Effect of Lysine, Tyrosine, Cysteine, and Glutathione on the Oxidative Cross-Linking of Feruloylated Arabinoxylans by a Fungal Laccase

Maria-Cruz Figueroa-Espinoza, Marie-Hélène Morel, and Xavier Rouau*

Unité de Technologie des Céréales et des Agropolymères, INRA, 2 Place Viala, 34060 Montpellier Cedex 01, France

The potential of a laccase from the fungus *Pycnoporus cinnabarinus* MIC11 to link covalently some amino acids (tyrosine, lysine, cysteine, and oxidized and reduced glutathione) to the ferulic acid esterified in wheat arabinoxylans was investigated, using capillary viscometry, SE-HPLC, and RP-HPLC of phenolic and thiol compounds. The laccase was compared to the system hydrogen peroxide/horseradish peroxidase. Both oxidative systems were able to gel arabinoxylan solutions by coupling feruloyl esters of adjacent chains into dehydrodimers. Cysteine and reduced glutathione hindered gelation, whereas tyrosine, lysine, and oxidized glutathione had no effect. Under the experimental conditions, in the presence of thiol compounds, a time delay proportional to the thiol concentration was observed. During this period, no esterified ferulic acid was consumed. Cysteine was not directly oxidized either by free ferulic acid or by laccase. When free ferulic acid, cysteine, and laccase reacted together, cysteine was readily oxidized into cystine. Similar results were obtained with reduced glutathione. Thus, ferulic acid oxidized by laccase into semi-quinone was regenerated by an oxidation−reduction reaction involving thiols. No direct coupling of thiol to semi-quinone by an addition reaction could be demonstrated.

Keywords: *Amino acids; arabinoxylan; dehydrodiferulic acids; ferulic acid; laccase; peroxidase*

INTRODUCTION

Arabinoxylans constitute the major cell-wall polysaccharides of wheat and rye endosperm and are partly extractable with water. Water-extractable pentosans (WEP) consist of a mostly protein-free linear β-(1−4) linked xylan backbone to which α-L-arabinofuranose units are attached as side residues via α-(1−3) and/or α-(1−2) linkages (Izydorczyk and Biliaderis, 1995). Ferulic acid is covalently linked via an ester linkage to the primary alcohol function of some of the arabinose residues (Smith and Hartley, 1983) (Figure 1). Arabinoxylans are able to form gels by oxidative coupling of their feruloyl groups (Geissmann and Neukom, 1973; Hoseney and Faubion, 1981; Izydorczyk et al., 1990). Although pure feruloylated arabinoxylan cross-linked upon oxidation, the participation of protein in pentosan gels is still unclear (Neukom et al., 1962; Neukom and Markwalder, 1978; Meuser and Suckow, 1986; Vinkx et al., 1991, 1993; Girhammar and Nair, 1995; Vinkx and Delcour, 1996).

According to Neukom and Markwalder (1978) and Meuser and Suckow (1986), ferulic acid may be linked to an N-terminal protein amino group, thus forming a pseudopeptide linkage. Upon oxidation, a diferulic acid bridge between protein and arabinoxylan could result. Ferulic acid bound to N-terminal protein amino groups was reported in barley proteins (Van Sumere et al., 1973).

Arabinoxylan-bound ferulic acid may also link to tyrosine residues of protein, which also contain a phenolic group (Neukom and Markwalder, 1978). In this respect, it is interesting to note that oxidative coupling of tyrosine with the formation of dityrosine occurs in cell walls (Andersen, 1966; Aechbach et al., 1976; Fry, 1986). However, according to Izydorczyk and Biliaderis (1995), links of esterified ferulic acid with protein-bound ferulic acid, or with tyrosine residues of protein, have not yet been isolated.

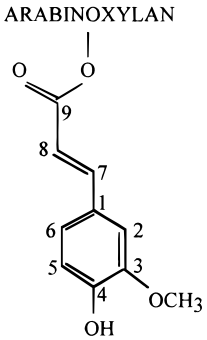

Figure 1. Structure of ferulic acid esterified to arabinoxylan. The numbered carbons allow one to deduce the structure of the dehydrodiferulic acids mentioned in the text.

Hoseney and Faubion (1981) and Moore et al. (1990) found that cysteine stopped the formation of the gel when hydrogen peroxide and peroxidase (H_2O_2/POD) were added to flour water soluble pentosans. Whereas Sidhu et al. (1980) and Hoseney and Faubion (1981) proposed that an activated thiol group of the protein (cysteine) could add to the double bond of the ferulic acid, Moore et al. (1990) suggested that cysteine reacts with the aromatic ring of the ferulic acid. However, this reaction mechanism is considered to be less likely since the addition of thiol blockers did not prevent gel formation (Neukom et al., 1962; Painter and Neukom,

* Author to whom correspondence should be addressed [telephone (33) 4 99 61 22 02; fax (33) 4 67 52 20 94; e-mail rouau@ensam.inra.fr].

S0021-8561(97)00977-1 CCC: $15.00
Published on Web 06/13/1998

1968; Vinkx et al., 1991). Vinkx et al. (1991) explained the inhibition of the gelation reaction by cysteine as an effect of competition for the H_2O_2.

Laccase, or *p*-diphenol-oxygen oxidoreductase (EC 1.10.3.2), is a copper-containing enzyme, widely distributed in plants and microorganisms, that catalyzes the oxidation of a wide variety of phenolic substrates. In the presence of oxygen, it induces a catalytic oxidation of phenols to free radical products (semi-quinones), which polymerize through nonenzymatic reactions, leaving water as the sole oxygen reduction product (Brown, 1967; Malmström et al., 1975; Holwerda et al., 1976).

According to Pierpoint (1970), quinones produced by the oxidation of diphenols can polymerize, be reduced, or undergo nucleophilic attack by substances having amino, thiol, or activated methylene groups. Cheynier et al. (1986) reported the addition of reduced glutathione to caftaric acid in the presence of grape polyphenol oxidase. Earlier, Painter and Neukom (1968) reported that the free amino groups of lysine residues did not appear to participate in the gelation reaction of a pentosan solution.

