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Effect of Cysteinyl Caffeic Acid, Caffeic Acid, and L-Dopa on the Oxidative Cross-Linking of Feruloylated Arabinoxylans by a Fungal Laccase

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To study a way to covalently link arabinoxylans and proteins using a fungal laccase from the fungus *Pycnoporus cinnabarinus*, the effect of cysteinyl caffeic acid on the cross-linking of wheat arabinoxylans was investigated by means of capillary viscometry and RP-HPLC of alkali labile phenolic compounds. Cysteinyl caffeic acid provoked a delay in gelation and in the consumption of the esterified ferulic acid on arabinoxylans. When reacting free ferulic acid and cysteinyl caffeic acid with laccase, the ferulic acid consumption and the dehydrodimers production were also diminished. These results suggest that cysteinyl caffeic acid is oxidized while reducing the semiquinones of ferulic acid produced by laccase. Thus, ferulic acid could not be oxidized into dimers until all cysteinyl caffeic acid was consumed, preventing the cross-linking of feruloylated arabinoxylan chains. A similar mechanism is proposed in the case of caffeic acid and of L-Dopa.

Keywords: *Arabinoxylan; caffeic acid; cysteinyl caffeic acid; dehydrodimers; ferulic acid; laccase; L-Dopa; polyphenol oxidase*

INTRODUCTION

In attempts to make composite covalent networks (polysaccharide/protein), the possible linkage between feruloylated arabinoxylans and some amino acids of proteins has been previously investigated (Figueroa-Espinoza et al., 1998). The direct covalent bonding between the esterified ferulic acid on arabinoxylans and amino acids of proteins (cysteine, lysine, tyrosine) was possible neither in the presence of laccase nor in that of the couple hydrogen peroxide/horseradish peroxidase. In this paper, an alternative way was investigated: the use of caffeic acid, a diphenol which can form quinones upon oxidation, as an intermediate between a thiol group of a protein and the ferulic acid of arabinoxylans.

Wheat water-extractable arabinoxylans (WEAX), nonstarch polysaccharides issued from the endosperm cell walls, consist of a chain backbone of (1-4)-linked β-D-xylopyranosyl residues to which α-L-arabinofuranose units are linked as side residues (Izydorczyk and Biliaderis, 1995). Some arabinoses carry a feruloyl group covalently linked via an ester linkage (Smith and Hartley, 1983). WEAX form highly viscous solutions and in the presence of free radical generating oxidants undergo oxidative gelation through the dimerization of their esterified ferulic acid (Geissmann and Neukom, 1973; Hoseney and Faubion, 1981; Izydorczyk et al., 1990; Figueroa-Espinoza and Rouau, 1998). Dehydrodimers, rising from the oxidative coupling of arabinoxylan ferulate esters in the presence of hydrogen peroxide/peroxidase or laccase, represent products of 8-5′, 8-8′, 8-*O*-4′ and 5-5′ radical coupling (Ng et al., 1997; Figueroa-Espinoza and Rouau, 1998).

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Laccase (*p*-diphenol: oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing enzyme (Barman, 1969). In the presence of oxygen, it oxidizes phenols into free radical products, which polymerize through nonenzymic reactions (Brown, 1967; Malmström et al., 1975; Holwerda et al., 1976). It was previously demonstrated that laccase from the fungus *Pycnoporus cinnabarinus* was able to gelate WEAX, yielding different dimers of ferulic acid esters (Figueroa-Espinoza and Rouau, 1998). Tyrosinase (*o*-diphenol:oxygen oxydoreductase, EC 1.10.3.1), also referred to as catechol oxidase, phenolase, or polyphenoloxidase (PPO), is able to act on monophenols by converting them into dihydroxyphenols (cresolase activity) and to oxidize diphenolic compounds into quinones (catecholase activity) (Brown, 1967; Barman, 1969; Mayer and Harel, 1979). Quinones can react with nucleophiles (e.g., amino acids, proteins, phenolic compounds) either to form molecular adducts or to oxidize compounds of lower oxidation−reduction potentials (Brown, 1967; Pierpoint, 1970; Vámos-Vigyázó, 1981). PPO oxidizes caffeic acid into the corresponding *o*-quinone, which can undergo condensation reactions producing brown polymeric pigments or be converted into 2-*S*-cysteinyl caffeic acid by the addition of the sulfhydryl of cysteine (Singleton et al., 1985; Cheynier et al., 1986; Cilliers and Singleton, 1990; Cheynier and Moutounet, 1992). Substitution is through the cysteine sulfur on the 2-position of the aromatic ring (Cheynier et al., 1986). This thiol adduct is no longer a substrate for PPO (Singleton et al., 1985), but it can be oxidized by laccase into quinones that further polymerize or form a doubly substituted product (2,5-di-*S*-cysteinyl caffeic acid) if more cysteine is present (Salgues et al., 1986).

