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Ferulic acid dehydrodimers from wheat bran: isolation, purification and antioxidant properties of 8–O–4-diferulic acid

M. T. Garcia-Conesa, G. W. Plumb, K. W. Waldron, J. Ralph*, G. Williamson

Biochemistry Department, Institute of Food Research, Norwich, UK;
*US Dairy Forage Research Centre, Agricultural Research Service, US Department of Agriculture, Madison, WI., USA

Summary Wheat bran contains several ester-linked dehydrodimers of ferulic acid, which were detected and quantified after sequential alkaline hydrolysis. The major dimers released were: *trans*-5-[(*E*)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid (5–8-BendiFA), (*Z*)-β–{4-[(*E*)-2-carboxyvinyl]-2-methoxy-phenoxy}-4-hydroxy-3-methoxycinnamic acid (8–O–4-diFA) and (*E,E*)-4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid (5–5-diFA). *trans*-7-hydroxy-1-(4-hydroxy-3methoxyphenyl)-6-methoxy-1,2-dihydro-naphthalene-2,3-dicarboxylic acid (8–8-diFA cyclic form) and 4,4′-dihydroxy-3,3′-dimethoxy-β,β′-bicinnamic acid (8–8-diFA non cyclic form) were not detected. One of the most abundant dimers, 8–O–4-diFA, was purified from de-starched wheat bran after alkaline hydrolysis and preparative HPLC. The resultant product was identical to the chemically synthesised 8–O–4-dimer by TLC and HPLC as confirmed by ^{1}H-NMR and mass spectrometry. The absorption maxima and absorption coefficients for the synthetic compound in ethanol were: λ_{max}: 323 nm, λ_{min}: 258 nm, $\varepsilon_{\lambda max}$ ($M^{-1}cm^{-1}$): 24800 ± 2100 and ε_{280} ($M^{-1}cm^{-1}$): 19700 ± 1100. The antioxidant properties of 8–O–4-diFA were assessed using: (a) inhibition of ascorbate/iron-induced peroxidation of phosphatidylcholine liposomes and; (b) scavenging of the radical cation of 2,2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate) (ABTS) relative to the water-soluble vitamin E analogue, Trolox C. The 8–O–4-diFA was a better antioxidant than ferulic acid in both lipid and aqueous phases. This is the first report of the antioxidant activity of a natural diferulate obtained from a plant.

Abbreviations

5–8-BendiFA: (*trans*-5-[(*E*)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid); 5–8-diFA open form: ((*E,E*)-4,4′-dihydroxy-3,5′-dimethoxy-β,3′-bicinnamic acid; 5–5-diFA: (*E,E*)-4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid); 8–O–4-diFA: ((*Z*)-β-{4-[(*E*)-2-carboxyvinyl]-2-methoxy-phenoxy}-4-hydroxy-3-methoxycinnamic acid); 8–8-diFA: (*trans*-7-hydroxy-1-(4-hydroxy-3methoxyphenyl)-6-methoxy-1,2dihydro-naphthalene-2,3-dicarboxylic acid; 8–8-diFA open form: 4,4′-dihydroxy-3,3′-dimethoxy-β,β′-bicinnamic acid; ABTS: 2,2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate); TEAC: Trolox C equivalent antioxidant capacity; IC_{50}: μM concentration at which 50% of lipid peroxidation inhibition is reached.

Correspondence to: G. Williamson, Biochemistry Department, Institute of Food Research, Norwich Research Park, Colney, Norwich. NR4 7UA, UK
Tel: +44 1603 255259 Fax: +44 1603 507723
email: gary.williamson(a)bbsrc.ac.uk

INTRODUCTION

Among the phenolic compounds present in plant cell walls, *trans*-ferulic acid is found in significant quantities and mainly ester linked to arabinoxylans (Graminaeae) and to pectins (Chenopodiaceae).[1] In the growing primary cell wall, such ferulate esters can undergo dimerization providing cross-linking of the cell wall polymers. This has a marked influence on various properties of the cell wall such as digestibility, extensibility and wall adherence.[2] 5–5-diFA, commonly referred to as 'diferulic acid', was the only diferulate identified for several years.[3,4]

Cross-linking of arabinoxylans by 5–5-diFA was first demonstrated after enzymic treatment of bamboo shoots.[5] Oxidative coupling of ferulate esters catalysed by cell wall bound peroxidases leads to the formation of several isomeric dehydrodimers (5–8, 8–O–4, 5–5 and 8–8 diFAs) that have now been chemically synthesized and identified by GC-MS in extracts of saponified grasses including cocksfoot, switchgrass and cultured corn walls.[6] The total amount of these more recently discovered dimers was higher than that of 5–5-diFA. A range of diferulates are found in a variety of materials: sugar beet pulp,[7] sugar beet and beetroot,[8] and Chinese water chestnut.[9] More recently, it has been shown that ferulate dehydrodimer cross-links are formed during peroxidase/H_2O_2 catalysed gelation of arabinoxylans[10] and sugar beet pectin.[11]

