Diferulates have varied roles as significant components of many plant fibers. The 4–O–5-coupled dimer has been found in cereal grain insoluble fiber fractions where total diferulate levels range from 2.3 to 12.6 mg/g (in maize). A new form of the 8–8-dimer has also been found. Disinapates are found in wild rice.

Introduction

Grasses have substantial amounts of hydroxycinnamic acids intimately associated with the cell wall. Ferulate, in particular, has a significant role in cross-linking as recently reviewed (Ishii 1997; Ralph et al. 1998; Hatfield et al. 1999). Polysaccharide–polysaccharide cross-linking is achieved by ferulate dimerization by either photochemical or, more importantly, radical coupling reactions of ferulate–polysaccharide esters. The whole range of ferulate dimers 1-9 are now routinely being found in a variety of samples. Radical cross-coupling of (polysaccharide-linked) ferulates with lignin monomers results in lignin-polysaccharide cross-linking. Even ferulate dehydrodimers cross-couple with lignin monomers/oligomers to incorporate into the lignin polymer resulting in extensive polysaccharide-polysaccharide-lignin cross-linking.

Methods of analysis

Although HPLC methods have been used for qualitative and quantitative analysis of diferulates (Waldron et al. 1996), HPLC really cannot afford the dispersion and resolution of GC. In addition, alkaline hydrolysates of plant materials produce a wide range of products that cannot be easily pre-fractionated. For example in grass stem samples, in addition to the diferulates from radical coupling of ferulates are the photochemical dimers and numerous ferulate-monolignol crossed dimers. There are many components still to be identified. As indicated below, two new diferulates have recently been found, along with dimers of another hydroxycinnamate, sinapate. Only cursory analyses are currently possible using HPLC; GC-FID and/or GC-MS should be used if component detail is required.

MS spectral data for the diferulates have been requested by many groups. Spectra from both a quadrupole El instrument and an Ion Trap are given in Figure 1. We have been impressed with the ion trap instruments for providing enhanced sensitivity in the high-mass region. The ion-trap spectra of all of the diferulates have a molecular ion peak that is quite abundant; quadrupole instruments tend to favor the uninformative trimethylsilyl peak at 73, and give only weak molecular ions.

A better GC standard for diferulates analyses

The standard used for the original quantification of diferulates and most subsequent studies has been o-coumaric acid (Ralph et al. 1994). Unfortunately this standard has neither ideal elution nor satisfactory response factors for the diferulates. A standard having better structural similarity was sought. Rather extensive surveys failed to unearth a satisfactory commercially available standard. We decided that the 5–5-coupled dimer which had been monomethylated might be a good candidate, and this has been used in our labs (Bunzel et al. 2000).
Figure 1. Structures and mass spectra from the nine diferulate products resulting from saponification of cereal insoluble fiber. The left column of spectra are traditional EI spectra from an HP 5970 bench-top quadrupole instrument. Note that spectra in which the TMS peak is noted as 73* have had all peaks but the 73 peak doubled in intensity for easier viewing of the important high-mass peaks. The right column of spectra are from a Thermoquest Polaris GCQ ion-trap instrument, and generally show superior molecular ion peaks. Spectrum labels include, in addition to the compound number and name, the nominal mass and the retention time relative to IS, the monomethylated 5-5-coupled ferulate dimer.
However, it was discovered somewhat late in the analysis process that the "standard" IS was contaminated with the di-methylated compound IS* (see the chromatograms in Figure 2), from which separation was extremely difficult. Also, it is possible that the compound does not fully survive base treatments (although perhaps as well as some of the true ferulate dimers do!). We therefore now favor the fully methylated dimer and rigorous trials are underway to test this standard. If it is found suitable, our lab will provide the reference compound to other labs interested in using it.

**Discovery of the 4-O-5-coupled ferulate dimer 9**

The sole ferulic acid dehydrodimer reported from plant cell walls before 1994 was 5-5-diferulic acid 7. 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-coupled diferulic acid 7, could produce other diferulic dimers 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 been more recently revealed in lignan biosynthesis, a dirigent protein (Davin et al. 1997), other dimers would be expected to be more prevalent than the 5-5-dimer. This has been found to be the case in every plant material subsequently examined. The only dehydrodimer not found until recently was 4-O-5-DFA 9 (Figure 1). This diferulic acid has also now been found (in rather small amounts) in several insoluble cereal fibers (Bunzel et al. 2000).

In extracts of saponified grain fiber 4-O-5-coupled diferulic acid 9 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. The mass spectrum of silylated 4-O-5-DFA 9 is in Figure 1; with originally one phenolic and two acid groups, its nominal molecular mass is 602. The relative retention time of 4-O-5-DFA against a new monomethylated 5-5-diferulate internal standard was 1.032. The amounts of 4-O-5-DFA9 were 33, 13 10 and 8 µg/g in maize (top chromatogram, Figure 2), spelt, wheat, and rice insoluble cereal fiber respectively – approximately 70–100 times lower than the amounts of the sum of 8-5-coupled diferulic acids 3 and 6.

