Identification of 4-O-5′-Coupled Diferulic Acid from Insoluble Cereal Fiber

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The extracts of saponified cereal fibers of whole grains of corn (*Zea mays* cv. microsperma KOERN.), wheat (*Triticum aestivum* L.), spelt (*Triticum spelta* L.), and rice (*Oryza sativa* L.) were investigated for dehydrodimers of ferulic acid using gas−liquid chromatography (GLC) with mass spectrometric detection (GLC−MS) and flame ionization detection (GLC−FID). In addition to the 8,5′-, 8,8′-, 5,5′-, and 8-O-4′-coupled diferulic acids previously identified from other plant materials the 4-O-5′-coupled diferulic acid (*E*)-3-[4-[(*E*)-2-carboxyvinyl]-2-methoxyphenoxy]-4-hydroxy-5-methoxycinnamic acid (4-O-5′-DFA) was identified in all fibers investigated. This new diferulate was authenticated by comparison of its mass spectrum and its relative GLC retention time with those of the synthesized compound. Semiquantitative determination of 4-O-5′-DFA showed that it is present at 8−30 μg/g, approximately 70−100 times lower concentrations than the sum of 8,5′-coupled diferulic acids, the major diferulic acids in the investigated fibers.

Keywords: *Ferulic acid; diferulic acid; diferulate; insoluble fiber; cereals; cell wall cross-linking; GC−MS*

INTRODUCTION

Ferulic acid, *p*-coumaric acid, and other phenolic acids have been found to be cell wall components of various monocots (Hartley and Ford, 1989) and also some dicots (Fry, 1982; Rombouts and Thibault, 1986). In grasses, ferulic acid is attached as an ester at the C-5 position of α-L-arabinose moieties of heteroxylans (Ishii, 1997).

Ferulates are thought to play an important role in modifying the mechanical properties of cell walls (Parker and Waldron, 1995) as well as in limiting polysaccharide degradation by exogenous enzymes (Grabber et al., 1998) by acting as a cross-link between polysaccharides and between polysaccharides and lignin (Ralph et al., 1994). Dimerization of ferulates is a mechanism for cross-linking cell wall polysaccharides. The formation of covalent cross-linkages between polysaccharides via ferulates was first investigated by Geissmann and Neukom (1971). Markwalder and Neukom (1976) isolated and identified 5,5′-diferulic acid from grass cell walls after saponification. Isolation and identification of 5,5′-diferuloyl oligosaccharides from the enzymatic hydrolyzate of bamboo shoot (Ishii, 1991) and from the acidic hydrolyzate of maize bran (Saulnier et al., 1999) proved that ferulate dimerization products act as cross-links.

The sole ferulic acid dehydrodimer reported from plant cell walls until recently was 5,5′-diferulic acid. The more recent determination (and authentication) of a range of diferulates from grasses (Ralph et al., 1994) stemmed from a recognition that radical coupling of ferulates, necessary to produce the 5,5′-dehydrodimer, could produce other dehydrodimers by anticipated 8,5′-, 8,8′-, 8-O-4′-, and 4-O-5′-coupling reactions, analogous to those observed for coniferyl alcohol during lignification. Unless the radical coupling was directly controlled by an enzyme or, as has been more recently revealed, a dirigent protein (Davin et al., 1997), other dimers would be expected to be more prevalent than the 5,5′ dimer, as was found in every plant material subsequently examined (see the Discussion). Prior to this study, the only dehydrodimer not found was 4-O-5′-DFA (Figure 1).

This paper reports its identification and semiquantitative determination in several insoluble cereal fibers.

MATERIALS AND METHODS

Internal Standard (IS) (*E*,*E*)-4-Hydroxy-4′,5,5′-trimethoxy-3,3′-bicinnamic Acid. Since the currently used internal standards (tetracosane, 2-hydroxycinnamic acid) have nonideal retention times or response factors that are too large, an internal standard more like the dimers being analyzed was sought. In this study, we used monomethylated 5,5′-DFA produced by methylation of diethyl 5,5′-diferulate using dimethyl sulfate, followed by column purification on silica gel and saponification. Although it worked well for this study, the standard cannot be recommended at this time for the following reasons. It was not discovered until well into this study that the standard was contaminated by its dimethylated analogue, peak IS* in Figure 2. Subsequent attempts to purify the compound failed. Since response factors were derived for IS against the authentic diferulates, the quantitative aspects of this study are sound, but in future studies it will be necessary to find or prepare a pure internal standard. Although we sought the mono-phenolic diacid as in IS to mimic the

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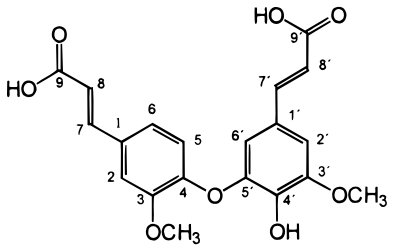

Figure 1. Structure of 4-O-5′-coupled diferulic acid.

properties of the DFA dimers most closely, the dimethylated product IS* may also be satisfactory. Certainly its relative retention time is appropriate and exhaustive methylation should produce it more cleanly.