Synthetic antioxidants are used in the food industry as preservatives. In particular, the addition of phenolic compounds minimizes the oxidation of lipids in food materials and their subsequent spoilage.[12] Phenolic compounds may also provide some health benefits and are used in several applications in the pharmaceutical industry. Many of these compounds exhibit antioxidant properties and are part of the diet. One of the most abundant plant phenolics, ferulic acid, has a high antioxidant potential owing to its resonance stabilized phenoxy radical structure. It is an effective scavenger of free radicals and it has been approved in certain countries as a food additive to prevent lipid peroxidation.[13] It also protects low density lipoprotein against metmyoglobin-induced oxidative damage.[14] A significant proportion of ferulate dimers are present in a range of plant materials. The antioxidant properties of two chemically synthesized ferulate dimers, 5–5-diFA and 5–8-BendiFA, have been reported.[15] In the aqueous phase, both dimers were less effective antioxidants than ferulic acid, although they were more efficient at inhibiting lipid peroxidation.

In this study, we determined the diferulic acid composition of wheat bran. One of the major dimers detected, 8–O–4-diFA, was purified and characterized in comparison to the chemically synthesized dimer. The antioxidant properties of the dimer in aqueous and lipid phase assays were determined.

MATERIALS AND METHODS

Materials

De-starched wheat bran was obtained from Agro-industrie Researches et Dévelopement (ARD), Pomacle, France. Trolox C and ABTS were supplied by Aldrich Chemical Company (Dorset, UK). Unless otherwise stated, all chemicals were of AnalaR or HPLC-grade purity.

Sequential alkaline hydrolysis

Wall bound phenolics were released by sequential alkaline hydrolysis of de-starched wheat bran under progressively more rigorous conditions as described by Parr et al.[9] Wheat bran (100 mg) was extracted with 0.1 M NaOH (6 ml) for 1 h at 20°C under N_2. The suspension was filtered through glass fibre paper (GF/C), and the residue retained for further extraction with, sequentially, 0.1 M NaOH (6 ml) for 24 h, 0.5 M NaOH (6 ml) for 24 h, 1.0 M NaOH (6 ml) for 24 h and 2.0 M NaOH (6 ml) for 24 h. *trans*-Cinnamic acid was added to all the filtrates as an internal standard, and the solutions were then acidified with HCl and extracted with ethyl acetate (× 3 volume). The phenolic extracts were evaporated to dryness and redissolved in 50% (v/v) aqueous MeOH prior to filtration (0.2 μm) and analysis by HPLC.[16] The alkali extractions were performed under N_2 and in darkness.

Purification and characterization of 8–O–4-diFA from wheat bran

Wheat bran (ca. 40 g) was hydrolysed with NaOH 0.1 M for 24 h at 20°C under N_2 in the dark. The suspension was filtered and acidified before precipitation of polymers with methanol (4°C, 16 h). The clear supernatant was concentrated and extracted with ethyl acetate (× 3 volume). The extract was evaporated to dryness, redissolved in 50% (v/v) aqueous methanol and filtered (0.2 μm) prior to purification by HPLC. Preparative HPLC was performed by adapting the analytical method described by Waldron et al[16] to an Inertpak ODS2 reverse phase column, 25 cm × 10 mm i.d., 5 μm, at a flow rate of 2.5 ml/min. Fractions containing the 8–O–4-diFA were collected and pooled together. The combined fractions were evaporated to dryness and redissolved in methanol.

8–O–4-diFA was also chemically synthesized following the method of Ralph et al.[6] The purity of synthetic and wheat bran purified 8–O–4-diFA was checked by analytical HPLC and TLC (silica gel 60 F_{254} pre-coated sheets, 0.2 mm, Merck, visualization by UV-light, ethyl acetate:methanol, 3:2, 100 μl of trifluoroacetic acid). ^{1}H-NMR of both dimers in methyl alcohol D_4 were run at 300 K on a Jeol GX-400 (^{1}H:400 Mhz). Both compounds were also analysed by mass spectrometry (EIMS probe, 70 eV).

Determination of absorption coefficients

The absorption coefficient for the 8–O–4-diFA was obtained from a solution of the synthetic compound in ethanol. An aliquot of the solution was added to a small aluminum vessel, dried (60°C) and weighed. In parallel,

the absorbance of an appropriate dilution was measured spectrophotometrically (DU-70, Beckman). Values are the mean and standard deviation of three determinations.

