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Association of non-starch polysaccharides and ferulic acid in grain amaranth (*Amaranthus caudatus* L.) dietary fiber

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The association of ferulic acid, an alkali-extractable phenolic acid in amaranth (*Amaranthus caudatus* L., Amaranthaceae) insoluble fiber (*trans*-ferulic acid: 620 µg · g^{-1}, *cis*-ferulic acid: 203 µg · g^{-1}), and non-starch polysaccharides was investigated. Enzymatic hydrolysis of insoluble amaranth fiber released several feruloylated oligosaccharides that were separated using Sephadex LH-20-chromatography and reversed phase-high performance liquid chromatography (RP-HPLC). Three compounds were unambiguously identified: *O*-(6-*O*-*trans*-feruloyl-β-D-galactopyranosyl)-(1→4)-D-galactopyranose, *O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1→5)-L-arabinofuranose, and *O*-α-L-arabinofuranosyl-(1→3)-*O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1→5)-L-arabinofuranose. These feruloylated oligosaccharides show that ferulic acid is predominantly bound to pectic arabinans and galactans in amaranth insoluble fiber. 5-*O*-*trans*-Feruloyl-L-arabinofuranose was the only compound isolated in pure form from an acid hydrolyzate. This compound may have its origin from pectic arabinans but also from arabinoxylans.

Keywords: Amaranth / Ferulic acid / Feruloylated oligosaccharides / Nuclear magnetic resonance / Pectins

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1 Introduction

The dicotyledonous plant amaranth was originally cultivated by pre-Columbians. Amaranth was an important part of the diets used as grain (seed) amaranth but also as vegetable (leaves) amaranth. Grain amaranth has now been rediscovered as a pseudocereal by the food industry. In Germany, grain amaranth products are sold primarily in health food shops but the food industry is increasingly becoming aware of the many uses of amaranth in various food preparations. For example, grain amaranth can be used as popped amaranth in breakfast cereals and snacks [1]. Up to 15% of wheat flour may be replaced by amaranth flour with little effect on the bread quality but a desirable change to a "nutty" flavor of these breads [1]. Furthermore, the high protein content and the balanced amino acid profile of amaranth with higher lysine contents than cereals renders an advantageous combination of cereals and amaranth [2], providing a better nutritional balance. More interesting is that the high protein content but also the low demands of the plant for climate and soil also render amaranth interesting as a major food source to fight malnutrition and underfeeding in developing areas. *Amaranthus caudatus* L. and *A. hybridus* L. are species used predominantly as grain amaranth, *A. cruentus* L. is used as grain and vegetable amaranth. *Amaranthus caudatus* L. species contain around 8% dietary fiber although there are also species containing twice as much [3]. According to its definition, dietary fiber contains indigestible polysaccharides, oligosaccharides, lignin, and associated plant substances [4]. Therefore, dietary fiber is comprised mainly of plant cell walls, especially plant cell wall polysaccharides. The polysaccharide composition of graminaceous plant cell walls usually differs considerably from that of dicotyledonous plants.

The polysaccharide fraction of cereal primary cell walls is usually dominated by arabinoxylans, cellulose and, in oats and barley, mixed-linked β-glucans whereas the dicotyledonous primary cell walls are rich in pectins, xyloglucans, and cellulose. Ferulate is a minor component of the cell wall that is bound to plant cell wall polymers, *e.g.*, polysaccharides and lignin. Dimerization of polysaccharide-bound fer-

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Abbreviations: GPC, gel permeation chromatography; **HMBC**, heteronuclear multiple bond correlation; **HSQC**, heteronuclear single quantum coherence; **TOCSY**, total correlation spectrocopy.

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ulates is a convenient mechanism to cross-link polysaccharides [5–9], thus influencing the physicochemical properties of the plant cell wall and as a consequence the physiological effects of the dietary fiber. In cell walls of monocotyledonous plants belonging to the families Poaceae and Bromeliaceae ferulic acid was found to be bound *via* an ester-linkage to arabinoxylans [10–15], whereas in dicotyledonous plants belonging to the family Chenopodiaceae ferulic acid was found to be associated with pectins also *via* an ester-linkage [13, 16, 17].

