JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Peroxidase-Catalyzed Oligomerization of Ferulic Acid Esters

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Valuable information about possible types of linkages, reaction mechanisms, and sequences for oxidative coupling of phenolic compounds in planta is available from in vitro model systems. Ferulate oligomers were generated in a system using ethyl ferulate, peroxidase, and hydrogen peroxide under various conditions. A molar ferulate/H_2O_2 ratio of 1:1, an ethanol level of 30% in an aqueous sodium phosphate buffer (pH 6.0), and a reaction time of 10 min were considered to be ideal to produce maximal proportions of ferulate trimers and tetramers from ethyl ferulate as starting material. The dominant trimer and tetramer were each isolated from the reaction mixture and identified as 8-*O*-4/8-5(cyclic)-dehydrotriferulic acid triethyl ester and 8-5(cyclic)/4-*O*-5/8-5(cyclic)-dehydrotetraferulic acid tetraethyl ester. The structure of the 8-*O*-4/8-5(cyclic)-dehydrotriferulic acid triethyl ester revealed that a third ferulate unit is bound to a preformed 8-*O*-4-diferulate dimer, a surprising reaction sequence considering the dominance of 8-5-coupled dimers among dehydrodiferulates in H_2O_2/peroxidase-based model reactions. As 4-*O*-5-coupling is not favored in the dimerization process of ferulates, the main tetramer isolated in this study is probably formed by 4-*O*-5-coupling of two preformed 8-5(cyclic)-diferulates, a logical step in analogy with reactions occurring in lignin biosynthesis.

KEYWORDS: Plant cell walls; cross-links; ferulic acid; ferulate; dehydrotriferulate; dehydrotrimer; dehydrotetraferulate; dehydrotetramer; peroxidase; NMR

INTRODUCTION

Polysaccharide-bound hydroxycinnamates occur widely in plant cell walls. In graminaceous plants such as cereals, but also in pineapple (family: Bromeliaceae), ferulic acid acylates the C5-OH of α-L-arabinosyl moieties of arabinoxylans (*1, 2*). Small amounts of arabinose-bound *p*-coumarates that probably also stem from arabinoxylans can also be detected following acidic hydrolysis of cereals (*3*). In dicotyledonous plants belonging to the family of Amaranthaceae, which now includes the formerly independent family of Chenopodiaceae (*4*), ferulates are attached to arabinans and galactans (*1, 5*). Dimerization of ferulates via oxidative coupling mechanisms (*6*−*8*) results in dehydrodiferulates that are now routinely identified in a variety of samples after alkaline hydrolysis (*9, 10*). Isolation and identification of diferuloyl arabinoxylan fragments after partial hydrolysis established that some dehydrodiferulates truly cross-link cereal arabinoxylans (*11*−*14*). More recently, dehydrotriferulic and dehydrotetraferulic acids were isolated from corn bran and were also identified in a variety of other plant samples (*15*−*21*).

The structures of some ferulate oligomers reveal their formation mechanisms, whereas the pathways for other oligomers remain open (*8*). Valuable information about possible types of linkages, reaction mechanisms, and sequences for oxidative coupling of phenolic compounds is usually available from in vitro model systems (*7, 22*−*24*). Ferulate model reactions have been performed primarily to study the formation of dehydrodiferulates. Only a few approaches exist to identify higher oligomers from oxidative oligomerization of hydroxycinnamates in model systems (*25*−*28*). These approaches used free hydroxycinnamic acids that model, for example, the detoxification of ferulic acid by some fungi (*29*). However, these approaches

Table 1. In Vitro Model Systems To Generate Ferulate Oligomers

system	ethyl ferulate (mg)	peroxidase (units)[a]	solvent: phosphate buffer (0.1 M, pH 6.0)/ethanol (v/v)	molar ratio of ethyl ferulate/H_2O_2	reaction time (min)
1	50	150	100/0	1/0.5	10
2	50	150	100/0	1/1	10
3	50	150	100/0	1/1.5	10
4	50	150	70/30	1/1	10
5	25	75	40/60	1/1	10
6	25	75	70/30	1/1	20
7	25	75	100/0	1/1	20

[a] One unit catalyzes the oxidation of 1.0 μmol of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) per minute at pH 6.0 at 27 °C.

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10.1021/jf801825z CCC: $40.75
Published on Web 10/08/2008

Table 2. NMR Data of the Isolated Ferulate Trimer **1** and Ferulate Tetramer **2**[a]