The aim of this work was to explore the possibility to link amino acids (cysteine, reduced and oxidized glutathione, lysine, or tyrosine), considered as models of functional groups of proteins, to the feruloyl esters of arabinoxylans, by the action of the laccase from the fungus *Pycnoporus cinnabarinus* MIC11 (Falconnier et al., 1994). Laccase was compared to the H_2O_2/POD system, used here as a reference.

MATERIALS AND METHODS

Arabinoxylans. WEP were isolated from a Thésée wheat cultivar flour according to the method of Faurot et al. (1995). 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 0.1 M sodium acetate buffer, pH 5.0, were used. The arabinoxylan (arabinose + xylose) content of WEAX was 82.5% db (95.2% of the total carbohydrates: arabinose + xylose + glucose), with a 0.55 arabinose-to-xylose ratio. Only traces of galactose were detected. These values were obtained by gas−liquid chromatography of alditol acetates obtained after sulfuric acid hydrolysis (2 M H_2SO_4, 2 h) on a DB-225 capillary column (J&W Scientific, Folsom, CA) according to the procedure of Blakeney et al. (1983). Inositol was used as internal standard. Protein content in WEAX (0.7%) was determined according to the Dumas method (AOAC, 1990; Bicsak, 1993).

Enzymes. Amyloglucosidase from *Aspergillus niger*, 75 units/mg (Merck, Darmstadt, Germany), and Pronase from *Streptomyces griseus*, 700 units (Boehringer, Mannheim, Germany), were used to purify arabinoxylans. Tyrosinase (monophenol dihydroxyphenilalanine:oxygen oxidoreductase EC 1.14.18.1) from mushroom, 4200 units/mg, and horseradish peroxidase (POD, donor:hydrogen-peroxide oxidoreductase EC 1.11.1.7) type I 96 PU/mg, were purchased from Sigma Chemical Co. (St. Louis, MO). Laccase (*p*-diphenol-oxygen oxidoreductase EC 1.10.3.2, solution in 35% glycerol) was obtained from a culture supernatant of *P. cinnabarinus* MIC11 kindly supplied by Dr. M. Asther from the Laboratoire de Biotechnologie des Champignons Filamenteux-INRA (Marseille, France).

Laccase activity was measured at 25 °C by mixing 5 μL of laccase solution with 895 μL of 0.1 M sodium acetate buffer (at different pH values) and 100 μL of syringaldazine (0.216 mM in methanol). Absorbance was read at 530 nm during 30 s, after 30 s of reaction. Under these reaction conditions, the optimum pH was around 5.0 with an activity of 0.04 nkat/μL. Laccase exhibited neither *endo*-xylanase nor protease activities and was able to oxidize catechol, *p*-coumaric acid, and guaiacol. The *endo*-xylanase activity was determined according to the method of McCleary (1992), by incubating the enzyme with a solution of wheat arabinoxylan (1% w/v, Megazyme, Australia) and measuring the rate of decrease in viscosity at 40 °C. The protease activity was measured according to the method of Peterson and Huffaker (1975) by reading the absorbance of the supernatant solution at 340 nm of samples incubated with azocasein (2 h, 40 °C).

Chemicals. Azocasein, bathophenanthroline disulfonic acid (BPDS), 1-fluoro-2,4-dinitrobenzene (DNFB), L-cystine, ferulic acid, hydrogen peroxide (H_2O_2) 30% (w/w), DL-lysine, syringaldazine, 3,4,5-trimethoxy-*trans*-cinnamic acid (TMCA), L-tyrosine, and oxidized and reduced glutathione were purchased from Sigma Chemical Co. L-Cysteine was obtained from Merck and phloroglucinol (1,3,5-trihydroxybenzene) from Fluka Chemie AG (Buchs, Switzerland). A mixture of dehydrodimers of ferulic acid in known amounts was kindly supplied by Dr. J. Ralph from the U.S. Dairy Forage Research Center USDA−ARS and Department of Forestry, University of Wisconsin, Madison (Ralph et al., 1994).

Reaction Mixtures. Reaction mixtures were prepared according to the formula 2 mL of WEAX 0.2% (w/v) + 50 μL of A + 50 μL of B.

In the control samples (WEAX + enzyme), A was 0.1 M sodium acetate buffer, pH 5.0, and B was the enzyme solution: 50 μL of laccase (0.05 nkat/mg of WEAX) or 25 μL of H_2O_2 (5×10^{-3} μmol/mg of WEAX) + 25 μL of POD (5.52×10^{-3} PU/mg of WEAX). H_2O_2 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 replaced by a solution of one of the model compounds (cysteine, reduced glutathione, oxidized glutathione, lysine, or tyrosine). Fifty microliters of A contained the model compound in a molar ratio (MR) of 1 or 10, compared to the ferulic acid initially present in 4 mg of arabinoxylan. B was one of the enzyme solutions. In blanks (WEAX without enzyme), enzyme solutions (B) were substituted by 0.1 M sodium acetate buffer, pH 5.0.

Depending on the further analyses, reactions were stopped in different ways: for RP-HPLC, 1 mL of 4 N NaOH was added to 1 mL of the reaction mixture (final pH around 12.0). For SE-HPLC or WEAX determination, 300 μL of reaction mixture was filtered (2.7 μm) before the reaction was stopped by freezing or by adding the same volume of 4 N NaOH, respectively.

WEAX Determination. WEAX concentration was determined according to the semiautomated colorimetric method of Rouau and Surget (1994), using an Evolution II autoanalyzer (Alliance Instruments, France). Samples were analyzed for their pentose content. The conversion of pentosan into furfural by hot acid treatment was followed by a color reaction with phloroglucinol. The filtered (2.7 μm) reaction mixture (200 μL) was mixed with 200 μL of 4 N NaOH to stop the reaction and then diluted 25-fold.