The aim of this study was to investigate the association of ferulic acid with polysaccharides in insoluble amaranth dietary fiber. Amaranth does not belong to the family of Chenopodiaceae but to the family of Amaranthaceae. Both families belong to the order of Caryophyllales and have been treated for a long time as separate entities, although there have been already suggestions to treat Chenopodiaceae and Amaranthaceae as one family in the past [18]. This suggestion was seized recently. In an "update of the Angiosperm Phylogeny group classification for the orders and families of flowering plants" the families Chenopodiaceae and Amaranthaceae are placed in the same broadly circumscribed family referred to as Amaranthaceae [19], although there are also arguments that do not favor such a merging [20].

2 Materials and methods

2.1 General

Heat-stable α-amylase Termamyl 120 L (EC 3.2.1.1, from *Bacillus licheniformis*, 120 KNU/g), the protease Alcalase 2.4 L (EC 3.4.21.62, from *Bacillus licheniformis*, 2.4 AU/g), and the amyloglucosidase AMG 300 L (EC 3.2.1.3, from *Aspergillus niger*, 300 AGU/g) were from Novo Nordisk (Bagsvaerd, Denmark). The carbohydrase mixture "Driselase" (from Basidiomycetes) was from Sigma (St. Louis, MO, USA). Amberlite XAD-2 was obtained from Serva (Heidelberg, Germany) and Sephadex LH-20 was from Pharmacia Biotech (Freiburg, Germany). The analytical Luna Phenyl-Hexyl HPLC column (250 × 4.6 mm, 5 µm) was from Phenomenex (Aschaffenburg, Germany), the analytical Nucleosil 100-5 C18 HD HPLC column (250 × 4 mm, 5 µm) and the semipreparative Nucleosil 100-5 C18 HD HPLC column (250 × 10 mm, 5 µm) from Macherey-Nagel (Düren, Germany). Analytical and semipreparative HPLC as well as gel permeation chromatography (GPC) equipments were from Merck/Hitachi (Darmstadt, Germany), HPLC-MS equipment from Hewlett Packard (Waldbronn, Germany) using a single-quadrupole MS (G1946A, ion-source: atmospheric pressure electrospray ionization, positive mode). GC was carried out on a Thermo Electron Focus GC-FID (Dreieich, Germany) using a 0.25 mm × 30 m HP-5MS capillary column (0.25 µm film thickness) (Hewlett Packard). 1-D and 2-D NMR experiments were performed on Bruker instruments (Rheinstetten, Germany): DMX-750, DRX-500 (with and without cryogenic probe). All extracts and eluates were concentrated under reduced pressure using a rotary evaporator at < 40°C.

2.2 Plant material

Seeds of amaranth (*Amaranthus caudatus* L., variety Oscar Blanco) were obtained from a health food shop in Hamburg, Germany. The botanical identity and origin (Peru, Cusco region) were confirmed by the supplier.

2.3 Preparation of insoluble amaranth dietary fiber

Preparation of insoluble amaranth dietary fiber was performed according to a preparative enzymatic isolation procedure described previously [7]. This procedure uses sequential application of the heat-stable α-amylase Termamyl 120 L, the protease Alcalase 2.4 L, and the amyloglucosidase AMG 300 L and washing steps with water, ethanol, and acetone. The fiber preparation process was repeated several times to get sufficient insoluble fiber to isolate feruloylated oligosaccharides as described below. As usual, these fiber preparations still contain proteins (~24%) and inorganic compounds (~4%).

2.4 Determination of *trans/cis*-ferulic acid contents and neutral carbohydrate composition of insoluble amaranth dietary fiber

Phenolic compounds were determined after alkaline hydrolysis by RP-(phenyl-hexyl)-HPLC with photodiode array detection. Amaranth insoluble fiber (100 mg) was hydrolyzed with degassed 2 M NaOH for 18 h under nitrogen and protected from light. *o*-Coumaric acid was used as the internal standard. Samples were acidified (pH < 2) and extracted into diethyl ether (three times). Extracts were combined and evaporated under a stream of nitrogen. Samples were redissolved in 0.5 mL MeOH/H_2O 50/50 v/v, sonicated, and membrane-filtered prior to HPLC analysis. Elution was carried out using a gradient of aqueous 1 mM trifluoroacetic acid (TFA) and MeOH: initially MeOH/1 mM TFA 28/72, held for 6 min, linear over 9 min to MeOH/1 mM TFA 55/45, linear over 3 min to MeOH/1 mM TFA 65/35, held isocratically for 12 min, followed by a rinsing and equilibration step (column temperature 45°C, flow rate 1.0 mL · min^{-1}). *trans/cis*-Ferulic acids were identified by comparison of their retention times and UV-spectra with those of authentic standard compounds. The carbohydrate composition of amaranth insoluble fiber was determined as detailed in [21] with some minor modifications. In brief,