ferulate trimer **1**			ferulate tetramer **2**		
trimer unit	^1H[b]	^{13}C[c,d]	tetramer unit	^1H[b]	^{13}C[c,d]
A1		129.8	A1		129.1
A2	7.47 (1H; d; 1.7)	112.0	A2	7.29 (1H; br s)[e]	112.7
A3		148.0	A3		145.5
A4		148.2	A4		150.4
A5	6.82 (1H; d; 8.3)	114.8	A5		126.7
A6	7.14 (1H; dd; 8.3/1.7)	122.2	A6	7.24 (1H; br s)	118.3
A7	7.59 (1H; d; 15.8)	144.3	A7	7.60 (1H; d; 15.8)[f]	144.2
A8	6.45 (1H; d; 15.8)	116.9	A8	6.394/6.398 (1H; d; 15.8)[g]	116.0
A9		166.6	A9		166.9
A3-OMe	3.80 (3H; s)	55.9	A3-OMe	3.88 (3H; s)[h]	55.9
B1		nd	B1		131.1
B2	7.44 (1H; br s)	115.4	B2	6.92 (1H; d; 1.7)	105.6
B3		144.7	B3		149.6
B4		149.7	B4		138.5
B5		126.8	B5		144.5
B6	7.46 (1H; br s)	120.1	B6	6.582/6.574 (1H; d; 1.7)	109.9
B7	7.40 (1H; s)	126.9	B7	5.98 (1H; d; 7.5)	87.3
B8		138.5	B8	4.36 (1H; d; 7.5)	55.7
B9		163.0	B9		170.6
B3-OMe	3.78 (3H; s)	55.9	B3-OMe	3.87 (3H; s)	55.9
C1	7.05 (1H; d; 1.9)	131.3	C1		136.2
C2		110.2	C2	7.19 (1H; d; 1.7)	111.3
C3		149.9	C3		151.2
C4		147.2	C4		150.4
C5	6.81 (1H; d; 8.2)	114.8	C5	6.83 (1H; d; 8.3)	118.7
C6	6.87 (1H; dd; 8.2/1.9)	119.5	C6	6.95 (1H; br d; 8.3)	118.8
C7	5.98 (1H; d; 8.1)	87.6	C7	6.09 (1H; d; 7.7)	87.3
C8	4.36 (1H; d; 8.1)	55.9	C8	4.43 (1H; d; 7.7)	55.7
C9		170.5	C9		170.6
C3-OMe	4.00 (3H; s)	56.1	C3-OMe	3.78/3.79 (3H; s)	55.9
			D1		129.1
			D2	7.34 (1H; br s)[e]	112.7
			D3		145.5
			D4		150.4
			D5		126.7
			D6	7.29 (1H; br s)	118.3
			D7	7.62 (1H; d; 15.9)[f]	144.4
			D8	6.42 (1H; d; 15.9)[g]	116.2
			D9		166.9
			D3-OMe	3.93/3.94 (3H; s)[h]	55.9

[a] Assignments follow the numbering shown in **Figure 4**; ethyl group data are not reported. [b] δ in ppm; J in hertz; s, singlet; br s, broad singlet; d, doublet; br d, broad doublet; dd, doublet of doublets. [c] δ in ppm. [d] Data from 2D HMQC/HMBC experiments; nd, not determined. [e] Assignments may be interchanged. [f] Assignments may be interchanged. [g] Assignments may be interchanged. [h] Assignments may be interchanged.

do not necessarily reflect the conditions in the plant cell wall, where ferulate does not exist as its free acid but in esterified form. Lack of esterification leads to partial decarboxylation of ferulic acid and its dimers; 8-5(decarboxylated)-dehydrodiferulic acid was shown to be one of its major dehydrodimers (*28*).

The aim of this study was to isolate and identify the major dehydrotriferulates and dehydrotetraferulates generated in a model system using ethyl ferulate, peroxidase, and hydrogen peroxide under various conditions. These oligomers are valuable for authenticating the structures formed in planta.

MATERIALS AND METHODS

General. Horseradish peroxidase (type II) was from Sigma (St. Louis, MO). The activity of the enzyme (1215 units/mg) was determined by a spectrophotometric assay (*30*) at 420 nm using the peroxidase substrate 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) in solution (0.1 M sodium phosphate buffer, pH 6.0, 27 °C). The molar extinction coefficient of the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) radical cation at 420 nm (31.5 L mM^{-1} cm^{-1}) was determined under the conditions used. Bio-Beads S-X3 was from Bio-Rad Laboratories (Munich, Germany), and the Bio-Beads size exclusion chromatography glass column ECO^{PLUS} was from Kronlab (Sinsheim, Germany). Size exclusion chromatography (SEC) instrumentation (L-2000 pump, L-7400 UV detector, preparative flow cell) was from Merck/Hitachi (Darmstadt, Germany). For more analytical SEC purposes, a Perkin-Elmer LC 90 UV detector (analytical flow cell) was used. Analytical and semipreparative phenyl-hexyl-HPLC-columns were purchased from Phenomenex (Aschaffenburg, Germany). Phenyl-hexyl-RP-HPLC was generally carried out using the following instrumentation: L-7150 intelligent pump, L-7300 column thermostat, L-7455 diode array detector (DAD) using an analytical or semipreparative flow cell, respectively (VWR, Darmstadt, Germany) or model 994 analytical photodiode array detector (Waters, Eschborn, Germany). HPLC-MS instrumentation was from Hewlett-Packard (Waldbronn, Germany): HP series 1100, autosampler G1313, pump G 1312A, mass spectrometer G 1946A. NMR experiments were performed on a Bruker DRX-500 instrument (Rheinstetten, Germany).

Ethyl Ferulate. Ethyl ferulate was prepared according to Fieser's esterification method (*31*) as described in Ralph et al. (*24*). In brief, ferulic acid (10 g) was dissolved in absolute ethanol (100 mL) to which acetyl chloride (5 mL) had been added. The solution was stirred overnight, and the solvents were removed by rotary evaporation at 40 °C. To ensure complete esterification, the procedure was repeated.

