

Oxidative Cross-Linking of Pentosans by a Fungal Laccase and Horseradish Peroxidase: Mechanism of Linkage Between Feruloylated Arabinoxylans¹

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ABSTRACT

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The potential of a laccase from the fungus *Pycnoporus cinnabarinus* to cross-link feruloylated soluble wheat arabinoxylans was investigated using capillary viscometry, size-exclusion HPLC, and reverse-phase HPLC of phenolic compounds. The laccase results were compared with those for a hydrogen peroxide/horseradish peroxidase system. The oxidants provoked an increase in viscosity of a 0.2% (w/v) arabinoxylan solution. A gel was formed after 30 min with laccase. Hydrogen peroxide was consumed rapidly before a gel could be formed. Free ferulic acid, methyl ferulate, and vanillic acid inhibited the gelation, whereas fumaric acid had no

effect. This suggests that the aromatic ring, and not the propenoic chain of ferulic acid, was the initiating site for arabinoxylan cross-linking. Ferulic acid and its 8-*O*-4', 8-5', and 5-5' dehydrodimers were present in nonoxidized arabinoxylans. Upon oxidation, the 8-8' and 8-5' benzofuran dehydrodimers appeared and the 8-*O*-4' and 8-5' dimers increased. The production of dimers was proportional to the consumption of ester-bound ferulic acid. In cross-linked arabinoxylans, the major dimers were 8-5' benzofuran, 8-8', and 8-*O*-4', whereas the 5-5' dehydrodimer remained at the same level as in the nonoxidized solution.

Pentosans, the major nonstarch polysaccharides of wheat flours, occur as water extractable (WEP) and water unextractable forms. They are mostly composed of arabinoxylans (Izydorczyk and Biliaderis 1995). The basic structure of arabinoxylans is a linear backbone of xylan that carries α -L-arabinose residues on C-2 or on both C-2 and C-3 of certain xylose residues. Some of the primary alcohol functions of arabinose can be esterified by ferulic acid (Fig. 1). WEP form highly viscous solutions and are able to gel through ferulic acid covalent cross-linking (Geissman and Neukom 1973) with some chemical and enzymic oxidizing systems (free-radical-generating agents). This unique property of WEP was first described by Durham (1925).

Different oxidizing agents (ammonium persulphate, ferric chloride, hydrogen peroxide/peroxidase [H_2O_2 /POD], and potassium periodate) can provoke arabinoxylan solutions to gel (Izydorczyk and Biliaderis 1995). Most of the studies in this field have used H_2O_2 /POD as oxidizing agents (Neukom and Markwalder 1978, Hosney and Faubion 1981, Izydorczyk et al 1990, Moore et al 1990, Girhammar and Nair 1995). Numerous hypotheses concerning the mechanism of oxidative gelation have been developed. Moore et al (1990) and later Vinkx et al (1991) found that ferulic acid, vanillic acid, and cysteine (but not fumaric acid) inhibited the oxidative gelation of soluble arabinoxylans in an H_2O_2 /POD system. These results support the hypothesis that the aromatic ring of ferulic acid (Geissman and Neukom 1973, Neukom and Markwalder 1978, Moore et al 1990, Vinkx et al 1991), and not the double bond (Sidhu et al 1980a,b; Hosney and Faubion 1981; Thibault et al 1987), serves as a cross-linking center for the arabinoxylan polymerization. Notwithstanding, Ralph et al (1994), Parr et al (1996), and Waldron et al (1996) have found different ferulic acid dimers in vegetable cell walls formed through phenoxy radical intermediates which allow ring and propenoic chain participation.

Laccase, or *p*-diphenol-oxygen oxidoreductase (EC 1.10.3.2), is a widely distributed enzyme in plants and microorganisms. It was discovered by Yoshida (1883) in the latex of the Japanese lacquer tree *Rhus vernicifera*. It is a copper-containing enzyme that catalyzes the oxidation of a wide variety of phenolic substrates. With oxygen, it induces a catalytic oxidation of phenols to free radical products (semiquinones, aryloxy radicals) that polymerize through

nonenzymic reactions, leaving water as the sole oxygen reduction product (Brown 1967, Malmström et al 1975, Holwerda et al 1976).

The aim of this work was to study the potential of laccase from the fungus *Pycnoporus cinnabarinus* MIC11 (Falconnier et al 1994) to cross-link arabinoxylans. The effect on gelation of methyl ferulate, ferulic, vanillic, and fumaric acids was studied to understand the mechanism of the reaction. These compounds were considered analogs of the ferulic acid of arabinoxylans. Results were compared to the H_2O_2 /POD system, used here as a reference.

MATERIALS AND METHODS

Arabinoxylans

WEP were purified from the wheat flour cultivar Thésée according to Faurot et al (1995). Protein-free water-extractable arabinoxylans (WEAX) were purified from WEP by amylase-protease treatment followed by 60% (v/v) ethanol precipitation (Rouau and Moreau 1993). WEAX solutions (0.2%, w/v) in 0.1M sodium acetate buffer (pH 5.0) were used.