Analysis of Alkali-Labile Phenolics. One milliliter of the reaction mixture was added to 1 mL of 4 N NaOH and incubated under argon during 2 h at 35 °C in the dark. TMCA (internal standard, 2.5 μg) was added, and pH was adjusted at 2.0 ± 0.02 with HCl. Phenolic acids were extracted twice with 2 mL of ether. Ether phase was transferred in amber test tubes and evaporated at 30 °C under argon. One hundred microliters of aqueous methanol 50% (v/v) was added, and samples were filtered (0.45 μm) and then injected (20 μL) in RP-HPLC, using an Interchrom (Interchim, Montluçon, France) Rsil C_{18} 5 μm column (250 × 4.6 mm). Detection was by UV absorbance at 320 nm. Gradient elution was performed using acetonitrile/0.05 M sodium acetate buffer, 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. The solvents were of HPLC grade, and the mobile phase was sparged with helium. A Waters 996 (Millipore Co., Milford, MA) photodiode array detector was used to record the spectra of ferulic acid

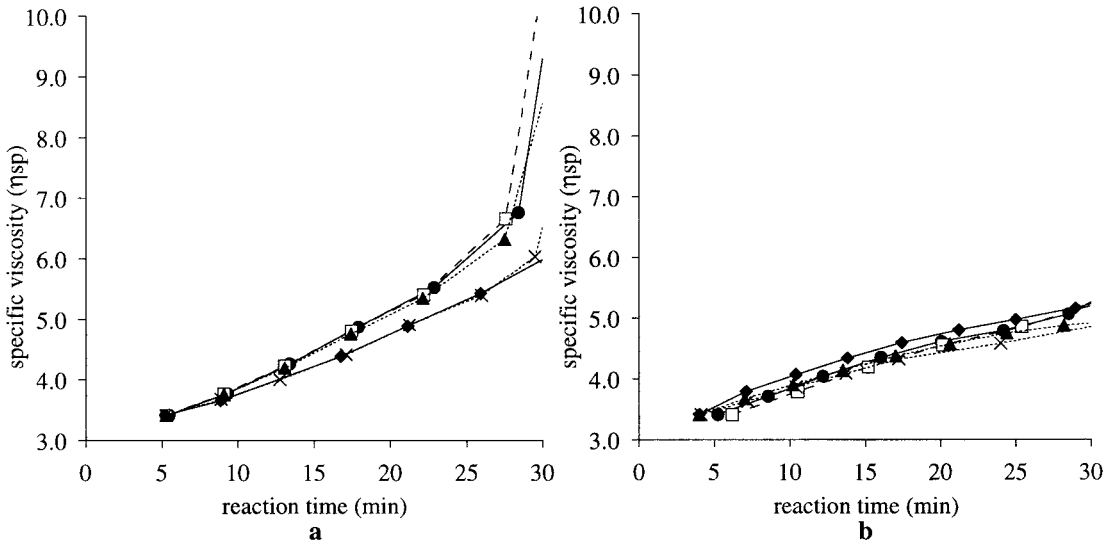

Figure 2. Capillary viscosimetry profiles of the effect of tyrosine MR = 1 (□) and 10 (●) and of lysine MR = 1 (▲) and 10 (×) on (a) WEAX−laccase solution and on (b) WEAX−H_2O_2/POD solution. (◆) represents the control sample (WEAX + enzyme).

and its dehydrodimers. Dimer identification and quantification were based on the mixture of standard dehydrodimers in known amounts. Each spectrum was analyzed by comparison with those already published (Parr et al., 1996; Waldron et al., 1996) and with those from the dimers mix. This mixture served to calculate the response factor for the main dehydrodimers under the conditions used.

Size Exclusion Chromatography (SE-HPLC). SE-HPLC was performed at 35 °C using a Waters Ultrahydrogel 1000, 10 μm column (7.8 × 300 mm), with a pullulan limit exclusion of 10^6 Da, eluted with 0.1 M sodium acetate buffer, pH 5.0, at 0.6 mL/min. Twenty microliters of the filtered (2.7 μm) reaction mixture was injected. The eluent was monitored with a Waters 410 differential refractometer.

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

Thiol and Disulfide Determination. A modification of the HPLC methods of Schofield and Chen (1995) and Siller-Cepeda et al. (1991) was used to quantify cysteine, cystine, and oxidized and reduced glutathione, after reaction with laccase, with and without ferulic acid. Reaction mixtures were prepared by mixing 1 mL of ferulic acid (4.2 mM) with 1 mL of thiol compound (4.2 mM) and 100 μL of laccase (20 nkat/mL). The reaction was stopped after 20, 40, and 60 min, by adding 400 μL of a 10% (v/v) perchloric acid−1 mM BPDS mixture, to 100 μL of the reaction mixture. A blank (without enzyme) was prepared for each compound. Samples were carboxymethylated with 50 μL of freshly prepared 200 mM iodoacetic acid. The acidic solution was brought to pH 9.0 by the addition of 450 μL of KOH (10 M)−$KHCO_3$ (3 M) mixture (20−80%) and incubated in the dark for 30 min. Samples were derivatized with 1 mL of DNFB 1% and kept in the dark at 4 °C overnight. Derivatized samples were filtered (0.2 μm) and then injected (20 μL) in RP-HPLC, using a Spherisorb S5 amino (Touzart & Matignon, Vitry sur Seine, France) 5 μm column (250 × 4.6 mm). Detection was by UV absorbance at 365 nm. The mobile phase, at a flow rate of 1 mL/min at 35 °C, comprised a mixture of two solvents: A, 0.05 M trihydrate sodium acetate in 80% (v/v) aqueous methanol, and B, 0.8 M trihydrate sodium acetate in 50% (v/v) aqueous methanol added by 120 mL/L of acetic acid. The following gradient was used: solvent B started at 0% and increased to 100% in 5 min, holding for 13 min, then decreased to 0% in 10 min, holding for 6 min. Both solvents were sparged with helium.

Repeatability. The coefficients of variation for the viscosimetry, the arabinoxylan, the ferulic acid, and the thiol−disulfide determination procedures were 3, 3, 4, and 10%, respectively. Results were expressed as mean values of at least duplicate analyses.