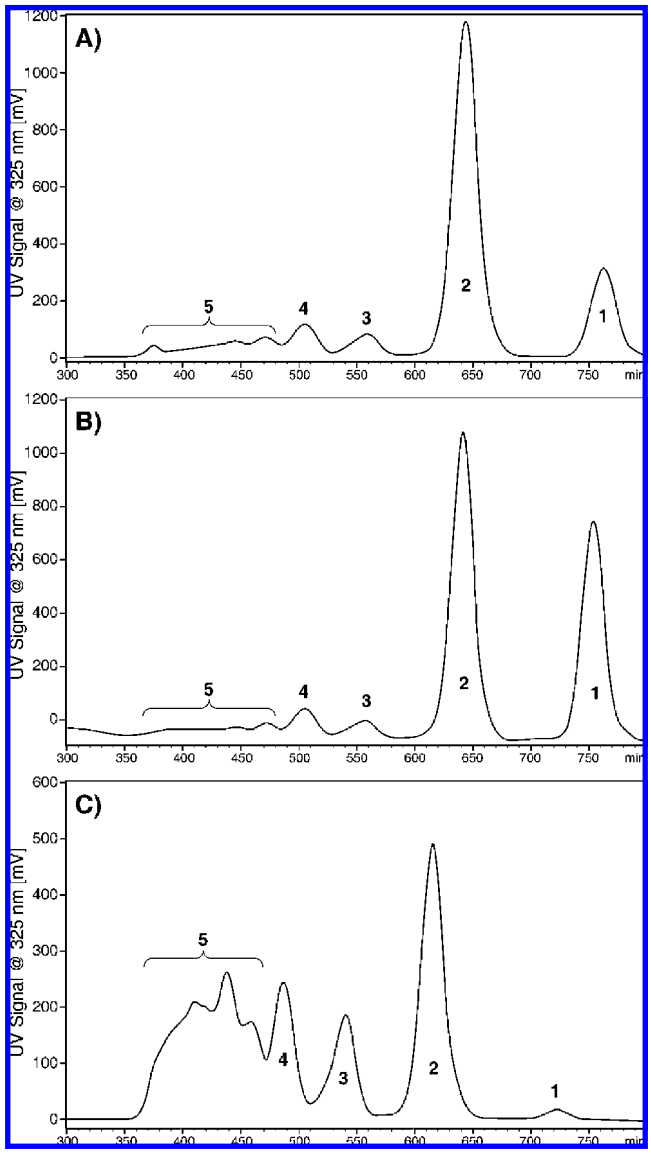

Figure 1. Size exclusion chromatograms of ferulate oligomers generated in different in vitro model systems: (**A**) ferulate oligomers from system 2 (**Table 1**) containing buffer and a ferulate/H_2O_2 ratio of 1:1; (**B**) ferulate oligomers from system 3 (**Table 1**) containing buffer and a ferulate/H_2O_2 ratio of 1:1.5; (**C**) ferulate oligomers from system 4 (**Table 1**) containing buffer/ethanol 70:30 (v/v) and a ferulate/H_2O_2 ratio of 1:1.

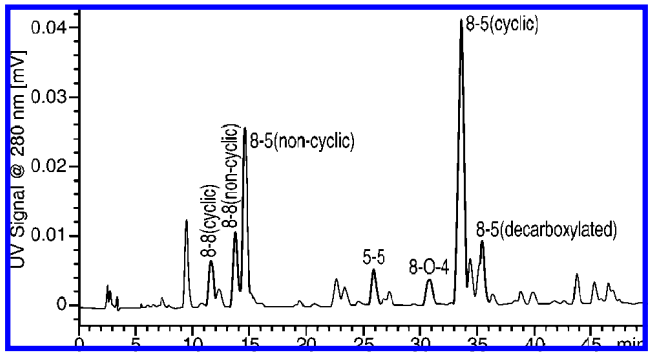

Figure 2. HPLC-DAD chromatogram monitored at 280 nm of the dimer fraction following a single saponification step. Peak assignment was performed by using authentic standard compounds.

Finally, generated HCl was removed by repeated addition and evaporation of ethanol. The product was crystallized from ethyl acetate−petroleum ether, and complete esterification was verified by NMR.

In Vitro Semipreparative Dehydrogenation To Generate Ferulate Oligomers. Seven model systems were tested to generate ferulate oligomers (**Table 1**). Ethyl ferulate (25 or 50 mg) was dissolved in sodium phosphate buffer (0.1 M, pH 6.0) at 65 °C or in mixtures of sodium phosphate buffer (0.1 M, pH 6.0) and ethanol at 40 °C (1 mL of solvent per milligram of ethyl ferulate). The solution was allowed to cool to below 35 °C before a solution (50 or 100 μL) of peroxidase was added, with a defined enzyme activity, in sodium phosphate buffer. The required volume of a carbamide−peroxide solution (1 M, hydrogen peroxide−urea adduct) was added in five aliquots at 2 min intervals. Carbamide−peroxide was used because it can be more precisely dosed than hydrogen peroxide solutions. Total reaction times were 10 min (additional 2 min after the final peroxide addition) or 20 min (additional 12 min after final peroxide addition). The reactions were stopped by extraction (three times) with ethyl acetate (7 or 15 mL). Extraction of the 60% ethanol system required the addition of a saturated NaCl solution to achieve phase separation. The organic layers were collected and extracted twice with water. The ethyl acetate layers were made up to 50 mL, and aliquots were used for SEC.

Ferulate Oligomerization on a Preparative Scale. Ethyl ferulate (500 mg) was dissolved in a sodium phosphate buffer (0.1 M, pH 6.0)/ethanol solution (70:30 v/v, 500 mL) and peroxidase (1520 units) in sodium phosphate buffer was added. Five aliquots (4.5 mL) of a carbamide−peroxide solution (2 M) were added in 2 min intervals. After a total reaction time of 10 min, the reaction was stopped by the addition of ethyl acetate (150 mL). Ethyl acetate extraction was performed three times; the organic layers were combined, washed twice with water (100 mL), and evaporated.