The aim of this work was to explore the possibility of using caffeic acid as a bridge to cross-link cysteine (used as a model of a functional group of proteins) and feruloylated WEAX, in the presence of the laccase of

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Pycnoporus cinnabarinus MIC11 (Falconnier et al., 1994). PPO was used to link cysteine to caffeic acid in the form of 2-*S*-cysteinyl caffeic acid. Laccase was used to make an attempt to link cysteinyl caffeic acid and caffeic acid to the ferulic acid on WEAX. The effect of caffeic acid and of its thiol adduct on the WEAX gelation and on the oxidation of ferulic acid was studied in model solutions. The effect of L-Dopa on arabinoxylans gelation was compared to that of caffeic acid, in order to study the effect of another diphenol on the WEAX gelation. L-Dopa is the product of the hydroxylation of tyrosine by an enzyme with a cresolase activity (Mayer and Harel, 1979; Vámos-Vigyázó, 1981). If L-Dopa can link to WEAX in the presence of laccase, it may represent a way to cross-link WEAX and proteins by starting from tyrosine using two different enzymes.

MATERIALS AND METHODS

Arabinoxylans. Wheat water-extractable pentosans (WEP) were isolated from a Thésée cultivar flour as described in 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 0.1 M sodium acetate buffer, pH 5.0, were used. The arabinoxylan (arabinose + xylose) content of WEAX was 82.9% db (92.3% total carbohydrates: arabinose + xylose + glucose), with a 0.57 arabinose−xylose ratio. Only traces of galactose were detected. These values were determined by gas−liquid chromatography as described in Figueroa-Espinoza et al. (1998). 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. Laccase (solution in 35% glycerol, 0.03 nkat/μL) 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). PPO from grape must was kindly supplied by Dr. V. Cheynier from the Institut des Produits de la Vigne-INRA (Montpellier, France). PPO from mushroom (tyrosinase, 3400 units/mg) was purchased from Sigma Chemical Co. (St. Louis, MO). Laccase activity on syringaldazine was measured as described in Figueroa-Espinoza et al. (1998).

Chemicals. Azocasein, caffeic acid (CA), L-cysteine (Cys), L-3,4-dihydroxyphenylalanine (L-Dopa), ferulic acid (FA), sodium sulfite, syringaldazine, and 3,4,5-trimethoxy-*trans*-cinnamic acid (TMCA) were purchased from Sigma Chemical Co. Phloroglucinol (1,3,5-trihydroxybenzene) was obtained 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, WI (Ralph et al., 1994).

Preparation of Cysteinyl Caffeic Acid (CCA). CCA was obtained at 25 °C using the following reaction mixture (RMa): 500 μL of CA 1.9 mM + 500 μL of Cys 1.9 mM + 1 mL of PPO (from grape must, 3 mg/mL; from mushroom, 1.5 mg/mL, 5100 units/mL). The solutions of CA, Cys, and both PPO were prepared using 0.05 M acetate buffer, pH 3.6. PPO solutions were sonicated for 4 min before use to break the protein aggregates and homogenize the suspension (Cheynier and Moutounet, 1992). The blank (RMa without enzyme) was made by replacing the PPO solution by 0.05 M acetate buffer, pH 3.6.

Reaction Mixtures. Reaction mixtures for the experiments using WEAX (RMb) were prepared at 25 °C according to the following (RMb): 2 mL of WEAX 0.2% (w/v) + 50 μL of A + 50 μL of B. In control samples (WEAX + enzyme), A was 0.1 M sodium acetate buffer, pH 5.0, and B was the laccase solution (3 nkat/mL). In blanks (WEAX without enzyme), the laccase solution (B) was replaced by 0.1 M sodium acetate buffer, pH 5.0. In the assays, A was substituted by one of the model compound solutions (CCA, CA, Cys or L-Dopa). Fifty microliters of A contained the model compound in a molar ratio (MR) of 0.25, 0.5, 1, 5, or 10, compared to the FA initially present in 4 mg of arabinoxylan (quantified by RP-HPLC). Fifty microliters of RMa diluted 2-fold are equivalent to CCA MR = 0.25. For RP-HPLC analyses, reactions were stopped after 15, 30, 60, and 90 min by adding 1 mL of 4 N NaOH to 1 mL of the reaction mixture RMb (final pH around 12).