RESULTS AND DISCUSSION

Both laccase and the couple H_2O_2/POD are able to gel WEAX solutions, by oxidizing their esterified ferulic acid into dehydrodimers (Figueroa-Espinoza and Rouau, 1998). The reaction can be monitored by the disappearance of ferulic acid and by the formation of dimers, by the increase in viscosity of the WEAX solution, and by the change in molecular weight distribution and loss of solubility of arabinoxylans.

Lysine, tyrosine, cysteine, and glutathione were used to clarify whether the ferulic acid esterified to WEAX could link to a protein by its amino, tyrosyl, or sulfhydryl group. In the case of linkage between ferulic acid and an amino acid, the gelation process was expected to be slowed or inhibited, with a similar ferulic acid consumption but with less dimer formation than in a control (WEAX + enzyme), because of the blocking of the reactive sites of ferulic acid.

SE-HPLC profiles and total ferulic acid and dimers contents were determined after different reaction times (5, 15, 25, 30, 60, and 90 min). Only the SE-HPLC profiles at 25 min of reaction and the quantification of phenolics at 25 and 90 min of reaction are presented.

Effect of Lysine on WEAX Oxidative Gelation. Addition of lysine at two different concentrations (molar ratio, MR = 1 and 10) did not modify the viscosity profiles compared to the control samples (Figure 2). This suggested that lysine did not interfere with WEAX gelation, whatever the oxidative system used, laccase (Figure 2a) or H_2O_2/POD (Figure 2b). These results agree with those of Painter and Neukom (1968).

Samples were filtered prior to SE-HPLC analysis, so that material insolubilized by the cross-linking reaction was removed. As expected, the SE-HPLC profile of the sample containing lysine (MR = 10) was similar to that of the control (Figure 3). The nonoxidized WEAX (blank, without enzyme) was eluted as a single peak at K_{av} around 0.2. Upon 25 min of oxidation, the amount of soluble material decreased, with the occurrence of a shoulder of excluded material that reflected the polym-

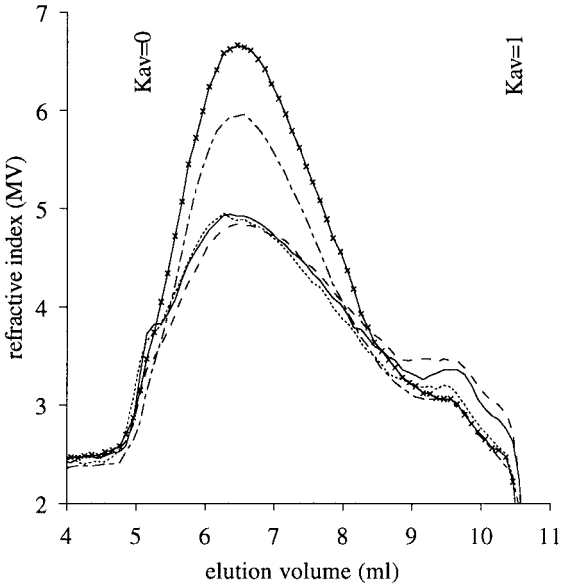

Figure 3. SE-HPLC elution profiles showing the effect of laccase on WEAX after 25 min of reaction in the presence of lysine MR = 10 (· · ·), tyrosine MR = 10 (— —), and cysteine MR = 10 (— - —). (×) represents the blank (without enzyme) and (—) the control (WEAX + enzyme).

Table 1. Contents of Ferulic Acid and Its Dehydrodimers in WEAX Solutions after 90 min of Reaction with Laccase or H_2O_2/POD in the Presence of Lysine and Tyrosine

	nmol/mg of WEAX						
sample	total FA[a]	8−8′	8−5′	8-O-4′	8−5′-benzo	5−5′	total dimers
WEAX	11.6	0.0	0.1	0.3	0.0	0.2	0.7
WEAX + laccase	2.4	1.5	0.2	1.6	2.7	0.1	6.2
+ lysine, MR[b] = 1	2.5	1.3	0.1	1.4	3.0	0.2	5.9
+ lysine, MR = 10	2.6	1.4	0.1	1.5	3.3	0.2	6.5
+ tyrosine, MR = 1	2.3	1.2	0.2	1.5	3.3	0.2	6.4
+ tyrosine, MR = 10	2.2	1.2	0.1	1.5	3.3	0.2	6.3
WEAX + H_2O_2/POD	3.1	2.4	0.9	1.8	2.0	0.2	7.3
+ lysine, MR = 1	2.8	2.5	0.8	1.7	2.1	0.1	7.2
+ lysine, MR = 10	2.9	2.3	0.7	1.6	2.2	0.1	6.8
+ tyrosine, MR = 10	3.2	2.4	0.8	1.8	2.3	0.1	7.3

[a] Total FA = *cis*- + *trans*-ferulic acid. [b] MR = molar ratio compound: esterified ferulic acid in 4 mg of WEAX.

erization of soluble arabinoxylans. Results were similar in the H_2O_2/POD system (not shown). Ferulic acid consumption and dimer formation were similar between lysine samples and the controls (laccase or H_2O_2/POD) (Table 1). In all samples, the 8−8′ and the 8−5′-benzo dehydrodimers appeared after oxidation, while in the blank WEAX they were not present. In the presence of lysine at both MR = 1 and 10, ferulic acid decreased by 78% after 90 min, similarly to the control WEAX + laccase (79%). The dimers that increased the most were the 8−5′-benzo and the 8−8′. The 5−5′ dimer remained constant. In the H_2O_2/POD system, ferulic acid decreases were similar in the control WEAX + H_2O_2/POD (73%) and in the samples containing lysine (75%). The 8−8′ and the 8−5′-benzo dimers were major. With both oxidizing systems, dimer contents were almost similar in the presence of lysine at either MR = 1 or 10.

Effect of Tyrosine on WEAX Oxidative Gelation. Tyrosine had no effect on gelation either in laccase or in the H_2O_2/POD system, as can be observed in Figure 2. The SE-HPLC profile in the presence of tyrosine (MR = 10) was similar to that of the control (Figure 3). Different concentrations of tyrosine (MR = 1 and 10) did not provoke significant differences either in the ferulic acid consumption or in the dehydrodimer production, as shown in Table 1. With laccase, ferulic acid decreased by 80 and 81% in the presence of tyrosine MR = 1 and 10, respectively. Dimers were produced in the same proportion in the two samples containing tyrosine, and these results were similar to those from the control WEAX + laccase. When tyrosine MR = 10 was added in the system H_2O_2/POD, ferulic acid consumption (72%) and dimer production were similar to those from the control.