Size Exclusion Chromatography. Aliquots (1 mL) of the semipreparative model reaction products were evaporated and redissolved in tetrahydrofuran (THF; 500 μL). SEC used Bio-Beads SX-3 as the stationary phase and THF as the mobile phase in a solvent-resistant column (1.5 cm × 100 cm; 94 cm gel bed). Samples were applied using a six-way valve and a 100 μL sample loop. The flow rate was maintained at 0.2 mL/min. Detection was performed at 325 nm using an analytical flow cell. A larger aliquot (40 mL) from model reaction 4 (**Table 1**) was treated as described, and the fractions were collected by hand according to the chromatogram and evaporated. For SEC separation of the preparative-scale reaction, batch aliquots (100 mg) were dissolved in THF (230 μL) and injected using a 200 μL sample loop. A flow rate of 0.25 mL/min was used, and detection at 325 nm used a preparative flow cell. Fractions were collected by hand according to the chromatograms.

Characterization of the SEC Fractions by Analytical HPLC-DAD. Aliquots of the SEC dimer, trimer, and tetramer fractions were evaporated and redissolved in MeOH. Separation was carried out by using a Luna phenyl-hexyl column (250 × 4.6 mm i.d., 5 μm). The column temperature was 35 °C, the flow rate was 0.6 mL/min, and elution was monitored at 280 and 325 nm. The following eluents (A−D, mostly aqueous eluents; E−G, mostly organic eluents) and gradient systems were used: eluent A, bidistilled water; eluent E, acetonitrile. Gradient a began at 50% A, 50% E, linear over 90 min to 20 % A, 80% E, following rinsing and equilibration steps. Gradient b was initially 80% A, 20% E, linear over 60 min to 20% A, 80% E, following rinsing and equilibration steps.

In addition, the separation of the free acids was tested. Saponification of the esters from the different SEC fractions was achieved by using 2 M NaOH for 18 h under N_2 in the dark. Following acidification (pH <2), the acids were extracted into diethyl ether. Following evaporation of the ether, the acids were dissolved in 70% aqueous methanol (v/v). Addition of a few drops of acetone was sometimes required to achieve complete solution for HPLC. Aliquots of the SEC fractions were also saponified twice to establish the completeness of the saponification process.

The following gradients were used to achieve separation of the acids using the HPLC system described above. Separation of ferulic acid trimers and tetramers: eluent C, 1 mM aqueous trifluoroacetic acid; eluent E, acetonitrile. Gradient c was initially 20% C, 80% E, linear over 60 min to 20% C, 80% E, following rinsing and equilibration steps. Separation of ferulic acid dimers (different flow rate = 1 mL/min): eluent C, 1 mM aqueous trifluoroacetic acid; eluent E, acetonitrile;

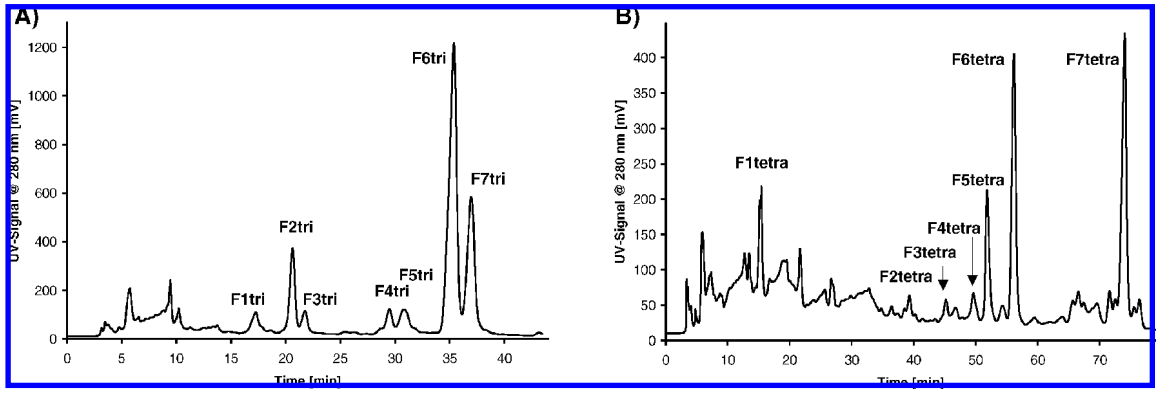

Figure 3. Semipreparative HPLC-DAD chromatogram monitored at 280 nm of the (**A**) trimer fraction and (**B**) tetramer fraction of the preparative ferulate oligomerization batch obtained from SEC.

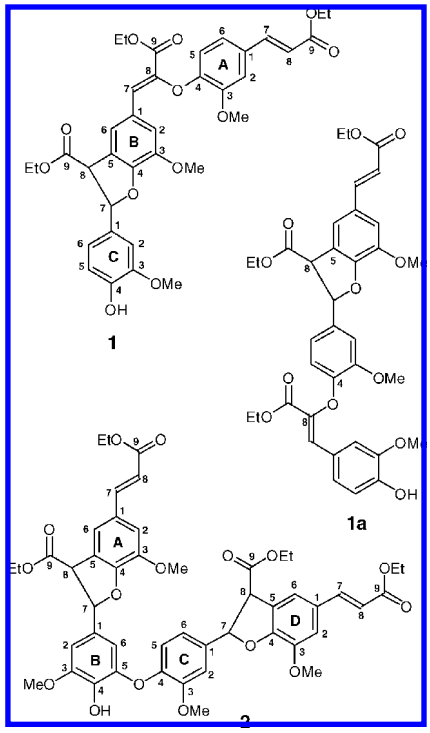

Figure 4. Structures and numbering system of the isolated trimer **1**, 8-*O*-4/8-5(cyclic)-dehydrotriferulic acid triethyl ester, and tetramer **2**, 8-5(cyclic)/4-*O*-5/8-5(cyclic)-dehydrotetraferulic acid tetraethyl ester. Trimer **1** was isolated from the HPLC fraction F6tri and tetramer **2** from fraction F7tetra (**Figure 3**). Structure **1a** shows an alternative trimer containing 8-*O*-4 and 8-5(cyclic) linkages. This structure, rather than **1**, was ruled out by interpreting the HMBC spectrum.