Synthesis of 4-O-5′-Coupled Diferulic Acid. 4-O-5′-DFA acid was synthesized as described by Ralph et al. (1994).

General Methods. All samples were analyzed in triplicate. Solvents 95% ethanol and acetone were from Merck, Germany; dioxane and diethyl ether were from Aldrich, Milwaukee, WI. NaOH and HCl were from Fischer Scientific, Fair Lawn, NJ; pyridine from Mallinckrodt, Paris, KY; and BSTFA from Pierce, Rockford, IL. Heat-stable α-amylase Termamyl 120L (from *Bacillus licheniformis*, 120 KNU/g), the protease Alcalase 2.4 L (from *B. licheniformis*, 2,4 AU/g), and the amyloglucosidase AMG 300 L (from *Aspergillus niger*, 300 AGU/g) were from Novo Nordisk, Bagsvaerd, Denmark.

Plant Material. Whole grains of corn (*Zea mays* cv. microsperma KOERN.), wheat (*Triticum aestivum* L.), spelt (*Triticum spelta* L.), and rice (*Oryza sativa* L.) were obtained from a local German supplier.

Preparation of Insoluble Fiber. Samples were milled to a particle size smaller than 0.5 mm. The sample material (10 g) was suspended in phosphate buffer (0.08 M, pH 6.0, 300 mL), and 750 μL of α-amylase was added. The beakers were placed in a boiling water bath for 20 min and shaken gently every 5 min. The pH was adjusted to 7.5, and samples were incubated with 300 μL of protease at 60 °C for 30 min with continuous agitation. After the pH was adjusted to 4.5, 350 μL of amyloglucosidase was added and the mixture was incubated at 60 °C for 30 min with continuous agitation. The suspension was centrifuged, and the residue was washed two times with hot water, 95% ethanol, and acetone and finally dried at 60 °C overnight in a vacuum oven.

Saponification of Insoluble Fiber and Extraction of Ester-Linked Phenolics. Insoluble fiber (40−90 mg) was weighed into a screw-cap tube, internal standard (5−50 μg) dissolved in dioxane was added, and saponification with NaOH (2 M, 5 mL) was carried out under nitrogen and protected from light for 18 h at room temperature. Samples were acidified with 0.95 mL of concentrated HCl (resulting pH < 2) and extracted into diethyl ether (4 mL, three times). Extracts were combined and evaporated under a stream of N_2. Finally, samples were dried under vacuum.

GC−FID and GC−MS Analysis of Dehydrodiferulic Acids. Dried extracts were silylated by adding 10 μL of pyridine and 40 μL of BSTFA and heating for 30 min at 60 °C in sealed vials. Trimethylsilylated derivatives of phenolic acids were separated by GLC (Hewlett-Packard 5980, Palo Alta, CA) using a 0.2-mm × 25-m DB-1 capillary column (0.33 μm film thickness) (J&W Scientific, Folsom, CA) and identified by their electron impact mass data collected on a Hewlett-Packard 5970 mass-selective detector (Palo Alta, CA). He (0.54 mL/min) was used as carrier gas. GLC conditions were as follows: initial column temperature, 220 °C, held for 1 min, ramped at 4 °C/min to 248 °C, ramped at 30 °C/min to 300 °C, held 40 min; injector temperature 300 °C, split 1/50. Mass spectra in the electron impact mode were generated at 70 eV.

Semiquantitative determination of 4-O-5′-DFA was carried out by GLC (Hewlett-Packard 5980, Palo Alto, CA) using the same column and GLC conditions and a flame ionization detector (detector temperature 300 °C). He (0.4 mL/min) was used as carrier gas.

RESULTS AND DISCUSSION

The isolation of insoluble cereal fiber was performed similarly to the AOAC method 991.42 (*Official Methods of Analysis*, 1992) for the determination of insoluble dietary fiber in food and food products, especially vegetables, fruits, and cereal grains. Dietary fiber, which can be divided into soluble and insoluble dietary fiber, is defined as that part of foodstuff that is not digested by secretions of the human gastrointestinal tract (Eastwood, 1992). Although there are other minor sources, plant cell walls constitute the major part of dietary fiber. This isolation procedure was chosen for investigations to determine the influence of diferulates on the structure of insoluble dietary fiber which will be published elsewhere.