Effect of Cysteine and Oxidized and Reduced Glutathione on WEAX Oxidative Gelation. Cysteine was added at different levels (MR = 1, 2, and 10) to a WEAX solution in the presence of the enzymes. With laccase (Figure 4a), cysteine had an inhibitory effect on gelation. It provoked a delay in the thickening of the solution, which increased with the cysteine concentration. Whereas the delay was 15 min for MR = 1 and 30 min for MR = 2, still no thickening was observed after 90 min of reaction for MR = 10. In the presence of H_2O_2/POD (Figure 4b), cysteine at MR = 1 provoked a delay and the pseudo-plateau was reached more rapidly than in the control. Gelation was totally inhibited when an excess of cysteine was added (MR = 10). Reduced glutathione also retarded gelation, but oxidized glutathione had no effect (not shown). It can be concluded that the thiol groups interfered with the cross-linking of feruloyl esters of arabinoxylans.

The viscosity results were confirmed by SE-HPLC (Figure 3). Laccase rendered WEAX insoluble due to polymerization (decrease of the peak at $K_{av} = 0.2$, with occurrence of a shoulder of excluded material). When cysteine was added at MR = 10, no material was insolubilized and the profiles were similar to the blank profile. Reduced glutathione provoked a similar effect (not shown).

When a thiol compound was added to a WEAX−enzyme solution, the ferulic acid consumption and the dehydrodimer production were retarded and no occurrence of new products was observed. In the control with laccase (Table 2), half of the ferulic acid disappeared after 25 min and 79% had disappeared after 90 min of reaction. In experiments with both cysteine and reduced glutathione at MR = 1, ferulic acid decreased only slightly after 25 min, whereas its consumption was close to that of the control after 90 min. The consumption coincided with the thickening observed in Figure 4. On the other hand, when the thiol compounds were added at MR = 10, ferulic acid was not consumed during the time of the experiment. The measured ferulic acid concentration was even slightly higher in samples than in the blank (WEAX), probably due to a protective effect of high levels of thiols during the saponification and extraction procedure. The retarding effect of thiol compounds was similar in the presence of H_2O_2/POD at MR = 10 (Table 3); at MR = 1 the consumption of ferulic acid took place in the first 25 min of reaction and then remained constant. These results agreed with those from viscosity (Figure 4), where the pseudo-plateau was reached after 25 min due probably to the depletion of H_2O_2. In conclusion, thiols did not establish linkages with the feruloyl esters of WEAX following laccase or H_2O_2/POD oxidation. These results disagreed with those already published which suggested that

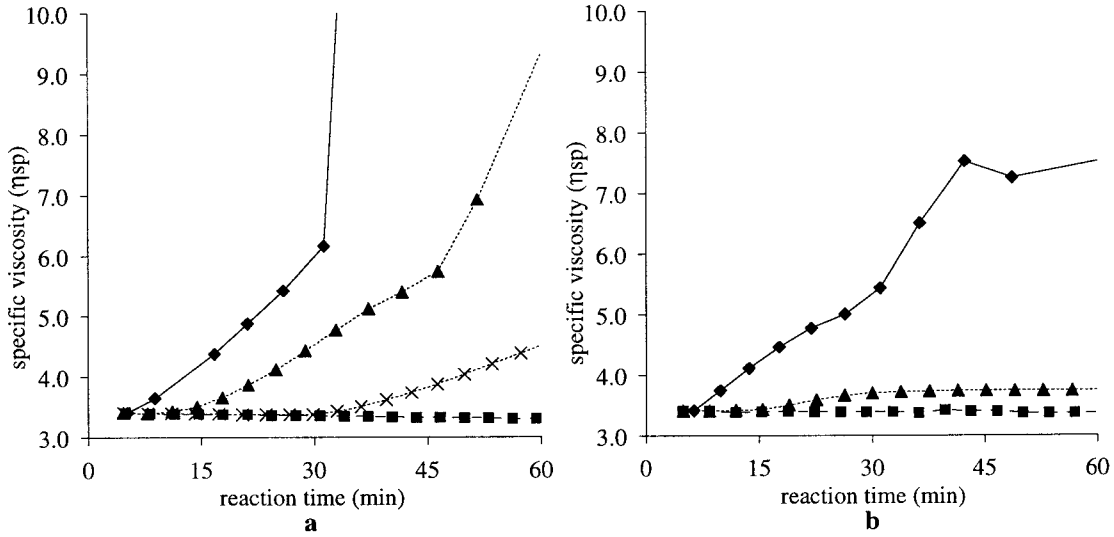

Figure 4. Capillary viscosimetry profiles of the effect of cysteine MR = 1 (▲), 2 (×), and 10 (■) on (a) WEAX−laccase solution and on (b) WEAX−H_2O_2/POD solution. (◆) represents the control sample (WEAX + enzyme).

Table 2. Ferulic Acid and Dehydrodimer Contents of WEAX Solutions after Reaction with Laccase in the Presence of Cysteine and Reduced Glutathione

		nmol/mg of WEAX						
sample	RT[a] (min)	total FA[b]	8−8′	8−5′	8-O-4′	8−5′-benzo	5−5′	total dimers
WEAX		11.6	0.0	0.1	0.3	0.0	0.2	0.7
WEAX + laccase	25	5.8	1.0	0.2	1.4	1.8	0.1	4.5
	90	2.4	1.5	0.2	1.6	2.7	0.1	6.2
+ cysteine, MR[c] = 1	25	10.3	0.5	0.2	0.7	0.6	0.2	2.2
	90	3.4	1.3	0.2	1.7	2.1	0.2	5.5
+ cysteine, MR = 10	25	12.6	0.0	0.2	0.0	0.3	0.2	0.7
	90	12.6	0.2	0.2	0.0	0.4	0.2	1.0
+ reduced glutathione, MR = 1	25	8.5	0.7	0.0	1.1	1.4	0.3	3.5
	90	3.5	1.3	0.1	1.4	2.3	0.2	5.4
+ reduced glutathione, MR = 10	25	12.3	0.0	0.0	0.3	0.1	0.2	0.6
	90	11.9	0.3	0.0	0.6	0.3	0.2	1.4

[a] Reaction time. [b] Total FA = *cis*- + *trans*-ferulic acid. [c] MR = molar ratio compound: esterified ferulic acid in 4 mg of WEAX.