Reaction mixtures for the experiments in model system RMc were prepared at 25 °C according to the following (RMc): 500 μL of filtered (0.45 μm) RMa + 500 μL of FA 0.47 mM + 500 μL of laccase solution (1.5 nkat/mL). FA and laccase solutions were prepared using 0.5 M acetate buffer, pH 5.75. The blank (RMc without enzyme) was made by replacing the enzyme solution by an equivalent volume of acetate buffer.

Determination of Alkali-Labile Phenolics. For the WEAX assays: 1 mL of RMb added to 1 mL of 4 N NaOH was treated as described in Figueroa-Espinoza et al. (1998). After the evaporation of the ether phase, 200 μL 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 Alltima Alltech (Alltech Associates, Inc., Deerfield, IL) C_{18} 5 μm column (250 × 4.6 mm). UV absorbance at 320 nm was used to detect the phenolic compounds. Gradient elution was performed using acetonitrile/0.05 M sodium acetate buffer, pH 4.0, at 1 mL/min and 35 °C, in isocratic elution for the first 3 min at 15/85 and then in linear gradients from 15/85 to 35/65 in 27 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. All solvents were of HPLC grade, and the mobile phase was sparged with helium. The spectra of ferulic acid and its dehydrodimers were recorded with a photodiode array detector Waters 996 (Millipore Co., Milford, MA).

For the model assays: To analyze the production of CCA by RP-HPLC, reactions were stopped at 4, 8, 12, 18, and 20 min by mixing 100 μL of RMa with 100 μL of sodium sulfite (38 mM) and 100 μL of TMCA (25 μg/mL), and then were filtered (0.45 μm) and injected (20 μL). CCA was identified by comparing its spectrum with that already published in Cilliers and Singleton (1990). To analyze by RP-HPLC the effect of CA and of CCA on the FA consumption by laccase, reactions were stopped at 4, 8, 12, 16, and 20 min by adding 300 μL of ether and 100 μL of TMCA (25 μg/mL) to 200 μL of RMc. Phenolics were extracted twice with 300 μL of ether. The ether phase was evaporated, and 200 μL of aqueous methanol (50% v/v) was added. Filtered samples (0.45 μm) were injected in RP-HPLC, using a Symmetry column from Waters (C_{18}, 5 μm, 250 × 4.6 mm) and using acetonitrile/0.05 M sodium acetate buffer, pH 4.0 (1 mL/min, 35 °C). Linear gradient started from 15/85 to 35/65 in 32 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.

Capillary Viscometry. Flow times of 2 mL of reaction mixture RMb 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: 29.99 s). Relative viscosities (ηrel) and specific viscosities (ηsp = ηrel − 1) were calculated using 0.1 M sodium acetate buffer, pH 5.0, flow time.

WEAX Determination. WEAX concentration was determined according to the semiautomated method of Rouau and Surget (1994), using an Alliance Instruments Evolution II autoanalyzer (Alliance Instruments, France). One hundred microliters of the WEAX solution diluted 50-fold were analyzed for their pentose content. The conversion of pentosan into furfural by hot acid treatment was followed by a color reaction with phloroglucinol.

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

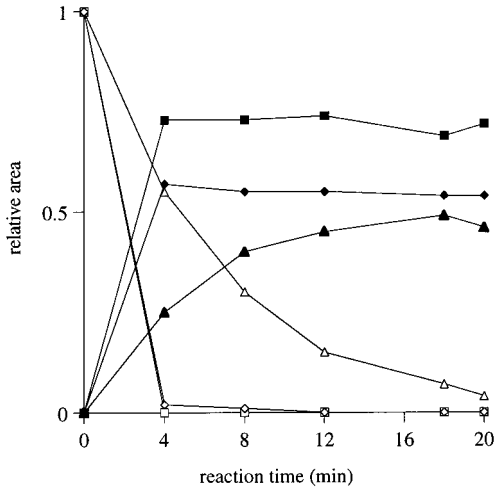

Figure 1. CA disappearance (unfilled symbols) and CCA formation (filled symbols) during the oxidation of CA in the presence of mushroom PPO and Cys MR = 1 (◆) and 10 (■) and of grape must PPO and Cys MR = 1 (▲). Results are expressed in percent of the initial relative area of CA with TMCA as internal standard.