In all investigated insoluble cereal fibers the 8,5′- and 8,8′-coupled diferulic acids as well as the 8-O-4′- and 5,5′-coupled diferulic acids were identified after saponification by their relative retention times and their mass spectra (Figure 2). These diferulic acids have previously been identified in cocksfoot (*Dactylis glomerata* L.), switchgrass (*Panicum virgatum* L.), and suspension-cultured corn (*Zea mays* L.) (Ralph et al., 1994), sugarbeet (*Beta vulgaris* L.) (Waldron et al., 1997; Micard et al., 1997), water chestnuts (*Eleocharis dulcis* TRIN.) (Parr et al., 1996), corn bran (*Zea mays* L.) (Ng et al., 1997; Saulnier et al., 1999), and carrots (*Daucus carota* L.) (Parr et al., 1997). Interestingly, the 8-O-4′-, 8,5′-, and 8,8′-diferulic acids but not the 5,5′-diferulic acid were identified in elongating pine hypocotyls (*Pinus pinaster* AITON) (Sánchez et al., 1996). In none of these investigations could the theoretically possible 4-O-5′-DFA be identified unambiguously.

In the extracts of saponified corn insoluble fiber, 4-O-5′-coupled diferulic acid was identified by comparison of its mass spectrum and its relative GLC retention time with that of the genuine compound, which was synthesized and authenticated by NMR. Figure 3 shows the MS spectrum of silylated 4-O-5′-DFA; with originally one phenolic and two acid groups, its nominal molecular mass is 602. The relative retention time of 4-O-5′-DFA against the internal standard was 1.032. From the insoluble fibers of wheat, spelt, and rice the 4-O-5′-DFA was identified by its relative GLC retention time, and detection of the molecular peak *m*/*z* 602 in selected ion chromatograms.

Semiquantitative determination of 4-O-5′-DFA was carried out by setting the response factor as 1.0. Determination of the accurate response factor was not possible because of the tiny amounts of synthesized 4-O-5′-DFA available. The response factors of the other diferulic acids, which could be synthesized in larger amounts, against the internal standard (*E*,*E*)-4-hydroxy-4′,5,5′-trimethoxy-3,3′-bicinnamic acid were close to 1 (0.91−1.18, with the exception of the response factor for 8,5-cyclic-coupled DFA, which is 2.20). The amounts of 4-O-5′-DFA in insoluble cereal fibers were in corn 33 ± 3 μg/g, in spelt 13 ± 1 μg/g, in wheat 10 ± 1 μg/g, and in rice 8 ± 2 μg/g. Consequently, the amounts of 4-O-5′-DFA are approximately 70−100 times lower than the amounts of the sum of 8,5′-coupled diferulic acids, which were identified as the major diferulic acids in the cereal fibers investigated. These results therefore provide evidence for the full range of possible ferulate radical coupling products in cereal and presumably in other plant cell walls containing ferulates and diferulates. They also confirm the prevalence for coupling at feru-