Enzymes

Amyloglucosidase from *Aspergillus niger* 75 U/mg (Merck, Darmstadt, Germany) and Pronase from *Streptomyces griseus* 700 U (Boehringer, Mannheim, Germany) were used to purify arabinoxylans. Horseradish peroxidase (POD) type I 96 PU/mg was purchased from Sigma Chemical Co. (St. Louis, MO). Laccase (solution in 35% glycerol) was obtained from a culture supernatant of *Pycnoporus cinnabarinus* MIC11 kindly supplied by M. Asther from the Laboratoire de Biotechnologie des Champignons Filamenteux-INRA (Marseille, France).

Laccase activity was measured at 25°C by mixing 5 μ L of laccase solution with 895 μ L of 0.1M sodium acetate buffer (at different pH levels) and 100 μ L of syringaldazine (0.216 mM in methanol). Absorbance was read at 530 nm after 1 min of reaction. Under these reaction conditions, the optimum pH was \approx 5.0 with an activity of 0.04 nkat/ μ L (nmol/sec/ μ L). Laccase presented neither protease nor arabinoxylanase activities, and it was able to oxidize catechol, *p*-coumaric acid, and guaiacol.

Reagents

Hydrogen peroxide (H_2O_2) 30% (w/w), ferulic acid, fumaric acid, syringaldazine, and vanillic acid, were purchased from Sigma Chemical Co. Methyl ferulate (0.49M in methanol) was kindly provided by C. B. Faulds from the Institute of Food Research (Norwich Laboratory, England). Mixture of standard dimers of ferulic acid in known amounts was kindly supplied by J. Ralph

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from the U.S. Dairy Forage Research Center, USDA-ARS, and Department of Forestry, University of Wisconsin.

Reaction Mixes

Reaction mixes were prepared as 2 mL of WEAX 0.2% (w/v) + 50 μ L of A + 50 μ L of B. In control samples, A was a 0.1M sodium acetate buffer (pH 5.0), B was an enzyme solution of 50 μ L of laccase (0.5 nkat/mg of WEAX) or 25 μ L of H₂O₂ (5 \times 10⁻³ μ mol/mg of WEAX) + 25 μ L of POD (5.52 \times 10⁻³ PU/mg of WEAX). H₂O₂ and POD concentrations were chosen to provoke the same increase in viscosity as laccase for the first 30 min of reaction.

To study the effect of different compounds on gelation, A was substituted by one of the model compound solutions (ferulic acid, methyl ferulate, fumaric acid, or vanillic acid). Solution A (50 μ L) contained the model compound in a molar ratio (MR) of 0.17, 1.7, 1, and 10, compared to the ferulic acid initially present in 4 mg of arabinoxylan (quantified by reverse-phase [RP] HPLC). B was one of the enzyme solutions. In blanks, B enzyme solutions were substituted by buffer.

Depending on subsequent analysis, reactions were stopped in different ways. For RP-HPLC, 1 mL of 4N NaOH was added to 1 mL of the reaction mix (final pH \approx 12.0). For size-exclusion [SE] HPLC, 300 μ L was filtered (2.7 μ m) before stopping reaction by freezing. For WEAX determination, the same volume of 4N NaOH was added.

WEAX Determination

WEAX concentration was determined according to the semi-automated method of Rouau and Surget (1994) using an Evolution

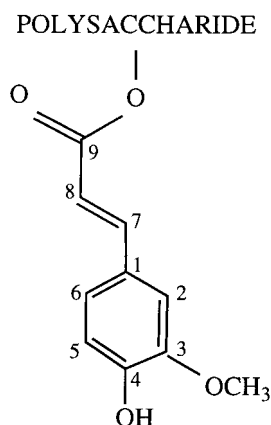


Fig. 1. Structure of ferulic acid esterified to arabinoxylan.

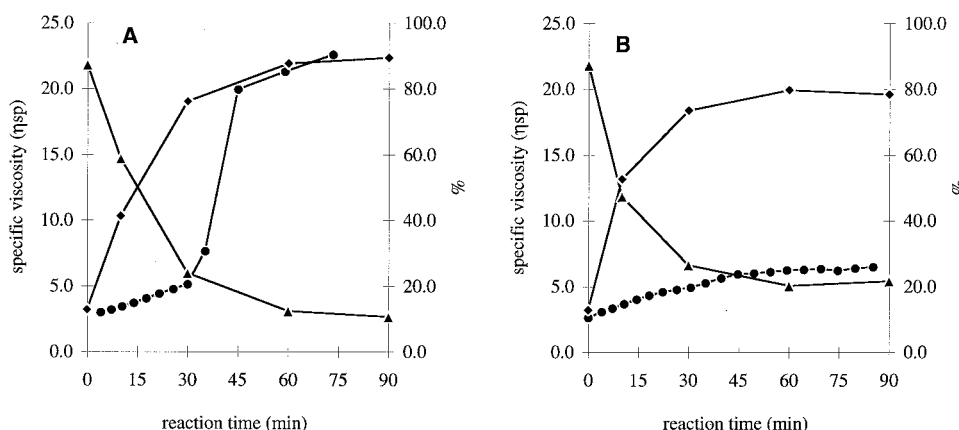


Fig. 2. Effect of laccase (A) and H₂O₂/POD (B) on arabinoxylans. Capillary viscometry (●), total ferulic acid consumption (-*cis* + -*trans* isomers) (▲), and total dimer apparition (◆).