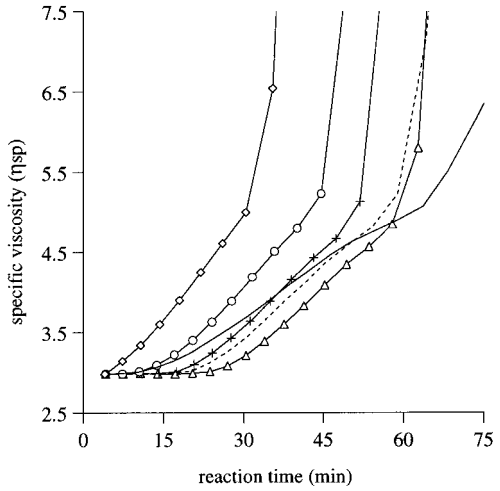

Figure 2. Capillary viscometry profiles of the effect on WEAX gelation of CA (—), Cys (○), CA + Cys (- - -), and CCA (+) at a MR = 0.25 and of CCA MR = 0.5 (△). ◇ represents the control sample (WEAX + laccase).

RESULTS

Production of CCA. Two different polyphenol oxidases were used to produce the CCA: one from grape must (used as a reference) (Cheynier et al., 1986), and one from mushroom. Both enzymes were able to produce CCA when incubated with CA and Cys. The RP-HPLC showed the decrease of CA and the appearance of a new compound, which increased as CA decreased (Figure 1). This compound was shown to be the thioether adduct CCA by comparison of its UV−visible spectrum (not shown) with that published by Cilliers and Singleton (1990). The spectrum of CA showed a maximum at 320.8 nm and a shoulder at 295 nm, and that of CCA showed only one at 316 nm. CCA was not a substrate for PPO, as published by Singleton et al. (1985).

An excess of Cys assured the total conversion of CA into CCA (Richard et al., 1991; Cheynier et al., 1986), as illustrated in Figure 1. All the CA was converted into CCA after 4 min of reaction when Cys was at a MR = 10 (MR between Cys and CA). As demonstrated previously (Figueroa-Espinoza and Rouau, 1998), Cys interferes with the oxidation of FA by laccase. Thus Cys at a MR = 1 was chosen to react with CA, in order to avoid an excess of thiol groups in the reaction mixture. The mushroom PPO was more efficient in producing CCA than the grape must PPO (Figure 1). Thus, the mushroom PPO was chosen to produce the thiol adduct to be used in the rest of the experiments. Eighteen minutes of reaction of CA and Cys MR = 1 with mushroom PPO (RMa) was considered enough to have mostly CCA and no CA left in the medium (Figure 1). RMa was filtered and used for the addition to the WEAX (RMb) or to the model system (RMc) mixtures.

Effect of CCA on WEAX Gelation and FA Consumption. CCA provoked a lag phase in the gelation process of WEAX. This delay without thickening increased with the CCA concentration (Figure 2). The specific viscosity of the control (WEAX + laccase) increased by 23% during the first 15 min. CCA MR = 0.25 and 0.5 provoked a delay before thickening of 14 and 22 min, respectively. Controls with CA, Cys, and CA + Cys, were made to compare the effect of these compounds with that of the thioether adduct. In all cases a delay on gelation was observed. CA MR = 0.25 and Cys MR = 0.25 provoked similar lag phases, whereas the delay for the control CA + Cys MR = 0.25 was similar to that of the CCA MR = 0.25. CCA MR = 0.5 produced a longer lag phase. From the analysis of phenolic compounds (Figure 3), it can be observed that in general FA consumption and dehydrodimers production were diminished. Whereas the FA content of the control was of 5 nmol/mg of WEAX after 15 min of reaction, in the rest of the assays (with CA, Cys, CA + Cys, or CCA) (Figure 3) the content of FA was of around 7 nmol/mg of WEAX. The delay in the FA consumption is in agreement with the viscosity results (Figure 2).

In the presence of CCA MR = 0.25 and 0.5 (Figure 3A), FA decreased by 7% after 15 min. The decrease was 33% in the control sample. After 90 min of reaction, for the control and experiments with CCA MR = 0.25 and 0.5, FA decreased by 90%, 83%, and 79%, and dehydrodimers increased by 9-, 7-, and 6-fold the initial concentration on the blank (WEAX without enzyme), respectively. Thus, since both FA consumption and dimers production were diminished and since FA consumption was proportional to the dimers production, it can be inferred that CCA did not link to the esterified FA on WEAX.