Table 3. Ferulic Acid and Dehydrodimer Contents on WEAX Solutions after Reaction with H_2O_2/POD in the Presence of Cysteine and Reduced Glutathione

		nmol/mg of WEAX						
sample	RT[a] (min)	total FA[b]	8−8′	8−5′	8-O-4′	8−5′-benzo	5−5′	total dimers
WEAX		11.6	0.0	0.1	0.3	0.0	0.2	0.7
WEAX + H_2O_2/POD	25	4.0	2.7	1.2	1.7	1.2	0.2	7.0
	90	3.1	2.4	0.9	1.8	2.0	0.2	7.3
+ cysteine, MR[c] = 1	25	10.4	0.0	0.3	0.6	0.2	0.2	1.2
	90	10.0	0.0	0.3	0.6	0.3	0.1	1.3
+ cysteine, MR = 10	25	11.2	0.0	0.2	0.4	0.2	0.3	1.0
	90	11.5	0.0	0.2	0.8	0.0	0.2	1.1
+ reduced glutathione, MR = 1	25	6.9	1.0	0.8	1.2	0.9	0.3	4.2
	90	6.9	0.0	0.9	1.3	0.7	0.2	3.1
+ reduced glutathione, MR = 10	25	11.3	0.0	0.2	0.4	0.1	0.2	0.8
	90	11.8	0.0	0.2	0.4	0.1	0.2	0.9

[a] Reaction time. [b] Total FA = *cis*- + *trans*-ferulic acid. [c] MR = molar ratio compound: esterified ferulic acid in 4 mg of WEAX.

cysteine reacted with the esterified ferulic acid on WEAX after reaction with H_2O_2/POD (Hoseney and Faubion, 1981; Moore et al., 1990) or with ultraviolet light (Sidhu et al., 1980). Our results agreed partly with those from Vinkx et al. (1991), who found that cysteine delayed the thickening of a pentosan−protein solution not by reacting with the ferulic acid on pentosans but by competing for the H_2O_2. According to the experiment described below, cysteine was not directly oxidized by the oxidant.

Effect of Laccase and Ferulic Acid on Sulfhydryl (−SH) Oxidation. According to Pierpoint (1970), the quinones produced by the oxidation of diphenols could suffer nucleophilic attack by substances possessing thiol

Table 4. Sulfhydryl and Disulfide Quantification in Solutions of Cysteine and Reduced Glutathione in the Presence of Ferulic Acid and/or Laccase

	−SH[a] (nmol)				−SS−[b] (nmol)			
compound	0 min	20 min	40 min	60 min	0 min	20 min	40 min	60 min
cysteine	1.9	nd[d]	1.9	1.7	0.1	nd	0.0	0.9
+ FA[c]	1.9	nd	2.0	1.9	0.0	nd	0.1	0.1
+ laccase	1.9	nd	1.8	1.7	0.1	nd	0.1	0.1
+ FA + laccase	1.9	0.9	0.6	0.0	0.1	0.5	0.6	0.9
reduced glutathione	1.9	nd	1.9	1.9	0.0	nd	0.0	0.0
+ FA	1.9	nd	1.8	1.9	0.0	nd	0.0	0.0
+ laccase	1.9	nd	1.8	1.9	0.0	nd	0.0	0.1
+ FA + laccase	1.9	1.4	1.0	0.8	0.0	0.2	0.4	0.5

[a] Sulfhydryl groups (cysteine or reduced glutathione). [b] Disulfide groups (cystine or oxidized glutathione). [c] Ferulic acid. [d] Not determined.

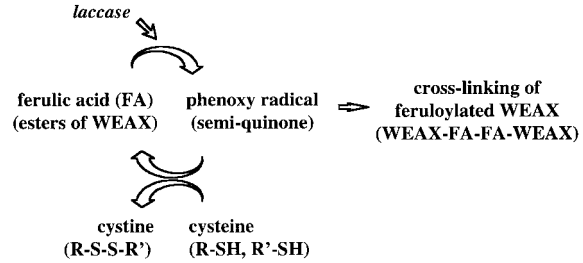

Figure 5. Hypothesis on the effect of laccase and ferulic acid on the oxidation of sulfhydryl groups.

groups. However, when ferulic acid is oxidized, laccase proceeds by a single electron-removing step and produces semi-quinones, which differ from quinones, because semi-quinones cannot react with thiols but they can be reduced by them (Prütz et al., 1983). Thus, reducing agents can immediately reduce the oxidized phenolics, thereby preventing the occurrence of secondary reactions (Whitaker, 1994).

To clarify the mechanism of reaction in the presence of thiols, sulfhydryl and disulfide groups were quantified by RP-HPLC after reaction with laccase, with and without addition of ferulic acid. Results are shown in Table 4. Neither ferulic acid nor laccase provoked separately the oxidation of cysteine. However, when ferulic acid and laccase were added together to the cysteine solution, a fast decrease in cysteine (sulfhydryl group), accompanied by a proportional increase in cystine (disulfide group), was observed: cysteine was depleted after 60 min of reaction and totally converted into cystine. Reduced glutathione was oxidized in similar conditions at a slower rate (58% oxidation in 60 min). These results showed that thiols were no substrate of laccase and that as readily as laccase oxidized the feruloyl esters of WEAX into semi-quinones (phenoxy radicals), cysteine reduced them into the original ferulic acids, while oxidizing into cystine (Figure 5). These coupled reactions continued until all of the sulfhydryls were consumed. The oxidation and dimerization of ferulic acid could then take place.