II auto-analyzer (Alliance Instruments, France). A portion (200 μ L) of the filtered (2.7 μ m) reaction mix was mixed with 200 μ L of 4N NaOH to stop the reaction and then diluted 25-fold.

Analysis of Alkali-Labile Phenolics

Reaction mix (1 mL) added to 1 mL of 4N NaOH was incubated under argon for 2 hr at 35°C in the dark. After adding 3,4,5-trimethoxy-*trans*-cinnamic acid (TMCA, internal standard, 5 μ g), the pH was adjusted to 2.0 \pm 0.02 with HCl. Phenolic acids were extracted twice with 2 mL of ether. The ether phase was transferred to an amber test tube and evaporated at 30°C under argon. Methanol (1 mL) was added and samples were filtered (0.45 μ m) then injected (20 μ L) into RP-HPLC using an Interchrom (Interchim, Montluçon, France) Rsil C₁₈ 5- μ m column (250 \times 4.6 mm). Detection was by UV absorbance at 320 nm. Gradient elution was performed using acetonitrile and sodium acetate buffer 0.05M (pH 4.0) at 1 mL/min at 35°C, in linear gradients from 15/85 to 35/65 in 30 min, from 35/65 to 60/40 in 0.5 min, from 60/40 to 15/85 in 4.5 min, and finally maintained at 15/85 for 5 min. A Waters 996 photodiode array detector (Millipore Co., Milford, MA) was used to record the ferulic acid and its dimer spectra. The solvents were of HPLC grade and mobile phase was sparged with helium. Dimer identification and quantification was performed by using the standard dimers in known amounts. This mixture served to calculate the response factor for the main dimers related to the system used and the internal standard (TMCA). Dimer spectra were analyzed by comparing them with those already published (Parr et al 1996, Waldron et al 1996) and with those from the dimers mixture.

SE-HPLC

SE-HPLC was performed at 35°C using a Waters (Millipore Co.) Ultrahydrogel 1000, 10- μ m column (7.8 \times 300 mm), with a pullulan limit exclusion of 10⁶ Da, eluted with 0.1M sodium acetate buffer (pH 5.0) at 0.6 mL/min. A 20- μ L of the filtered (2.7 μ m) reaction mix was injected. The eluent was monitored with a Waters 410 differential refractometer.

Capillary Viscometry

Flow times of reaction mixes (2 mL) were measured at 25°C using an AVS 400 (Schott Geräte, Hofheim/Ts, Germany) capillary viscometer equipped with an Oswald capillary tube (water flow time 29.41 sec). Relative viscosities (η_{rel}) and specific viscosities ($\eta_{sp} = \eta_{rel} - 1$) were calculated using 0.1M sodium acetate buffer (pH 5.0) flow time.

General

Protein content in WEAX was determined according to the Dumas method (AOAC 1990, Bicsak 1993), using an NA 2000

nitrogen and protein analyzer (Fisons Instruments, Arcueil, France) ($N \times 5.7$). Moisture content was determined by oven drying at 130°C for 2 hr.

The coefficients of variation for the viscometry, arabinoxylan, and ferulic acid determination procedures were 3, 3, and 4%, respectively. Results were expressed as mean values of replicates.

RESULTS AND DISCUSSION

Effect of Laccase on WEAX Solutions

The arabinoxylan content of WEAX was 82.5% db (95.2% total carbohydrates) with a 0.55 arabinose-to-xylose ratio, the ferulic acid content was 11.2 nmol/mg of WEAX, and the protein content was 0.7%.

The oxidative cross-linking of WEAX catalyzed by the laccase was evidenced by the thickening of WEAX solutions, changes in WEAX molecular weight and solubility, and consumption of ferulic acid accompanied by the formation of ferulic acid dimers. Capillary viscometry, SE-HPLC, and RP-HPLC were used to follow these changes.

The effect of laccase on WEAX solutions was greatly influenced by arabinoxylan and enzyme concentrations and by the pH of the reaction mixture. At pH 5.0 (optimum for laccase activity), the specific viscosity (η_{sp}) increased with enzyme concentration. In a 0.2% (w/v) WEAX solution, 0.5 nkat of laccase/mg of WEAX gave a clear increase in η_{sp} for 25 min without gelation. With 1 nkat, after a rapid increase in η_{sp} , a gel was formed in 14 min. A dose of 0.05 nkat caused almost no thickening. WEAX solutions with concentrations <0.2% (w/v) thickened very slowly, whereas at >0.2% (w/v) a fast gelation occurred. To observe significant variations in viscosity before gelation and to study the

effect of different model compounds on WEAX cross-linking, all further experiments were performed at pH 5.0 with 0.2% (w/v) WEAX solutions and 0.5 nkat of laccase/mg of WEAX.