eluent G, methanol. Gradient d began at 69% C, 4% E, 27% G, held for 5 min, linear over 11 min to 65% C, 6% E, 29% G, linear over 24 min to 60% C, 25% E, 15% G, held isocratically for 6 min, following rinsing and equilibration steps.

Characterization of the SEC Fractions by Analytical HPLC-MS. Molecular mass determination was achieved by HPLC-MS using atmospheric pressure−electrospray ionization (AP-ESI) in positive and negative modes (fragmentor voltage, 60, 95, or 100 V; scan range, *m*/*z* 150−950). Separation was performed on a Luna phenyl-hexyl column (250 × 4.6 mm i.d., 5 μm). The column temperature was 35 °C, the flow rate 0.6 mL/min, and the injection volume 20 μL. Elution was also monitored by UVD. HPLC conditions: eluent D, 0.1% aqueous formic acid; eluent E, acetonitrile. Gradient e (used for trimeric and tetrameric ferulate esters) began at 40% D, 60% E, linear over 33 min to 30% D, 70% E, linear over 20 min to 10% D, 90% E, held isocratically for 7 min, following an equilibration step.

Isolation of Dominant Ferulate Trimers and Tetramers by Semipreparative HPLC. Isolation of the dominant ferulate trimers and tetramers was achieved by separation of the ethyl esters on a Luna phenyl-hexyl column (250 × 10 mm i.d., 5 μm) at 35 °C using a flow rate of 2.8 mL/min. The injection volume was 70 μL using a 100 μL sample loop, and elution was monitored at 280 nm. Solvents were (B) water/acetonitrile 90:10 (v/v), (F) acetonitrile/water 90:10 (v/v), and (G) methanol. Gradient f (used for the separation of trimers) was initially 39% B, 51% F, 10% G, linear over 60 min to 26% B, 64% F, 10% G, linear over 3 min to 100% F, held isocratically for 10 min, following an equilibration step. Gradient g (used for the separation of tetramers) began at 51% B, 39% F, 10% G, linear over 15 min to 39% B, 51% F, 10% G, linear over 60 min to 26% B, 64% F, 10% G, linear over 3 min to 100% F, held isocratically for 10 min, following an equilibration step.

Structural Identification by NMR. Structural identification of samples dissolved in 0.7 mL of acetone-d_6 was performed using one- and two-dimensional NMR experiments (^{1}H, H,H-COSY, HMQC, HMBC). ^{13}C data were taken from the two-dimensional HMQC and HMBC experiments. Chemical shifts (δ) (**Table 2**) were referenced to the central solvent signals (δ_H 2.04 ppm; δ_C 29.8 ppm). Coupling constants (*J*) are given in hertz.

RESULTS AND DISCUSSION

Size Exclusion Chromatography of Ferulate Oligomers. In a preliminary study we tested several stationary and mobile phases to separate ferulate oligomers according to their molecular weights. A test mixture made up of ferulic acid, 8-5(cyclic)-diferulic acid and 5-5/8-*O*-4-triferulic acid (*32*) was used. Neither Sephadex LH-20 using 2-propanol as eluent nor Sephadex G-10 or Sephadex G-25 using 30% methanol as eluent was a suitable chromatographic system to separate the mixture. We were able to achieve a respectable separation of our test mixture using Bio-Beads SX-3 as stationary phase and THF as eluent. A second test mixture made up of ferulic acid ethyl ester and 8-5(cyclic)-diferulic acid diethyl ester was also well separated by using Bio-Beads SX-3/THF, whereas the use of Sephadex LH-20/2-propanol did not result in a baseline separation. Therefore, Bio-Beads SX-3/THF as the SEC system was chosen for all further SEC steps.

In Vitro Models To Generate Ferulate Oligomers. Because most ferulate in the plant cell wall acylates cell wall polymers we used the ethyl ester of ferulic acid as our model. Esterification prevents the ferulates from being decarboxylated during the oxidative radical coupling process; decarboxylation occurs extensively when using free acids (*25, 27, 28*). We are aware of the fact that simple ethyl esters in homogeneous systems do not fully model reactions of ferulates that are linked to polymers in the cell wall; however, in the case of ferulate dimers, this model was quite useful to prepare and predict the possible linkage types for ferulate dimers (*7, 24*). Several reaction conditions to optimize the yields of ferulate trimers and tetramers