CONCLUSIONS

Neither lysine nor tyrosine had an effect on the oxidative cross-linking of feruloylated WEAX, suggesting that amino and tyrosyl groups of proteins could not react with ferulic acid, either in the presence of laccase from *P. cinnabarinus* MIC11 or in the presence of H_2O_2 and horseradish peroxidase. According to Brown (1967), laccase is not able to oxidize tyrosine. Thus, when tyrosine is added to a WEAX−laccase solution, the only substrate for the enzyme is ferulic acid. In the presence of both laccase and H_2O_2/POD, ferulic acid is oxidized into semi-quinones but cannot be hydroxylated into a diphenol to yield a quinone upon oxidation. Semi-quinones polymerize through free radical reactions but do not form adducts with compounds containing amino or thiol groups as do quinones, issued from the oxidation of diphenols (Pierpoint, 1970).

Cysteine and reduced glutathione, but not oxidized glutathione, inhibited the gelation of feruloylated WEAX with both laccase and H_2O_2/POD systems. Thiols provoked a lag time before thickening, proportional to the initial concentration in thiols, during which no ferulic acid was consumed. It was demonstrated that in a cysteine (or reduced glutathione)−laccase solution, cysteine (or reduced glutathione) was rapidly converted into cystine (or oxidized glutathione) when ferulic acid was present. Thus, instead of linking to ferulic acid, thiols were oxidized into disulfides with a concomitant reduction of the phenoxy radicals (semi-quinones) into the original ferulic acid, until total consumption of thiols.

In conclusion, during the gelation process of WEAX, ferulic acid is oxidized by laccase or by H_2O_2/POD into a semi-quinone that cannot react either with amino or with tyrosyl or with thiol groups. Cysteine and reduced glutathione inhibited the gelation by reducing the formed phenoxy radicals and not by blocking the sites of dimerization of ferulic acid.

ABBREVIATIONS USED

8-*O*-4′, (*Z*)-*β*-{4-[(*E*)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxycinnamic acid; 8−5′, (*E*,*E*)-4,4′-dihydroxy-3,5′-dimethoxy-*β*,3′-bicinnamic acid; 8−8′, 4,4′-dihydroxy-3,3′-dimethoxy-*β*,*β*′-bicinnamic acid; 8−5′-benzo, *trans*-5-[(*E*)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-di-hydrobenzofuran-3-carboxylic acid; 5−5′, (*E*,*E*)-4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid (see Figure 1).

ACKNOWLEDGMENT

We thank Dr. M. Asther (Laboratoire de Biothechnologie des Champignons Filamenteux-INRA, Marseille, France) for supplying the laccase from the fungus *Pycnoporus cinnabarinus* MIC11 and Dr. J. Ralph (U.S. Dairy Forage Research Center USDA−ARS and Department of Forestry, University of Wisconsin, Madison) for providing the mixture of standard dimers of ferulic acid. We also thank Ms. A. Surget for excellent technical assistance.

LITERATURE CITED

Aeschbach, R.; Amado, R.; Neukom, H. Formation of dityrosine cross-links in proteins by oxidation of tyrosine residues. *Biochim. Biophys. Acta* **1976**, *439*, 292−301.

Andersen, S. O. Covalent cross-links in a structural protein, resilin. *Acta Physiol. Scand.* **1966**, *66* (Supp. 263), 1−81.

AOAC (Association of Official Analytical Chemists). *Official Methods of Analysis*, 15th ed., 1st suppl.; AOAC International: Arlington, VA, 1990; sec. 990.03.

Bicsak, R. C. Comparison of Kjeldahl method for determination of crude protein in cereal grains and oilseeds with generic combustion method: collaborative study. *J. AOAC Int.* **1993**, *76* (4), 780−786.

Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.* **1983**, *113*, 291−299.

Brown, B. R. Biochemical aspects of oxidative coupling of phenols. In *Oxidative Coupling of Phenols*; Taylor, W. I., Batlersby, A. R., Eds.; Dekker: New York, 1967; Vol. 1.

Cheynier, V. F.; Trousdale, E. K.; Singleton, V. L.; Salgues, M. J.; Wylde, R. Characterization of 2-*S*-glutathionylcaftaric acid and its hydrolysis in relation to grape wines. *J. Agric. Food Chem.* **1986**, *34* (2), 217−221.

Falconnier, B.; Lapierre, C.; Lesage-Meessen, L.; Yonnet, G.; Brunerie, P.; Colonna-Ceccaldi, B.; Corrieu, G.; Asther, M. Vanillin as a product of ferulic acid biotransformation by the white-rot fungus *Pycnoporus cinnabarinus* I-937: Identification of metabolic pathways. *J. Biotechnol.* **1994**, *37*, 123−132.

Faurot, A.-L.; Saulnier, L.; Bérot, S.; Popineau, Y.; Petit, M.-D.; Rouau, X.; Thibault, J.-F. Large scale isolation of water-soluble and water-insoluble pentosans from wheat flour. *Lebensm.-Wiss. -Technol.* **1995**, *28*, 436−444.

Figueroa-Espinoza, M. C.; Rouau, X. Oxidative cross-linking of pentosans by a fungal laccase and horseradish peroxidase. Mechanism of linkage between feruloylated arabinoxylans. *Cereal Chem.* **1998**, *75*, 259−265.

Fry, S. C. Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Annu. Rev. Plant Physiol.* **1986**, *37*, 165−186.

Geissman, T.; Neukom, H. On the composition of the water-soluble wheat flour pentosans and their oxidative gelation. *Lebensm.-Wiss. -Technol.* **1973**, *6*, 59−62.

Girhammar, U.; Nair, B. M. Rheological properties of water soluble non-starch polysaccharides from whole grain rye flour. *Food Hydrocolloids* **1995**, *9* (2), 133−140.

Holwerda, R. A.; Wherland, S.; Gray, H. B. Electron-transfer reactions of copper proteins. *Annu. Rev. Biophys. Bioeng.* **1976**, *5*, 363−396.