Comparison of the Effects of Laccase and H₂O₂/POD

Laccase was compared with the H₂O₂/POD system already used in numerous studies for the oxidative effect on arabinoxylan solutions. The dose of H₂O₂/POD was chosen so that the slope of viscosity increase was similar to the one obtained in laccase experiment for the first 30 min of reaction (Fig. 2). From this point, viscosity profiles differed. With laccase, η_{sp} increased linearly and rapidly until gelation at 45 min, whereas with H₂O₂/POD, η_{sp} raised slowly until it reached a pseudo-plateau after 45 min of reaction. Between 0 and 30 min, η_{sp} increased approximately twofold for both systems, and the ferulic acid content decreased similarly by 6.2 nmol/mg of WEAX (Table I) (55.4% of initial ferulic acid concentration) (Fig. 2A) for laccase and by 7.3 nmol/mg of WEAX (Table I) (65.2% of initial ferulic acid concentration) (Fig. 2B) for H₂O₂/POD. Between 30 and 60 min, laccase increased η_{sp} 4.2-fold by consuming less ferulic acid than between 0 and 30 min by 2.6 nmol/mg of WEAX (Table I) (78.6% of initial ferulic acid concentration) (Fig. 2A). During the same period, H₂O₂/POD increased η_{sp} only 1.3-fold with a ferulic acid consumption of 0.9 nmol/mg of WEAX (Table I) (73.2% of initial ferulic acid concentration) (Fig. 2B). The increase in phenolic dimers was proportional to the consumption of ferulic acid for both systems (Fig. 2).

For both oxidizing systems, the results obtained between 0 and 30 min reflected the initial formation of covalent linkages between ferulic acid residues of adjacent arabinoxylan chains that provoked constant increases in viscosity. For the second 30 min, in

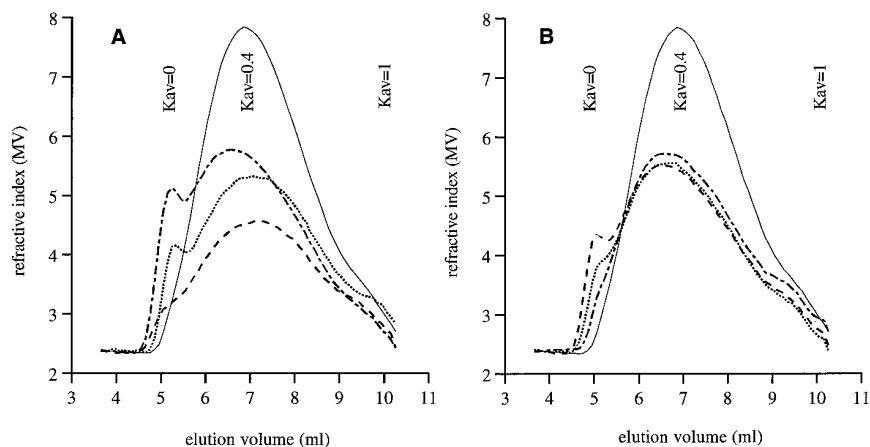


Fig. 3. Size-exclusion HPLC elution profiles showing the effect of laccase (A) and H₂O₂/POD (B) on water-extractable arabinoxylans. Blank samples (without enzyme) (—). Reaction times: 10 (· · · · ·), 30 (— — —), and 90 (— — —) min.

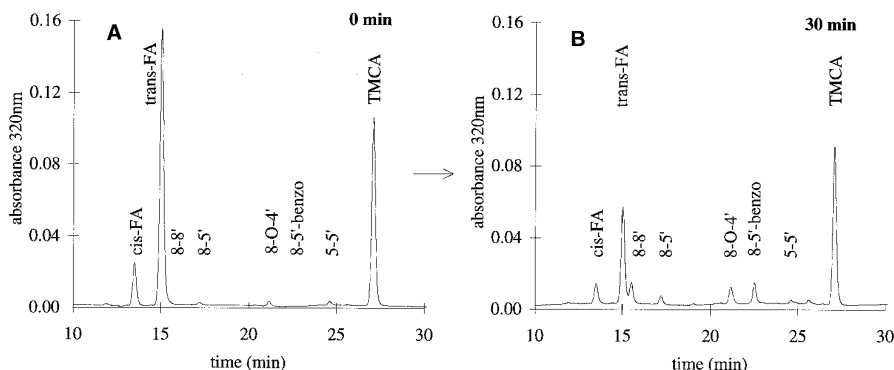


Fig. 4. Reverse-phase HPLC profiles of alkali-labile phenolics of native water-extractable arabinoxylans (WEAX) (0 min of reaction) (A) and WEAX with laccase (30 min of reaction) (B). FA = ferulic acid, TMCA = 3,4,5-trimethoxy-*trans*-cinnamic acid, benzo = benzofuran.

the case of laccase, once sufficient cross-links were formed, a few additional linkages between soluble cross-linked material provoked sharper increases in viscosity and formed a gel. In the H₂O₂/POD system, the pseudo-plateau was reached when all H₂O₂ was consumed. The H₂O₂ limiting factor was demonstrated by adding different amounts of H₂O₂ (data not shown).