In the model experiments with free FA, results were similar (Figure 4A). In a FA−laccase solution, FA consumption presented a lag phase during the first 4 min, when CCA MR = 1 was added; then FA started to decrease at a slow rate until 8 min. From this point, FA diminished similarly as in the control (FA + laccase) to reach a total decrease of 97% after 20 min of reaction versus 99.8% in the control. Dehydrodimers production was also delayed. During the 4 min lag phase, no dimers were produced.

Effect of CA on WEAX Gelation and FA Consumption. CA was used to clarify whether the effect of CCA on WEAX gelation was due to the CA molecule itself, or to the presence of Cys in CCA.

CA inhibited gelation of WEAX in the presence of laccase (Figure 5). It provoked a delay which increased with the CA concentration. An 8 min delay was observed for a MR = 0.25, 16 min for a MR = 0.5, and 23 min for a MR = 1. No thickening occurred during the first 90 min of reaction for MR = 5 and 10.

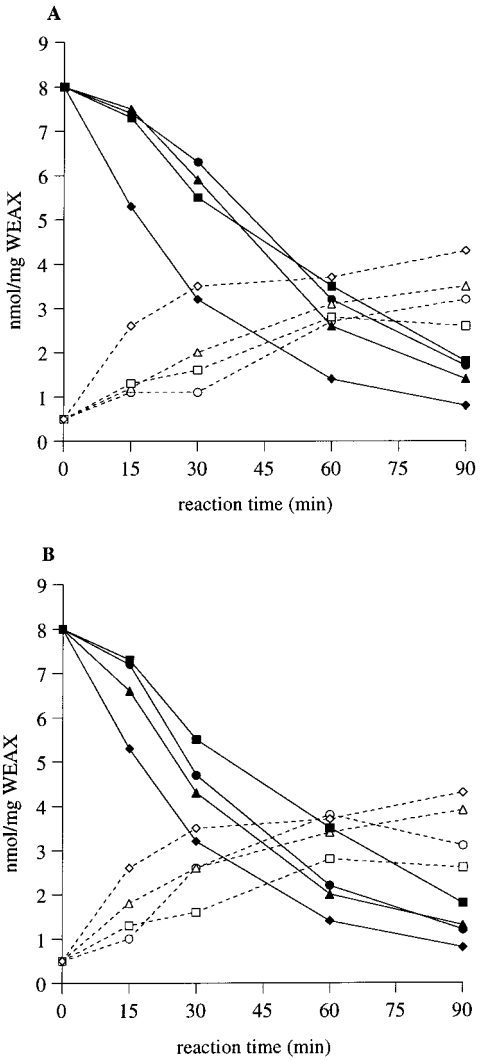

Figure 3. FA (filled symbols) and dehydrodimer (unfilled symbols) contents of WEAX solutions after reaction with laccase in the presence of CCA, CA, and Cys. Panel A: CCA MR = 0.25 (▲) and 0.5 (●). Panel B: Cys MR = 0.25 (▲) and CA + Cys MR = 0.25 (●). ◆ represents the control sample (WEAX + laccase) and ■ the sample with CA MR = 0.25.