Hoseney, R. C.; Faubion, J. M. A mechanism for the oxidative gelation of wheat flour water-soluble pentosans. *Cereal Chem.* **1981**, *58* (5), 421−424.

Izydorczyk, M. S.; Biliaderis, C. G. Cereal arabinoxylans: advances in structure and chemical properties. *Carbohydr. Polym.* **1995**, *28*, 33−48.

Izydorczyk, M. S.; Biliaderis, C. G.; Bushuk, W. Oxidative gelation studies of water-soluble pentosans from wheat. *J. Cereal Sci.* **1990**, *11*, 153−169.

Malmström, B. G.; Andréasson, L.-E.; Reinhammar, B. Copper-containing oxidases and superoxide dismutase. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: Orlando, FL, 1975; Vol. XII.

McCleary, B. V. Measurement of *endo*-1,4-β-D-xylanase. In *Xylans and Xylanases*; Visser, J., Beldman, G., Kusters-van Someren, M. A., Voragen, A. G. J., Eds.; Elsevier Science Publishers: Amsterdam, The Netherlands, 1992.

Meuser, F.; Suckow, P. Non-starch polysaccharides. In *Chemistry and Physics of Baking*; Blanshard, J. M. V., Frazier, P. J., Galliard, T., Eds.; The Royal Society of Chemistry, Burlington House: London, 1986.

Moore, A. M.; Martínez-Muñoz, I.; Hoseney, R. C. Factors affecting the oxidative gelation of wheat water-solubles. *Cereal Chem.* **1990**, *67* (1), 81−84.

Neukom, H.; Markwalder, H. U. Oxidative gelation of wheat flour pentosans: A new way of cross-linking polymers. *Cereal Foods World* **1978**, *23* (7), 374−376.

Neukom, H.; Kündig, W.; Dfuel, H. The soluble wheat flour pentosans. *Cereal Sci. Today* **1962**, *7* (4), 112−125.

Painter, T. J.; Neukom, H. The mechanism of oxidative gelation of a glycoprotein from wheat flour. *Biochim. Biophys. Acta* **1968**, *158*, 363−381.

Parr, A. J.; Waldron, K. W.; Ng, A.; Parker, M. L. The wall-bound phenolics of chinese water chestnut (*Eleocharis dulcis*). *J. Sci. Food. Agric.* **1996**, *71*, 501−507.

Peterson, L. W.; Huffaker, R. C. Loss of ribulose 1,5-diphosphate carboxylase and increase in proteolytic activity during senescence of detached primary barley leaves. *Plant Physiol.* **1975**, *55*, 1009−1015.

Pierpoint, W. S. Formation and behaviour of *o*-quinones in some processes of agricultural importance. *Rothamsted Rep.* **1970**, Part 2, 199−218.

Prütz, W. A.; Butler, J.; Land, E. J. Phenol coupling initiated by one-electron oxidation of tyrosine units in peptides and histone. *Int. J. Radiat. Biol.* **1983**, *44* (2), 183−196.

Ralph, J.; Quideau, S.; Grabber, J. H.; Hatfield, R. D. Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *J. Chem. Soc., Perkin Trans. 1* **1994**, 3485−3498.

Rouau, X.; Moreau, D. Modification of some physicochemical properties of wheat flour pentosans by an enzyme complex recommended for baking. *Cereal Chem.* **1993**, *70* (6), 626−632.

Rouau, X.; Surget, A. A rapid semi-automated method for the determination of total and water-extractable pentosans in wheat flours. *Carbohydr. Polym.* **1994**, *24*, 123−132.

Schofield, J. D.; Chen, X. Analysis of free reduced and free oxidised glutathione in wheat flour. *J. Cereal Sci.* **1995**, *21* (2), 127−136.

Sidhu, J. S.; Hoseney, R. C.; Faubion, J.; Nordin, P. Reaction of ^{14}C-cysteine with wheat flour water solubles under ultraviolet light. *Cereal Chem.* **1980**, *57* (6), 380−382.

Siller-Cepeda, J. H.; Chen, T. H. H.; Fuchigami, L. H. High performance liquid chromatography analysis of reduced and oxidized glutathione in woody plant tissues. *Plant Cell Physiol.* **1991**, *32* (8), 1179−1185.

Smith, M. M.; Hartley, R. D. Occurrence and nature of ferulic acid substitution of cell-wall polysacharides in graminaceous plants. *Carbohydr. Res.* **1983**, *118*, 65−80.

Van Sumere, C. F.; De Pooter, H.; Ali, H.; Degrauw-van Bussel, M. N-Feruloylglycyl-L-phenylalanine: a sequence in barley proteins. *Phytochemistry* **1973**, *12*, 407−411.

Vinkx, C. J. A.; Delcour, J. A. Rye (*Secale cereale* L.) Arabinoxylans: A critical review. *J. Cereal Sci.* **1996**, *24*, 1−14.

Vinkx, C. J. A.; Van Nieuwenhove, C. G.; Delcour, J. A. Physicochemical and functional properties of rye nonstarch polysaccharides. III. Oxidative gelation of a fraction containing water-soluble pentosans and proteins. *Cereal Chem.* **1991**, *68* (6), 617−622.

Vinkx, C. J. A.; Reynaert, H. R.; Grobet, P. J.; Delcour, J. A. Physicochemical and functional properties of rye nonstarch polysaccharides. V. Variability in the structure of water-soluble arabinoxylans. *Cereal Chem.* **1993**, *70* (3), 311−317.

Waldron, K. W.; Parr, A. J.; Ng, A.; Ralph, J. Cell wall esterified phenolic dimers: identification and quantification by reverse phase high performance liquid chromatography and diode array detection. *Phytochem. Anal.* **1996**, *7*, 305−312.

Whitaker, J. *Principles of Enzymology for the Food Sciences*; Dekker: New York, 1994.

Received for review November 17, 1997. Accepted April 15, 1998. M.-C.F.-E. is grateful to the Consejo Nacional de Ciencia y Tecnología (CONACYT, Mexico) for providing a doctoral financial support in a cooperation program with the Société Française d'Exportation des Ressources Educatives (SFERE, France).

JF970977W