The available ferulic acids esterified to arabinoxylans are the initiation site for polymerization. They are converted by laccase into very reactive semiquinones that react nonenzymatically to give different dimers (8-8', 8-5', 8-O-4', 8-5' benzofuran, and 5-5') (Table I). Although compounds of higher molecular weight could be produced with free ferulic acid, the steric hindrance by arabinoxylan chains probably limits the coupling products to the dimer form. The enzyme continues to oxidize all accessible ferulic acids

until the three-dimensional network impedes further polysaccharide chains or enzyme mobilities (Izydorzyc et al 1991, Izydorzyc and Biliaderis 1995). In the H₂O₂/POD system, the peroxidase catalyses the oxidation reaction. H₂O₂ acts as the acceptor and the esterified ferulic acid as the donor of hydrogen atoms (Whitaker 1994). Phenoxy radicals are formed that can further dimerize. The reaction proceeds until H₂O₂ or ferulic acid are consumed or until the mobility of the reagents is depressed by the gelation.

The viscosity results (Fig. 2) were confirmed by SE-HPLC (Fig. 3A and B) which measured the molecular weight distribution and the insolubilization of arabinoxylans. Samples were filtered before injecting the column so that material insolubilized by the cross-linking reaction was removed. The nonoxidized WEAX was eluted as a single peak at K_{av} ≈ 0.4. Upon oxidation, the amount of soluble material decreased dramatically. The K_{av} of the peak first shifted toward a lower value (10 min, K_{av} ≈ 0.3), with the occurrence of a second peak of excluded material reflecting the polymerization of soluble arabinoxylans. Then the loss of material by insolubilization slowed down (30, 90 min), and the molecules remaining in solution could not participate in the gel formation.

TABLE I

Ferulic Acid and Dimer Contents of Water-Extractable Arabinoxylan (WEAX) and Laccase or H₂O₂/POD Solutions After Reaction with Free Ferulic Acid

Reaction Time (min)	nmol/mg of WEAX					
	tot-FA ^a	8-8'	8-5'	8-O-4'	8-5' benzo	5-5'
WEAX + laccase						
Blank	11.2	0.0	0.2	0.4	0.0	0.3
10	9.2	0.9	0.3	1.0	0.9	0.2
30	5.0	2.0	0.6	2.2	2.6	0.1
60	2.4	2.0	0.8	2.5	2.9	0.1
90	1.7	1.9	0.8	1.9	2.4	0.1
+ Free ferulic acid (MR = 0.17) ^b						
Blank	14.9	0.0	0.1	0.4	0.0	0.2
10	8.8	1.0	0.2	0.7	1.1	0.1
30	4.9	1.7	0.6	1.7	2.0	0.2
60	2.3	1.8	0.7	1.8	2.3	0.3
90	1.6	2.0	0.9	2.0	2.6	0.1
+ Free ferulic acid (MR = 1.7)						
Blank	30.7	0.0	0.2	0.4	0.0	0.2
10	9.5	2.6	0.8	0.8	4.7	0.1
30	4.5	2.5	0.7	1.1	3.6	0.1
60	2.1	2.1	0.7	1.1	2.6	0.1
90	1.4	2.1	0.6	0.9	2.3	0.0
+ Free ferulic acid (MR = 1.7) (no saponification)						
Blank	19.2	0.0	0.0	0.0	0.0	0.0
10	0.5	0.2	0.0	0.2	1.0	0.0
30	0.0	0.2	0.0	0.0	0.9	0.0
60	0.0	0.3	0.0	0.2	0.5	0.1
90	0.3	0.0	0.0	0.2	0.0	0.0
WEAX + H ₂ O ₂ /POD						
Blank	11.2	0.0	0.2	0.4	0.0	0.3
10	7.0	1.1	0.4	1.1	1.1	0.2
30	3.9	1.6	0.5	1.5	1.6	0.2
60	3.0	1.7	0.7	1.7	1.8	0.2
90	3.6	1.6	0.7	2.0	2.1	0.2
+ Free ferulic acid (MR = 0.17)						
Blank	14.9	0.0	0.1	0.4	0.0	0.2
10	7.4	1.4	0.4	1.1	1.2	0.2
30	4.7	1.9	0.7	1.7	1.9	0.2
60	3.9	1.9	0.6	1.6	1.8	0.2
90	4.2	1.5	0.6	1.7	2.0	0.2
+ Free ferulic acid (MR = 1.7)						
Blank	30.7	0.0	0.2	0.4	0.0	0.2
10	17.3	0.0	0.5	0.5	3.2	0.1
30	15.2	1.8	0.4	0.4	3.1	0.1
60	15.3	2.1	0.4	0.6	3.3	0.1
90	16.9	2.3	0.4	0.6	3.8	0.2
+ Free ferulic acid (MR = 1.7) (no saponification)						
Blank	19.2	0.0	0.0	0.0	0.0	0.0
10	6.4	0.0	0.0	0.0	0.9	0.0
30	5.8	0.0	0.0	0.0	1.5	0.0
60	5.9	0.8	0.0	0.0	1.8	0.0
90	5.9	1.1	0.0	0.0	1.7	0.0

^a totFA = *cis* + *trans* ferulic acid.