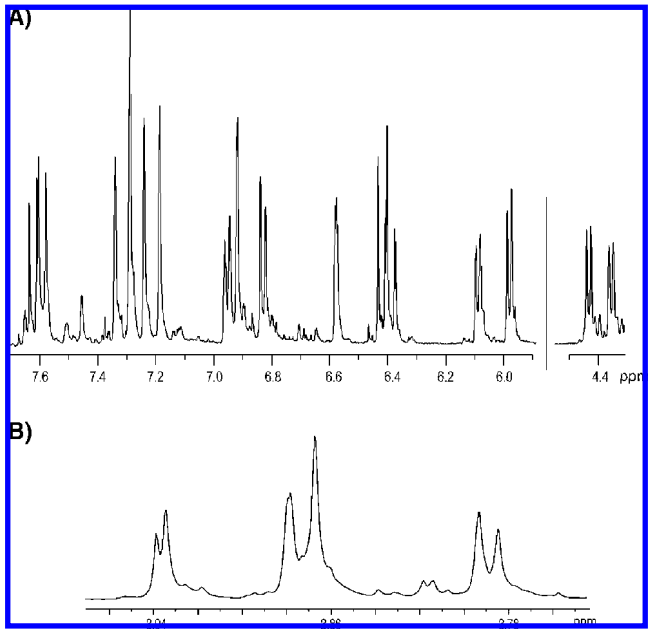

Figure 5. ^{1}H NMR spectrum (500 MHz) of the isolated ferulate tetramer **2**, 8-5(cyclic)/4-*O*-5/8-5(cyclic)-dehydrotetraferulic acid tetraethyl ester: (**A**) section showing the aromatic and side-chain signals; (**B**) zoomed section showing the signals from the guaiacyl methoxyl groups and indicating that the isolate is a mixture of two isomers (see text for explanation).

were tested. As the pH optimum for the used peroxidase was described to be in the range of 6.0−6.5, we used an aqueous pH 6.0 buffer solution. Molar ratios of ethyl ferulate to H_2O_2, solvents, and reaction times were as listed in **Table 1**. To prevent H_2O_2-dependent enzyme inactivation (*33*), H_2O_2 was added in five portions at 2 min intervals. The reaction batches were separated by SEC as shown in **Figure 1**. Molar ferulate/H_2O_2 ratios of 1:0.5 (not shown) and 1:1 (**Figure 1A**) using an aqueous buffer as solvent and a reaction time of 10 min resulted in similar SEC profiles. Ferulate dimers (peak 2, **Figure 1A**) clearly dominate the profile, and considerable amounts of monomeric ferulate (peak 1, **Figure 1A**) were not transformed into oligomers. Using a higher ferulate/H_2O_2 ratio of 1:1.5 led to decreased oligomerization (**Figure 1B**), possibly because higher amounts of H_2O_2 led to a partial inactivation of the enzyme (*33*). The amounts of trimers and higher oligomers were generally low. It was assumed that the low solubility of the ferulate dimers in the aqueous buffer prevented substantial conversion of the formed dimers to higher oligomers. To enhance the solubility of the intermediate dimers, buffer/ethanol solutions with ethanol proportions of 30 and 60% were chosen as solvents (ferulate/H_2O_2 ratio 1:1). As shown in **Figure 1C** the use of ethanol in the reaction medium led to enhanced proportions of higher oligomers. An ethanol proportion of 30% was slightly more effective than an ethanol proportion of 60% (not shown). Partial inactivation of the enzyme due to incipient protein denaturation at higher ethanol proportions in the solvent may explain this result. Finally, we tested whether or not prolongation of the reaction time from 10 to 20 min improved the formation of higher oligomers. However, an enhanced reaction time did not increase the yield of higher oligomers using a ferulate/H_2O_2 ratio of 1:1 and an ethanol portion of 30% in the solvent. Therefore, a ferulate/H_2O_2 ratio of 1:1, an ethanol portion of 30% in the solvent, and a reaction time of 10 min was used in the preparative batch using 500 mg of ethyl ferulate as substrate.

Isolation of the Dominant Ferulate Trimers and Tetramers. The preparative batch was fractionated by SEC. The molecular weight fractionation into monomers, dimers, trimers, tetramers, and higher oligomers was confirmed by application of analytical HPLC-MS to the different SEC fractions. Further fractionation of the SEC fractions was possible by semipreparative RP-HPLC using a phenyl-hexyl stationary phase. Saponification of the dimer fraction led to the chromatogram shown in **Figure 2**. By using authentic standard compounds (*32*) the expected dimer profile was verified, although the chromatogram was more complex than expected: 8-5-coupled dimers dominated, whereas only small amounts of 8-8-, 8-*O*-4-, and 5-5-coupled dimers were found (*24, 34*). A second saponification step of the dimer fraction reduced the total number of minor peaks, demonstrating the rather high stability of some ester linkages as has been seen previously (*35*). Some smaller signals in the dimer fraction remained unassigned even after a second saponification. As demonstrated for 5-5-dehydrodiferulate (*16*), alkaline saponification may lead to small amounts of aldehydes despite the use of a nitrogen atmosphere under the experimental conditions described.