These results therefore provide evidence for the full range of possible ferulate radical coupling products in cereal grains, and presumably in other plant cell walls containing ferulates and diferulates. They also confirm the prevalence for coupling at ferulate'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. 1994). 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.

Another 8-8-coupled diferulate?

Most alkaline hydrolysates of grass cell walls also contain another previously unidentified peak. MS analysis suggests that it is the tetrahydrofuran dimer 4, Figure 1 (Grabber et al., 2000). As can be seen in the chromatogram in Figure 2, it is a substantial component that should also be quantified as resulting from 8-8-dimerization. Work is currently underway to isolate sufficient amounts of the compound for structural elucidation by NMR and to synthesize it.

**Levels of diferulates in cereal grain insoluble fiber**

Grain fiber is known to be beneficial for human nutrition. The levels of diferulates in a range of cereal grain fibers was recently surveyed (Bunzel et al. 2000) where the following were found: maize 12.6, wheat 2.4, spelt 2.6, rice 4.0, wild rice 2.8, barley 3.7, rye 4.0, oat 3.6 and millet 5.7 mg/g of insoluble fiber. Very low levels were found in the soluble fiber fraction as might be anticipated. The high levels in maize make this an ideal secondary standard to check column performance and variations in the analyses over longer periods of time.

**Disinapates cross-linking cell wall polysaccharides?**

Although sinapic acid has been identified in plant extracts and can be released in small quantities from grass cell walls by low-temperature base, it has not been determined if it acylates polysaccharides or other components. Nor has sinapate been shown to be involved in radical coupling reactions to produce dehydrodimers. Preliminary identification of such radical dimerization products in the insoluble fiber fraction from wild rice samples is presented here. GC-MS total ion chromatograms of saponified extracts from wild rice (Zizania palustris L.) insoluble fiber showed additional peaks in the dimer region; two peaks were, especially predominant. The mass spectra were analogous to those of ferulate dimers (with masses of various peaks offset by 30 or 60 mass units (one or two additional methoxyls). The products could also be obtained by saponification of dimers prepared by oxidative coupling of ethyl sinapate. However, only two of the dimers are striking in the wild rice samples, whereas a range of dimers seems to be produced in significant amounts in vitro. We first assumed that they were the two 8-8-
Figure 2. Top. GC-MS total ion chromatogram of the dimers region from saponification of maize grain insoluble fiber showing diferulate products 1-9. The crossed out peaks are apparently artifacts that are not present in more recent chromatograms from such samples. IS is the mono-methylated derivative of the 5–5-dimer 7; the di-methylated derivative is also present, IS*. Middle. Similar data (different machine and conditions!) for wild rice insoluble fiber hydrolysate showing diferulates and two new disinapates. Bottom. Ion-trap mass spectra of disinapates. Only the first peak has been authenticated as 1*; the second looks like 2* (the sinapate analog of 2), but a synthetic sample of apparent 2* does not have the same retention time or spectrum - it is therefore currently unknown.
coupled disinapate analogs of the 8–8-diferulates 1 and 2, one ring form and one open-chain. Currently, one of the disinapates has been firmly identified as the cyclic 8–8-product 1* (the sinapate analog of 1), but the other remains unidentified at this moment. It has been found in radical coupling products using Mn(OAc)₃, which unfortunately produces a plethora of products. We are currently isolating the component to identify it conclusively by NMR and MS. Although it is logical that sinapoylated polysaccharides might be cross-linked by radical dimerization of the sinapates in an analogous way to the ferulates, we have not demonstrated that they are in fact esterified to polysaccharides. It is also curious that disinapates would form with little evidence (from MS data so far) of any cross-coupling products between ferulate and sinapate; however, we have not examined the propensity for such cross-coupling. If disinapates are found in cell wall fractions from stems or grains of plants other than wild rice, such issues should be clarified by further research.

Conclusions

Diferulates continue to emerge in varied roles as significant components of many plant fibers and have interesting implications for human and animal health as well as for their properties in limiting cell wall digestibility by ruminants. The 4–O–5-coupled dimer has now been found in small quantities in many cereal grains in which the insoluble fiber fraction contains high levels of total diferulates, up to 12.6 mg/g in maize. A new form of the 8–8-dimer (a tetrahydrofuran) has also been found. Many dimers of ferulate cross-coupled with monolignols (not detailed here) can also be found in grain and stem fibers. Sinapates in wild rice, like ferulates in all masses, appear to dimerize via radical coupling reactions to produce at least two sinapate dehydrodimers, which can be detected by GC(-MS). We assume new roles for such hydroxycinnamate dehydrodimers will continue to be discovered and researchers will learn more about their impact on other physiological processes.

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Literature