^b MR = molar ratio between compound and esterified ferulic acid in 4 mg of WEAX.

Formation of Ferulic Acid Dimers

Several peaks with typical cinnamic acid spectra occurred along with *trans*-ferulic acid on RP-HPLC chromatograms (Fig. 4). Comparing these spectra with those obtained from the mixture of standard dimers (Ralph et al 1994) and with those reported by Parr et al (1996) and Waldron et al (1996) allowed us to identify ferulic acid dehydrodimers. In the native WEAX, the dimers 8-O-4' (major), 8-5' acyclic form, and 5-5' were detected (Table I). These results agree with Ralph et al (1994), Parr et al (1996), and Bartolomé et al (1997), who found that different ferulic acid dehydrodimers occur naturally in plant cell walls, including benzofuran and acyclic forms of 8-5', 8-8', 8-O-4', and 5-5' (always present in small amounts). In the nonoxidized WEAX, the 8-5' benzofuran and 8-8' dimers were not detected.

Upon oxidation of WEAX by either laccase or H₂O₂/POD, a decrease in ferulic acid occurred that was balanced by the formation of dehydrodimers (Fig. 4, Table I). The concentration of the

TABLE II

Ferulic Acid and Dimer Contents of Water-Extractable Arabinoxylan (WEAX) and Laccase Solutions After Reaction with Fumaric and Vanillic Acids

Reaction Time (min)	nmol/mg of WEAX					
	tot-FA ^a	8-8'	8-5'	8-O-4'	8-5' benzo	5-5'
Blank	11.2	0.0	0.1	0.3	0.0	0.2
WEAX + laccase						
15	5.1	0.6	0.2	0.4	0.4	0.1
30	5.5	1.0	0.2	1.1	2.0	0.2
60	3.5	1.3	0.2	1.2	2.7	0.2
+ Fumaric acid (MR = 1) ^b						
15	8.3	0.8	0.1	1.1	1.7	0.3
30	5.9	1.2	0.2	1.6	2.6	0.1
60	3.4	1.2	0.1	1.6	2.9	0.1
+ Fumaric acid (MR = 10)						
15	8.7	0.9	0.1	1.2	1.7	0.1
30	5.8	1.2	0.1	1.4	2.5	0.1
60	3.6	1.3	0.2	1.7	3.3	0.1
+ Vanillic acid (MR = 1)						
15	8.2	0.6	0.1	0.9	1.3	0.1
30	5.3	0.8	0.2	1.2	1.9	0.1
60	2.5	0.9	0.1	1.2	1.9	0.1
+ Vanillic acid (MR = 10)						
15	6.6	0.3	0.3	0.6	0.5	0.0
30	4.2	0.4	0.3	0.6	0.6	0.0
60	1.9	0.3	0.1	0.4	0.5	0.0

^a totFA = *cis* + *trans* ferulic acid.

^b MR = molar ratio between compound and esterified ferulic acid in 4 mg of WEAX.

5-5' remained almost constant, whereas the 8-5' and 8-O-4' increased. Two other dimers appeared: the 8-8' and the 8-5' benzofuran form, which were the major oxidation products.

Mechanism of Cross-Linking

Free ferulic acid and methyl ferulate (a model of esterified ferulic acid) were used to confirm the central role of feruloyl groups in the covalent cross-linking of arabinoxylans. They were separately added to solutions of WEAX (0.2%, w/v) and laccase or H₂O₂/POD. Both compounds inhibited the thickening of a WEAX solution treated by laccase (Figs. 5A and 6A) or H₂O₂/POD (Figs. 5B and 6B). Free ferulic acid was added in different concentrations (Fig. 5, Table I). For the laccase system, after 90 min of reaction, the total ferulic acid was similar with and without the addition of free ferulic acid (MR = 0.17, Table I). When free ferulic acid was added in excess (MR = 1.7), dimer formation was not proportional to total ferulic acid consumption, probably due to the formation of polymers. For H₂O₂/POD, total ferulic acid decreased during the first 30 min, then reached a constant value, corresponding to the consumption of the limited amount of H₂O₂. In both oxidizing systems, dimers 8-8' and 8-5' benzofuran were not present in blanks when free ferulic acid was added, and after oxidation they were the major products. The addition of methyl ferulate essentially confirmed the results obtained with free ferulic acid with methyl ferulate (MR = 1), the consumption of esterified ferulic acid (≈50% at 30 min) (data not shown) and the formation of dimers were similar to those of the control sample (oxidized WEAX). At high levels (MR = 10), methyl ferulate completely inhibited the thickening of the solution. Nevertheless, esterified ferulic acid was still consumed (≈85% at 30 min).