Scavenging of ABTS radicals

The TEAC was measured by the method of Salah et al.[17] Values are expressed relative to a standard of Trolox C, the water soluble analogue of vitamin E. The extent of quenching of the ABTS radical was measured spectrophotometrically at 734 nm and compared to standard amounts of Trolox C. Positive controls were performed as described previously.[18,19]

Inhibition of lipid peroxidation

Peroxidation of phospholipid liposomes was performed as described previously with a range of 8–O–4-diFA concentrations.[20] Results are expressed as percentage inhibition of peroxidation, where 100% inhibition is baseline peroxidation of liposomes without added iron/ascorbate, and 0% inhibition is peroxidation of liposomes with added iron/ascorbate. Calculation of IC_{50} value was performed by fitting a third order polynomial curve to the data.

RESULTS AND DISCUSSION

Dimers of ferulic acid in wheat bran

The results of sequential alkaline release of phenolic compounds from wheat bran are shown in Table 1. The main monomeric phenolic acid released was *trans*-ferulic acid (total level: 4.5 mg g^{-1}). *cis*-Ferulic acid, *trans-p*-coumaric acid and vanillin were present only in low amounts. The main three dimers detected were identified as 5–8-BendiFA, 8–O–4-diFA and 5–5-diFA. 5–8-BendiFA was the most abundant diferulate (57%) whereas the 5–5-dimer accounted for a much smaller fraction (15%). In the conditions of our analysis, we were not able to detect 8–8-coupled diferulates. Over 20% of the quantified esterified phenolics comprise ferulic acid dehydrodimers, indicating a high degree of polymer cross-linking. 5–5-diFA constitutes only a small fraction of the total dimers present in wheat bran, in agreement with the results obtained by other authors in other plant cell walls.[6,8,9]

An unidentified compound ('unknown', Fig. 1(a)), with spectral properties similar to those of the other diferulate dimers, was also detected. A similar component has been detected in Chinese water chestnut and suggested to be an oxidized product.[9] In the conditions of our analysis care was taken to avoid oxidation. More recently an unidentified compound has also been detected in arabinoxylans gels formed by peroxidase.[10]

After extraction with NaOH (0.1 M for 24 h), there was no further significant release of monomers or dimers of phenolic compounds at higher concentrations of NaOH. The differences observed in the release of the phenolic acids with increasing alkali concentration suggests differences in the accessibility of the alkali to the ester link, possibly caused by the presence of other cell wall components. This heterogeneity in the alkali-lability of phenolic esters has been reported in other plant materials.[8,9]

Purification and characterization of 8–O–4-diFA from wheat bran

In order to prevent solubilization of unwanted components with high alkali concentrations, NaOH 0.1 M (24 h) was used for the large scale extraction of 8–O–4-diFA from wheat bran. Figure 1(a) shows the phenolic profile of the concentrated extract from wheat bran. The fractions corresponding to 8–O–4-diFA were collected and pooled. The purified and concentrated fraction was reanalysed by HPLC and showed a single peak (Fig. 1(b)) with the same retention time as the synthetised compound. HPLC detection at 280 nm of the synthetic 8–O–4 diferulate indicated that the compound was 75–85% pure. There were minor impurities, also detected by NMR, none of which accounted for more than 2%. These impurities probably arise from side reactions during the synthesis. They were not observed in the sample

Table 1 Phenolic compounds from wheat bran (μg.g^{-1}) after sequential extraction with cold NaOH

	0.1 M 1 h	0.1 M 24 h	0.5 M 24 h	1.0 M 24 h	2.0 M 24 h	Total
Monomeric components						
Vanillin	28	21	9	3	6	67
t-p-Coumaric acid	55	50	13	2	–	120
t-Ferulic acid	1303	2872	266	37	16	4494
c-Ferulic acid	88	144	12	2	–	246
Dimeric components						
5–5-diFA	41	152	8	–	–	201
8–O–4-diFA	157	182	17	–	–	356
5–8-BendiFA	424	267	55	–	–	746

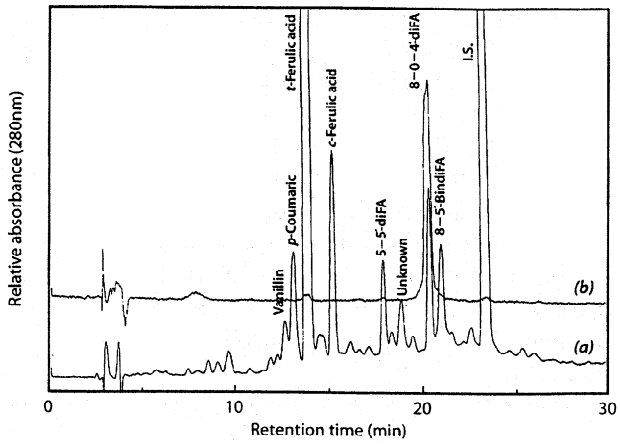

Fig. 1 (a) HPLC elution profile of phenolic monomers and dimers present in saponified extracts from wheat bran; (b) HPLC trace of the purified and concentrated fraction containing 8–O–4-diFA. Detection at 280 nm.