Saponification of the trimer fraction led to a highly complex chromatogram. Following a second saponification step, the number of peaks was slightly reduced, but the chromatogram was still too complex to isolate pure compounds by RP-HPLC. Because this was also the case for the tetramer fraction, it was decided to separate the trimers and tetramers as their ethyl esters. We initially tried to avoid separation of the esters because RP-HPLC of the esters requires high portions of organic solvents in the gradient system, decreasing the options to optimize the chromatographic system. Chromatograms of the semipreparative fractionation of the ethyl ferulate trimers and tetramers are shown in **Figure 3**. Peaks F6tri and F7tri (**Figure 3A**) were isolated and analyzed by ^{1}H NMR. Peak F7tri clearly contained a mixture of several compounds. Although not a single compound, the purity of F6tri (around 75%) was sufficient to structurally elucidate the main component by NMR (**Table 2**). The 1D proton spectrum revealed three singlets at 3.78, 3.80, and 4.00 ppm, which were assigned to nine protons from three methoxyl groups. Two 15.8 Hz doublets indicated one unsubstituted propenyl side chain. Two 8.1 Hz doublets at 4.36 and 5.98 ppm (especially in conjunction with their carbon data, derived from 2D HMQC spectra, at 55.9 and 87.6 ppm) were characteristic for 7- and 8-protons of a ferulate side chain involved in an 8-5(cyclic)-linkage (*7*). Two doublets of doublets at 6.87 ppm and 7.14 ppm with coupling constants of 8.2 and 1.9 Hz and 8.3 and 1.7 Hz, respectively, indicate two guaiacyl units with protons in their 2-, 5-, and 6-positions. The third guaiacyl unit is linked at the 5-position as already mentioned and as also shown by the two broad singlets at 7.44 and 7.46 ppm. A singlet at 7.40 ppm (in conjunction with the carbon shift at 126.9 ppm) is an indicator for 8-coupling of a second side chain. Couplings were verified by interpreting the H,H-COSY spectrum. In conjunction with carbon data deduced from the HMQC spectrum, the guaiacyl units within the trimer were determined to be coupled via an 8-5(cyclic)- and an 8-*O*-4-linkage, indicating two possible structures (compounds **1** and **1a**, **Figure 4**). Differentiation between those two structures was possible by interpreting the HMBC spectrum. HMBC data revealed that the unsubstituted propenyl side chain is bound to the guaiacyl unit containing protons at its 2-, 5-, and 6 positions, thus ruling out structure **1a**. The HMBC spectrum also gave evidence for the 8-5(cyclic)-linkage between the units B and C, thus proving compound **1** to be 8-*O*-4/8-5(cyclic)-dehydrotriferulic acid triethyl ester. Compound **1** differs substantially from ferulate trimers (*27, 33*) that were previously isolated from model reactions using free

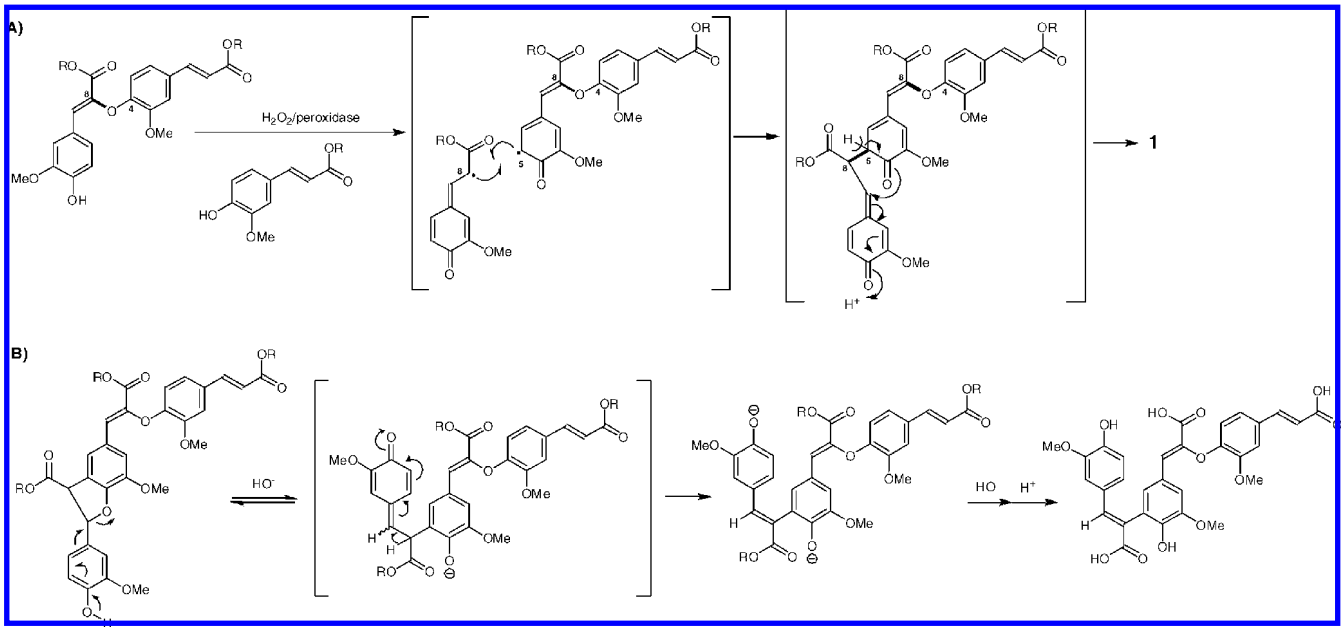

Figure 6. (**A**) Formation mechanism of ferulate trimer **1**, 8-*O*-4/8-5(cyclic)-dehydrotriferulic acid triethyl ester. R = ethyl. (**B**) Base-catalyzed ring opening and formation of the 8-*O*-4/8-5(noncyclic)-dehydrotriferulic acid that was isolated from corn bran (*16*). R = arabinoxylans.

ferulic acid as a substrate. All ferulic acid derived trimers isolated from model reactions to date contain a decarboxylated moiety; oligomerization of free ferulic acids clearly favored the decarboxylation of the dehydrodimer intermediates and therefore does not properly model the pathways for ferulate esters in the plant cell wall.