Excess of free ferulic acid provoked a lag time before the thickening of a WEAX-laccase solution (MR = 1.7 and 10) (Fig. 5A) where total ferulic acid continued to be consumed (MR = 1.7) (Table I). To study how the added free ferulic acid was consumed, samples were injected on RP-HPLC omitting the saponification step. After 10 min of reaction with laccase, the free ferulic acid disappeared without an equivalent formation of dimers (Table I). In the same period of time, H₂O₂/POD provoked an important disappearance and reached a constant value after total consumption of H₂O₂. From these results, it seemed that both oxidizing systems preferentially used the more accessible free ferulic acid rather than the ester arabinoxylan-linked ferulic acid by linking the free ferulic acid to the feruloyl esters of arabinoxylan or by forming polymers not detectable with the HPLC method used.

Vanillic acid and fumaric acid were used to clarify whether the aromatic ring (Geissman et al 1973, Neukom and Markwalder 1978, Moore et al 1990, Vinkx et al 1991) or the double bond of the propenoic chain (Sidhu et al 1980a,b; Hosoney and Faubion 1981; Thibault et al 1987) of the ferulic acid were the center of WEAX cross-linking. When two different concentrations of fumaric acid (containing a double bond but no aromatic ring) were added to a WEAX-laccase solution, the ferulic acid consumption was similar to that obtained with the control (Table II). Similar viscosity (Fig. 7A) profiles showed that fumaric acid did not interfere in oxidative arabinoxylan cross-linking. On the other hand, vanillic acid (containing an aromatic ring but no double bond) inhibited the gelation at low and high concentrations (Fig. 7B), with a larger esterified ferulic acid consumption than with the control (Table II), probably because some vanillic acid residues can bind feruloyl esters of arabinoxylans. Results are in agreement with those ob-

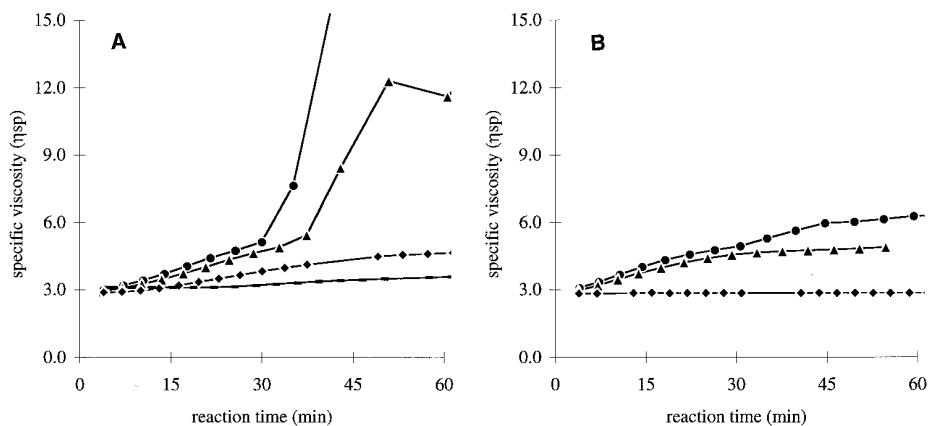


Fig. 5. Capillary viscometry profiles of the effect of different molar ratio (MR) of free ferulic acid on water-extractable arabinoxylans (WEAX) with laccase (A) and WEAX with H₂O₂/POD (B). Control (●). MR = 0.17 (▲), 1.7 (◆), and 10 (◻).

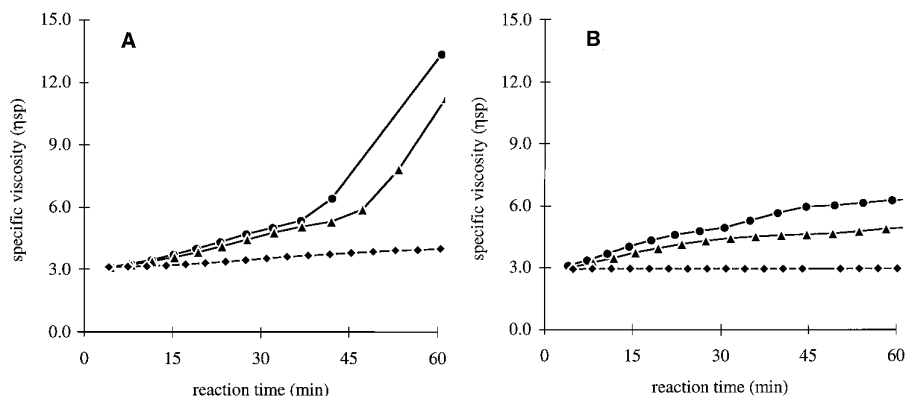


Fig. 6. Capillary viscometry profiles of the effect of different molar ratio (MR) of methyl ferulate on water-extractable arabinoxylans (WEAX) with laccase (A) and WEAX with H₂O₂/POD (B). Control (●). MR = 1 (▲) and 10 (◆).