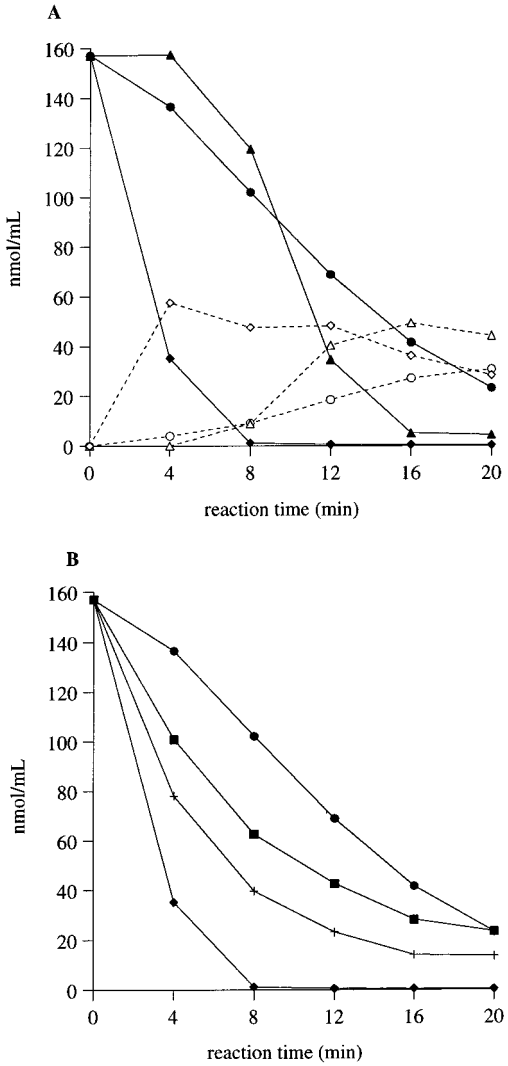

Figure 4. Panel A: FA (filled symbols) and dehydrodimer (unfilled symbols) contents of model solutions of FA after reaction with laccase in the presence of CCA MR = 1 (▲) and CA MR = 1 (●). ◆ represents the control sample (AF + laccase). Panel B: CA content of model solutions of CA after reaction with laccase (■) and with FA MR = 1 + laccase (+). ◆ and ● represent the same samples as in A.

FA consumption and dehydrodimer production were also retarded in the presence of CA (Figure 6). During the first 15 min, FA was consumed 4 times faster in the control sample (WEAX + laccase) (0.2 nmol/min) than in the experiment with CA MR = 0.25 (0.05 nmol/min). This coincided with the delay in viscosity (Figure 5), where ηsp increased by 23% for the control and by 4% for CA MR = 0.25. After 90 min of reaction, FA decreased by 90% in the control sample and by 77%, 65%, and 42% in the samples with CA MR = 0.25, 0.5, and 1, respectively. No changes were observed with CA MR = 5 (not shown) and 10 (Figure 6). Dehydrodimer production was also retarded. After 90 min of reaction, dimers increased 9-fold in the control sample compared to the initial content in the blank (WEAX without enzyme) and 5-, 2-, and 1-fold in the experiments with CA MR = 0.25, 0.5, and 1, respectively. Dimers did not change when CA MR = 5 (not shown) and 10 were added.

Both CA and FA were substrates for laccase (Figure 4B). In model solutions, for a similar initial concentration of both phenolic acids, FA was more rapidly consumed (99.8%) by laccase than CA (85%), after 20 min of reaction. When CA was added to a FA−laccase solution, FA consumption was diminished (85%) whereas that of CA was accelerated (91%) for the same reaction time. Dehydrodimer production was also delayed (Figure 4A). While in the sample AF + CA + laccase, dimers increased up to 30.8 nmol/mL after 20 min; in the control (FA + laccase) the dimers content reached a maximum of 57.6 nmol/mL after 4 min. The dimers decrease in the control after 4 min of reaction is probably due to a polymerization process. From these results, it is suggested that CA acted like CCA and did not link to FA either in WEAX solutions or in model solutions.

Effect of L-Dopa on WEAX Gelation. As observed in Figure 7, L-Dopa added at different levels (MR = 0.25, 0.5, 1, and 10) provoked a delay on the WEAX thickening, which increased with the L-Dopa concentration. Viscosity profiles were similar to those from the samples WEAX + CA + laccase for equivalent MR (Figures 5 and 7).

In the WEAX solutions with L-Dopa, the esterified FA was quantified after 90 min of reaction (Table 1). It was observed that the presence of L-Dopa provoked a delay

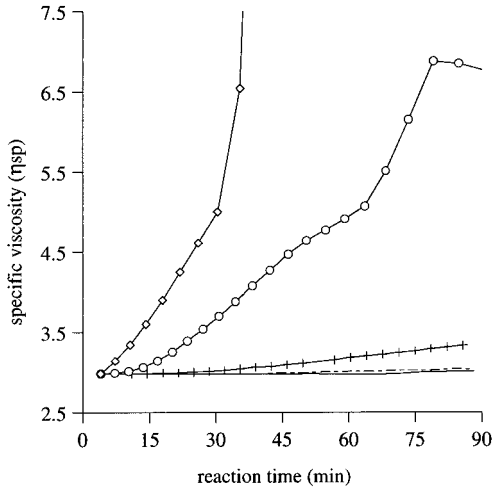

Figure 5. Capillary viscometry profiles of the effect on WEAX gelation of CA MR = 0.25 (○), 1 (+), 5 (- - -), and 10 (—). ◇ represents the control sample (WEAX + laccase).