The chromatogram of the semipreparative fractionation of the ethyl ferulate tetramers (**Figure 3B**) revealed three major peaks at 280 nm (F5tetra, F6tetra, F7tetra). Peaks F5tetra, F6tetra, and F7tetra were analyzed by ^{1}H NMR showing that F5tetra and F6tetra were each composed of several compounds, whereas F7tetra was fairly clean (>90%) (**Figure 5A**). Proton signals between 3.7 and 4.0 ppm, the region where methoxyl signals of guaiacyl units usually are located, showed an unexpected splitting (**Figure 5B**). Although integration of these signals revealed 12 protons, which is in accordance with a ferulate tetramer, we did not observe the expected four singlets, as will be explained later. Four 7.5 or 7.7 Hz doublets at 5.98 and 4.36 ppm as well as 6.09 and 4.43 ppm (in conjunction with their carbon shifts at 87.3 and 55.7 ppm) are again characteristic for 7- and 8-protons of a cinnamate side chain involved in an 8-5(cyclic)-linkage. Four doublets with coupling constants of 15.8 or 15.9 Hz indicate two unsubstituted propenyl side chains. The aromatic region of the proton spectrum also revealed that only one guaiacyl unit contains protons at its 2-, 5-, and 6-positions (with $J_{5,6}$ = 8.3 Hz), whereas the other three guaiacyl units seem to be linked at their 5-positions as indicated by the lack of 8 Hz couplings. Apparently next to the methoxyl signals two further proton signals (H8, unit A, and H6, unit B) are further split, and some other signals are broader than usual. This is the result of having the possibility of two stereoisomers for this tetramer. In conjunction with the carbon data from the HMQC experiment (**Table 2**) the proton data indicate a mix of stereoisomers of compound **2**, an 8-5(cyclic)/4-*O*-5/8-5(cyclic)-dehydrotetraferulate (**Figure 4**) making up the fraction F7tetra. The proposed structure is in accordance with the HMBC data that clearly demonstrated 8-5(cyclic)-coupling of the ring B and A as well as C and D.

NMR of compound **2** revealed that two stereoisomers of this compound exist that could not be separated using RP-HPLC of the esters. However, separation of the derived free acid stereoisomers was possible, confirming their existence; the proton NMR spectra differed only subtly. Although the 7,8-stereochemistry of each unit is *trans*, and fixed relative to each other, the two units are independent; compound **2** behaves as a compound with two asymmetric carbons.

To the best of our knowledge this is the first ferulate tetramer isolated from a model reaction. However, Liu et al. (*25*) isolated a tetramer that was formed from free sinapic acid in a peroxidase/H_2O_2-catalyzed reaction. As described for oligomerization of free ferulic acid, decarboxylation reactions were favored by using free sinapic acid as a substrate, thus inadequately modeling the reactions from plant-derived sinapate or ferulate esters in the plant cell wall.

Implications. It was assumed that the major trimers would be formed by addition of a third ferulate to a preformed 8-5(cyclic)-diferulate due to its dominant role shown here and in comparable in vitro reactions (*7*). The 8-*O*-4-diferulate usually makes up only a small portion of the dimer fraction. The isolation of 8-*O*-4/8-5(cyclic)-triferulate **1** as a dominant trimer was therefore somewhat surprising. Formation of trimer **1** requires that the 8-*O*-4-diferulate is formed first, and the 8-5(cyclic)-linkage is formed by addition of the third ferulate (**Figure 6A**); a preformed 8-5(cyclic)-diferulate will not couple via the propenylic 8-position because the particular moiety's phenolic hydroxyl group is blocked, precluding the formation of a radical with single-electron density at the required 8-position. Formation of an 8-5-linkage as a chain elongation step is known from lignin chemistry, where hydroxycinnamyl alcohol monomers are predominantly coupled to preformed oligomers or polymers by the formation of β-*O*-4- or β-5-linkages (*36, 37*).

An analogous compound, the 8-*O*-4/8-5(noncyclic)-dehydrotriferulic acid, was isolated from corn bran (*16*). The justified assumption that 8-5(cyclic)-structural units in ferulate trimers show the same behavior under alkaline conditions as do the dimers (*7*) suggests that compound **1** is the precursor of the corn-derived 8-*O*-4/8-5(noncyclic)-dehydrotriferulic acid in the plant. The ring-opening mechanism under alkaline conditions is shown in **Figure 6B**.

The main tetramer is likely formed by 4-*O*-5-coupling of two preformed 8-5(cyclic)-diferulates. Formation of 4-*O*-5-linkages is not favored in the dimerization process of ferulates (*38*). However, in analogy to lignin biosynthesis, the formation of a 4-*O*-5-linkage between preformed dimers is logical; 5-5- and 4-*O*-5-linkages in lignin predominantly stem from oligomer−oligomer coupling (*36*). Two ferulate tetramers have been isolated from corn bran to date (*20*). Both tetramers contained a central 5-5-linkage, but both tetramers also contained a 5-5/8-*O*-4-dehydrotriferulate moiety, probably the predominant trimer in corn bran. Therefore, it was hard to deduce whether those tetramers were formed by coupling of a fourth unit to a preformed trimer or whether two preformed dimers were coupled by the formation of a 5-5-linkage. In our model system the dominant tetramer most likely stems from 4-*O*-5-coupling of two dimers, possibly giving a hint that also the corn bran tetramers were formed by 5-5-dimer−dimer coupling. Whereas a trimer analogous to compound **1** was already isolated from a natural source, the race will now be to isolate or identify a tetramer analogous to compound **2** from a natural source.

ABBREVIATIONS USED

DAD, diode array detector; SEC, size exclusion chromatography; THF, tetrahydrofuran.

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Received for review June 13, 2008. Revised manuscript received August 17, 2008. Accepted August 22, 2008.

JF801825Z