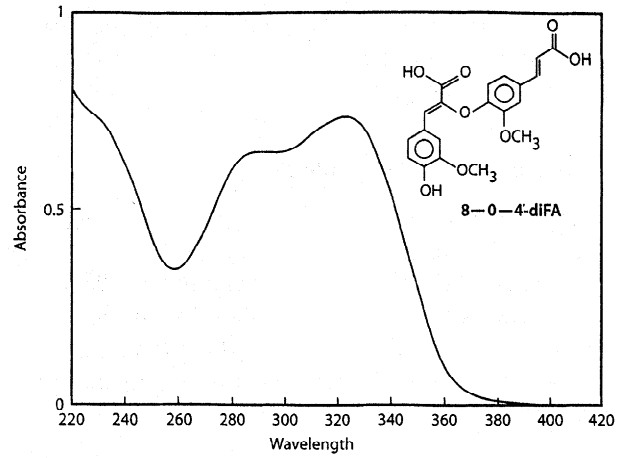

Fig. 2 Absorption spectrum in ethanol and structure of 8–O–4-diFA.

purified from wheat bran. Both dimeric products showed a single UV-absorbing spot on TLC ($R_f = 0.28$). The identity of the purified 8–O–4-diFA from wheat bran was confirmed by ^{1}H-NMR and by mass spectrometry, m/z (rel. int.): 386 (14), 368 (4), 342 (100), 298 (11), 281 (44), 263 (19), 194 (51), 177 (20), 150 (56).

Absorption coefficients

The absorption coefficients calculated for 8–O–4-diFA in ethanol were: $\lambda_{max} = 323$ nm, $\lambda_{min} = 258$ nm, $\varepsilon_{\lambda max} = 24800 \pm 2100$ and $\varepsilon_{280} = 19700 \pm 1100$ (Fig. 2).

Aqueous and lipid phase antioxidant capacity of 8–O–4-diFA

The results of the antioxidant capacity of the 8–O–4-dimers, both synthetic and purified from wheat bran, are presented in Table 2. In the aqueous phase, the 8–O–4-diFA was a better antioxidant than ferulic acid (TEAC: 1.96) although the calculated TEAC was still lower than the predicted value from the addition of two molecules of ferulic acid. The TEAC value for 8–O–4-diFA was higher than for 5–8-BendiFA (1.49 ± 0.02) and similar to that calculated for 5–5-diFA (2.19 ± 0.01).[15] The antioxidant activity in this assay is partially dependent on the number of free phenolic hydroxyls. 8–O–4-DiFA and 5–8-BendiFA each have a single phenolic hydroxyl. These results support the expectation that the antioxidant properties of the diferulates are also affected by the type of linkage between the two ferulic monomers. 8–O–4-DiFA, like ferulic acid, has a fully conjugated system; the 5–8-BendiFA does not.

Table 2 Aqueous and lipid phase antioxidant capacity of 8–O–4-diFA

	TEAC	IC_{50} (μM)
8–O–4-diFA (synthetic)	2.6 ± 0.1	14.0 ± 0.6
8–O–4-diFA (wheat bran)	2.9 ± 0.2	15.0 ± 0.7

In the lipid phase, 8–O–4-diFA was less efficient than 5–5-diFA (6.87 ± 0.68 μM) and 5–8-BendiFA (9.11 ± 0.45 μM). These results show that dimers of ferulic acid are more effective inhibitors of lipid peroxidation than ferulic acid itself (IC_{50} = 26.6 ± 0.5 μM). This is partly owing to the more hydrophobic nature of the dimers (as shown by their retention time on HPLC) which leads to a higher concentration in the lipid phase.

Wheat bran is a good source of esterified phenolics. It contains a large proportion of ferulate and feruloylated dimers. Ferulic acid has very good antioxidant properties.[13] Some dimers of ferulic acid have been reported to have good antioxidant capacity.[15] The 8–O–4-diFA, which is predominant in wheat bran, is a good antioxidant in both lipid and aqueous phase and may be an effective 'natural' antioxidant.

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