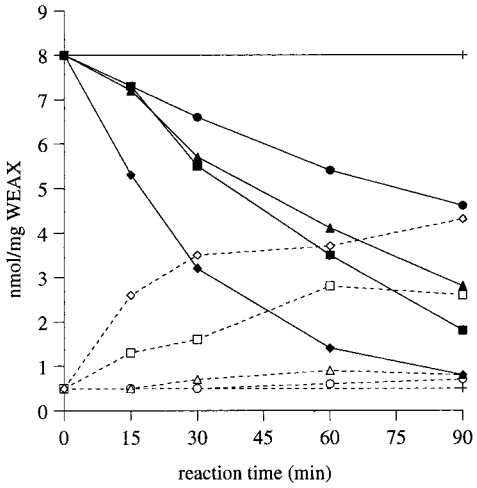

Figure 6. FA (filled symbols) and dehydrodimer (unfilled symbols) contents of WEAX solutions after reaction with laccase in the presence of CA MR = 0.25 (■), 0.5 (▲), 1 (●), and 10 (+). ◆ represents the control sample (WEAX + laccase).

on the consumption of the FA on WEAX, which coincided with the viscosity results (Figure 7). FA decreased by 81%, 66%, and 22% when L-Dopa was added at MR = 0.25, 0.5, and 1, respectively. In the control (WEAX + laccase), the esterified FA decreased by 90%. The production of dehydrodimers was also retarded. In the control, dimers increased 9-fold compared to the initial concentration in the blank, and the increase was 7-, 6-, and 2-fold for the samples with L-Dopa MR = 0.25, 0.5, and 1, respectively. In the sample with L-Dopa MR = 10, neither FA nor dimers changed. These results are in agreement with those observed in the presence of CA.

DISCUSSION

The objective of studying the effect of CCA on WEAX gelation was based on the possibility to use CA as a linkage agent between a feruloyl group of WEAX and a Cys of proteins. In case of the establishement of a covalent linkage between CCA and a FA on WEAX, the gelation process was expected to be slowed or inhibited, with an important FA consumption and with little or no dimer production. It was shown that CCA provoked a delay in the thickening of a WEAX−laccase solution (Figure 2), and the more CCA was added, the less FA

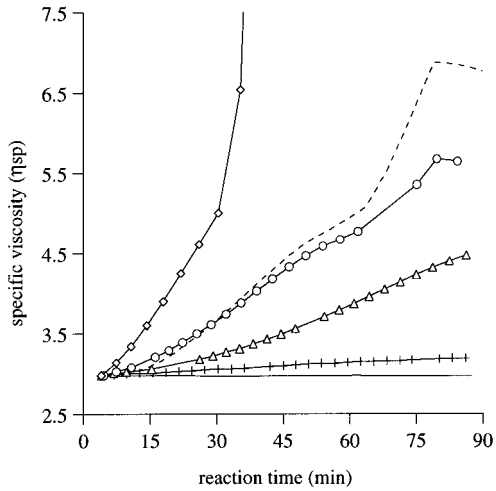

Figure 7. Capillary viscometry profiles of the effect on WEAX gelation of L-Dopa MR = 0.25 (○), 0.5 (△), 1 (+), and 10 (—). ◇ represents the control sample (WEAX + laccase), and - - - the sample with CA MR = 0.25, so it can be compared with the effect of L-Dopa.

Table 1. Ferulic Acid and Dehydrodimer Contents of WEAX Solutions, after 90 min of Reaction with Laccase in Presence of L-Dopa

sample	nmol/mg WEAX	
	tot-FA[a]	sum dim[b]
blank	8.0	0.5
WEAX + laccase	0.8	4.3
+ L-Dopa MR[c] = 0.25	1.5	3.6
+ L-Dopa MR = 0.5	2.7	2.9
+ L-Dopa MR = 1	6.2	1.2
+ L-Dopa MR = 10	8.0	0.3

[a] tot-FA = *cis*- + *trans*-ferulic acid. [b] Sum of dehydrodimers of ferulic acid. [c] MR = molar ratio between compound and the esterified ferulic acid in 4 mg of WEAX.

was consumed and the less dimers were produced (Figure 3A). CCA also delayed the consumption of FA and the production of dimers when added to a model FA−laccase solution (Figure 4A). These results suggest that CCA does not link to the feruloyl groups of WEAX in the presence of laccase.