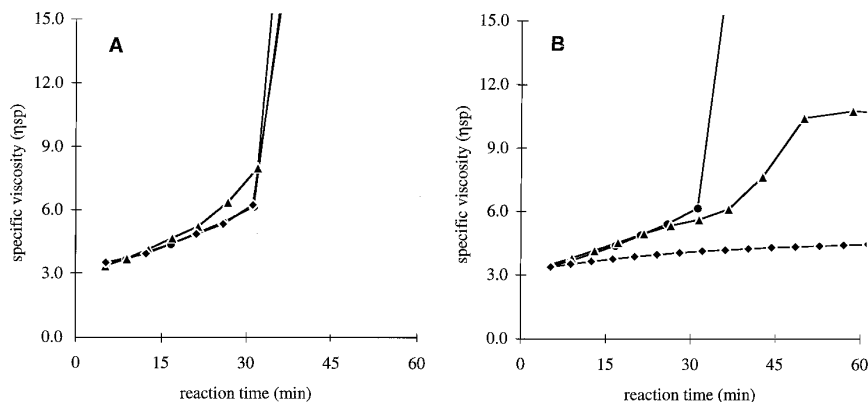


Fig. 7. Capillary viscometry profiles of the effect of different molar ratio (MR) of fumaric acid on water-extractable arabinoxylans (WEAX) with laccase (A) and of vanillic acid on WEAX with laccase (B). Control (●). MR = 1 (▲) and 10 (◆).

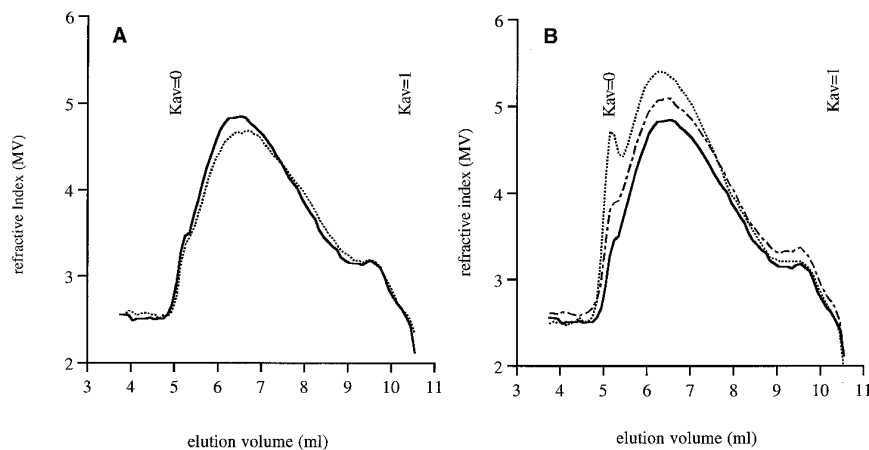


Fig. 8. Size-exclusion HPLC profiles of water-extractable arabinoxylans (WEAX) with laccase after 30 min of reaction. Fumaric acid (A) and vanillic acid (B) at molar ratio (MR) = 1 (---) and 10 (···). (MR = 1 is superimposed on the control sample [—] in A).

tained by Moore et al (1990) and Vinx et al (1991) with the H_2O_2/POD system.

SE-HPLC profiles confirmed the viscosity results. Fumaric acid showed the same profile that the control did at two different concentrations after 30 min of reaction (Fig. 8A). Vanillic acid delayed the insolubilization of WEAX. A comparison of Figs. 3A and 8B shows that the more vanillic acid was added, the more the SE-HPLC profile was similar to the profiles of the short-time reaction control.

These results confirmed the importance of the aromatic ring on gelation. The aromatic ring, and more specifically the phenol function, seems to act as the initiating site of the reaction rather than the cross-linking center. However, the double bond of the propenoic chain of ferulic acid was also involved in dimerization as evidenced by the 8-8', 8-O-4', and the 8-5' dimers in oxidized WEAX, which is in accordance to the dimer structures identified in plant cell walls by Ralph et al (1994). The mechanism of the reaction is: laccase catalyses the dehydrogenation of the hydroxyl group at C-4 of ferulic acid into a very reactive phenoxy radical. The latter can also isomerize into a radical with a trivalent carbon atom with an unpaired electron at C-5 or C-8, that can further dimerize or react with another radical to form C-C (5-5', 8-5', and 8-8') or C-O (8-O-4') linkages (Thomson 1964, Ralph et al 1994).

CONCLUSIONS

Laccase from *Pycnoporus cinnabarinus*, like the H_2O_2/POD system, provoked the cross-linking of feruloylated arabinoxylans. Free ferulic acid and methyl ferulate inhibited the gelation in reacting with the feruloyl esters, confirming that these are the site of

cross-linking. Vanillic acid inhibited the gelation but fumaric acid did not, so the aromatic ring is necessary, and the phenolic function is the initiation site of the reaction. However, the propenoic chain is also involved in the cross-linking due to the phenoxy radical isomerization, which is confirmed by the occurrence of different ferulic acid dehydrodimers containing C-C and C-O linkages. Following oxidation by laccase or H_2O_2/POD , the 8-8' and 8-5' benzofuran dimers appeared, and the 8-5' and 8-O-4' dimers, already present in the nonoxidized arabinoxylan, increased. The amount of 5-5' dimer, also present in native arabinoxylan, did not change upon oxidation. The predominant dimers in an arabinoxylan gel were 8-8', 8-5' benzofuran, and 8-O-4'.

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