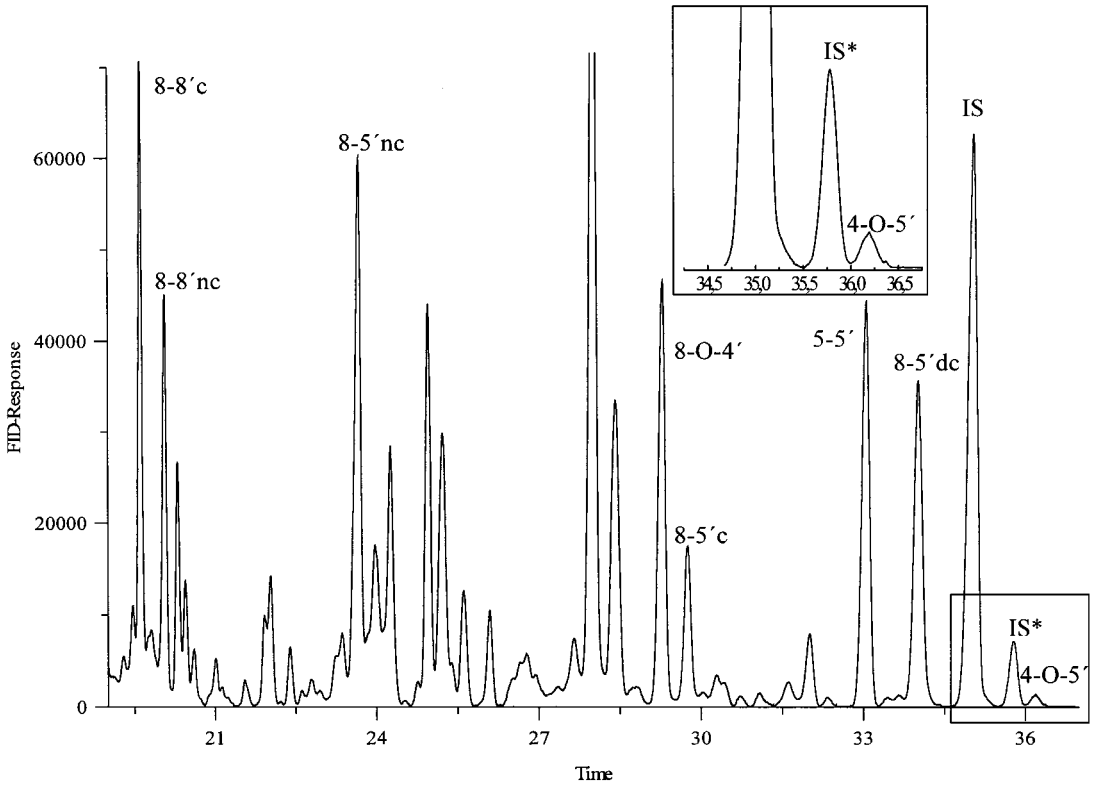

Figure 2. GC−FID chromatogram of the extract of saponified spelt insoluble fiber. 8,8′-Coupled diferulic acids: c, cyclic; nc, noncyclic. 8,5′-Coupled diferulic acids: nc, noncyclic; c, cyclic; dc, decarboxylated. Many of the unlabeled peaks have been assigned as ferulate cross-products, as will be detailed elsewhere.

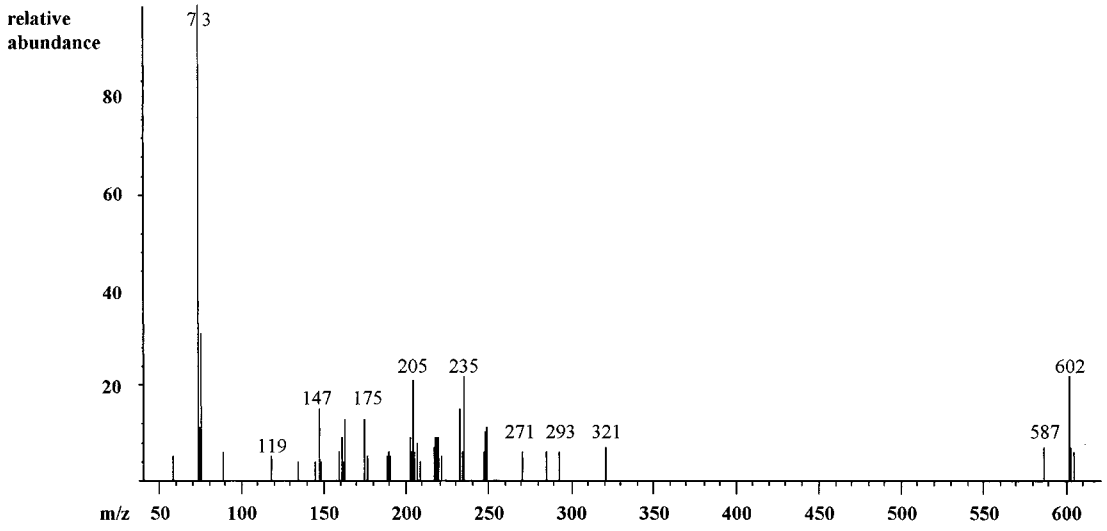

Figure 3. MS spectrum of trimethylsilylated 4-O-5′-coupled diferulic acid.

late's 8-position (to give the more predominant 8,5′-, 8,8′-, and 8-O-4-dimers), as also observed in ferulate cross-coupling into lignins (Ralph et al., 1992).

CONCLUSIONS

Alkali-releasable 4-O-5′-DFA has been detected in plant materials. The relatively high signal-to-noise chromatograms from the methods described here allow the product to be definitively identified. Its identification as an, albeit minor, diferulate supports its role in the important cell wall cross-linking reactions achieved by ferulate dehydrodimerization. The finding completes the spectrum of ferulate dehydrodimers to be found in plants and supports the concept of free-radical coupling of cell wall components independently of enzymes or proteins which might otherwise confer a strict regiochemical course, i.e., produce only a single diferulate.

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