The effects of CA on WEAX gelation (Figures 2 and 5) and on FA and dimers evolution (Figures 3 and 6) were similar to those observed with CCA (Figures 2 and 3). To better understand this effect, the evolution of both FA and CA was followed using model solutions (Figure 4B). It was observed that CA slowed the consumption of FA, and FA accelerated the consumption of CA, in the presence of laccase. Thus, no covalent linkage occurred between CA and FA.

As shown in Figures 2 and 3, the effect of CCA and CA on WEAX gelation and on FA and dimers evolution was similar to that provoked by Cys. In a previous paper (Figueroa-Espinoza et al., 1998), it was shown that Cys provoked a delay in the thickening of WEAX−laccase solutions and on FA consumption by reducing the FA semiquinones formed by laccase. Results with L-Dopa were similar to those observed with CA (Figure 7, Table 1). It is thus suggested that these three diphenolic compounds (CCA, CA, L-Dopa) provoke an effect similar to that of Cys.

A proposed mechanism of reaction is shown in Figure 8. Laccase oxidizes FA into a phenoxyl radical (semiquinone) (reaction 1), which undergoes nonenzymatic dimerization (reaction 2). When a diphenol like CA is

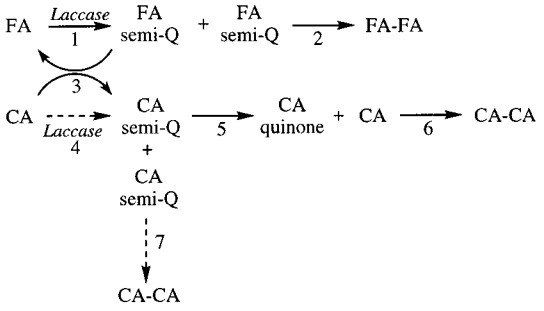

Figure 8. Hypothesis of the mechanism of reaction of the effect of caffeic acid on the oxidation of ferulic acid by laccase; semi-Q = semiquinone.

present, it immediately reduces FA radicals formed by laccase into the original FA (reaction 3). This protective effect of CA may be explained because when the number of hydroxyl groups of phenolic compounds increases, their redox potential decreases with an increasing susceptibility to oxidation (Thomson, 1964). Then CA is oxidized into a very unstable semiquinone which immediately disproportionates into an *o*-quinone (reaction 5) that undergoes further condensations leading to browning products (reaction 6). When an excess of CA was added (MR = 5 and 10), the WEAX−laccase solution presented a yellow color after 90 min of reaction, confirming the oxidation of CA into oligomers (Cilliers and Singleton, 1991). It is therefore suggested that both semiquinones of FA and CA would have to be present simultaneously to obtain a FA−CA compound. However, disproportionation of the CA semiquinone (reaction 5) is faster than the direct dimerization (reaction 7) (Hapiot et al., 1996).

In the presence of CA, the thickening of the WEAX−laccase solution was slower than that in the presence of Cys, although lag phases were similar (Figure 2). As shown in Figure 4B, both FA and CA are substrates for laccase, whereas Cys is not (Figueroa-Espinoza et al., 1998). Thus, CA could also compete with FA esterified to WEAX for the active site of laccase (Figure 8, reaction 4), explaining the slower thickening. It is also likely that some of the dimers produced from CA acted like CA by reducing the FA semiquinones because the phenyl substituent, as an electron-supplying group, lowers the redox potential of CA to which it is attached (Singleton, 1980). The mechanism is supposed to be the same in the case of CCA and L-Dopa. The effect of CCA is clearer than that of CA, probably because it reacts more rapidly due to a lower redox potential than CA (Cilliers and Singleton, 1990).

CONCLUSIONS

CCA and CA provoked similar effects on the WEAX gelation by laccase: a lag phase before the thickening of the solution, with a delay on the FA consumption and on the dehydrodimers formation. This effect is explained by the oxidation of CCA and CA with concominant reduction of the FA semiquinones into the original FA immediately as they are formed. In the presence of laccase, it was not possible to produce a covalent linkage between CCA and FA and therefore to use CA as an intermediate between a thiol group of protein and a FA of WEAX. L-Dopa provoked a similar effect as CA on the WEAX gelation by laccase. L-Dopa did not covalently link to FA in the presence of laccase. Consequently, linking feruloylated WEAX to proteins through hydroxylation of tyrosine by cresolase followed by oxidation by laccase did not appear feasible.

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.

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