Cα), 110.50 (C2), 115.50 (C5), 119.56 (C6), 134.06 (C1), 146.77 (C4), 148.26 (C3).

3.3. Preparation of sinapyl aldehyde homodehydrodimer models

β,β′-Di-sinapyl aldehyde was purified by TLC (from 0.58 g of sinapyl aldehyde) from a HRP-catalyzed oxidative dimerization of sinapyl adehyde. The reaction was performed at pH 8 without internal standard in a 77-fold concentrated medium (containing 18.5% (v/v) acetone) compared to the standard oxidation reaction.

β,β′- Di -sinapyl aldehyde (Fig. 2, **7**) (yield 22%): ^{13}C NMR (62.9 MHz, acetone-d_6), δ 56.63 (OCH_3), 109.19 (C2,C6), 125.95 (Cβ), 134.15 (C1), 140.56 (Cα), 149.09 (C3,C5), 155.20 (C4), 194.52 (Cγ).

3.4. Standard oxidation reactions

To 200 μl of 2 mM phenolic substrate (in 25 mM NaH_2PO_4–Na_2HPO_4 buffer), 200 μl of 25 mM NaH_2PO_4–Na_2HPO_4 buffer (or 200 μl of second phenolic substrate), 50 μl of 4 mM aqueous internal standard (3,4,5-trimethoxybenzoic acid), 50 μl of 8 mM H_2O_2 and 100 μl of HRP type II solution in 25 mM NaH_2PO_4–Na_2HPO_4 buffer (typically 100 ng/ml) were added. Controls containing 25 mM NaH_2PO_4–Na_2HPO_4 buffer in place of the enzyme were used for time zero. Mixtures were kept at 30 °C and aliquots of the oxidation reaction medium (50 μl) were regularly taken, mixed with 50 μl to stop the reaction and placed at −20 °C prior to capillary electrophoresis.

The apparent turnover numbers (k) of HRP towards different starting compounds was defined as the number of substrate molecules that can be oxidized by one enzyme molecule per second. It was calculated as the ratio of the apparent V_{max} (expressed in mol l^{-1} of substrate used per sec) to the molar concentration of enzyme (the HRP molecular mass used for calculation is 44 kDa).

3.5. Capillary zone electrophoresis

Capillary zone electrophoresis was performed as previously described for the quantitative determination of coniferyl alcohol oxidation products (Fournand and Lapierre, 2001). The same conditions were used for the quantitative determination of sinapyl aldehyde or coniferyl alcohol/sinapyl aldehyde mixture oxidation products. Using 3,4,5-trimethoxybenzoic acid as an internal standard (IS), the molar response factors of compounds **1–7** were 1.40, 2.35, 1.90, 0.67, 0.97, 0.77, and 2.00, respectively. The different compounds were detected in the following order: **5** (*erythro* form), **4**, **5** (*threo* form), **1**, **6**, **2**, IS, **3**, and **7**, as shown in Fig. 5.

Acknowledgements

We are grateful to the Institut National de la Recherche Agronomique (INRA, France) for the special ANS grant received for this study.

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