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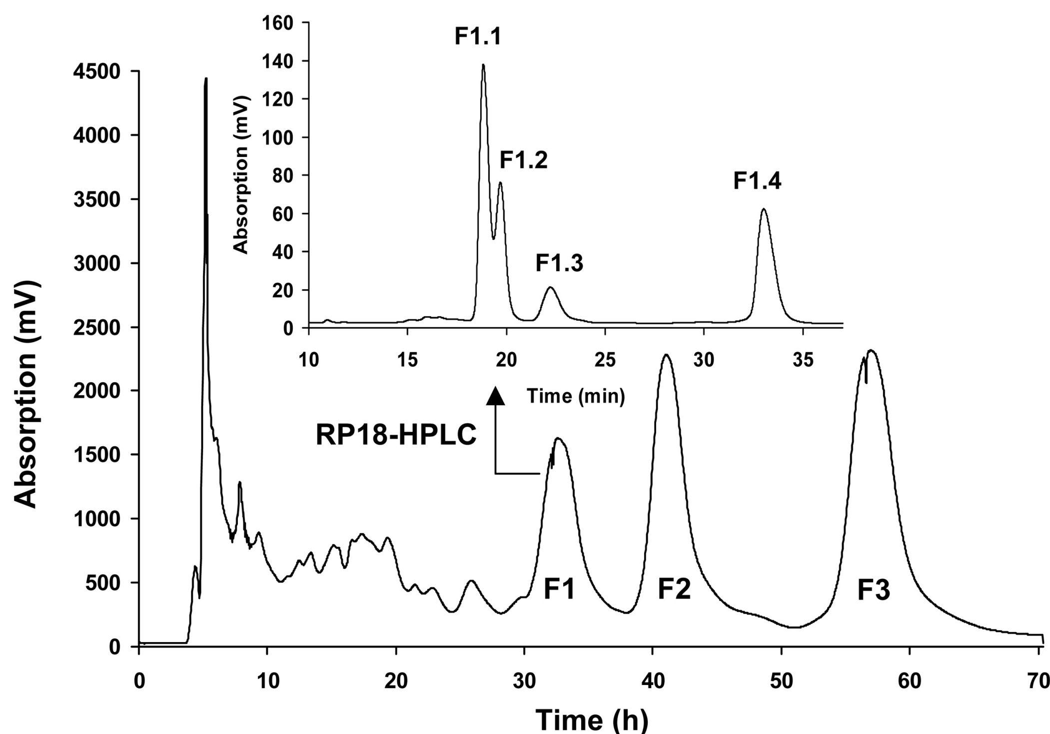

Figure 1. Isolation of feruloylated oligosaccharides from enzymatically hydrolyzed amaranth insoluble fiber using Sephadex LH-20- (bottom) and RP-chromatography (top).

amaranth insoluble fiber was pretreated with 12 M H_2SO_4 at 35°C for 30 min. Hydrolysis was performed with 2 M H_2SO_4 for 1 h at 100°C. Neutral monosaccharides were reduced with $NaBH_4$. Resulting alditols were acetylated with acetic anhydride using 1-methylimidazole as a catalyst. Myo-inositol was used as the internal standard. Alditol acetates were separated by gas liquid chromatography (GLC) under the following conditions: initial column temperature, 150°C, held for 3 min, ramped at 5°C min^{-1} to 220°C, held for 10 min, ramped at 20°C min^{-1} to 300°C, held for 5 min; split injection (1:10); detector temperature 250°C. He (1.6 mL · min^{-1}) was used as carrier gas.

2.5 Partial acid hydrolysis and enzymatic degradation of insoluble amaranth dietary fiber

Partial acid hydrolysis of the insoluble fiber was carried out as described previously [10, 14], refluxing 9.3 g fiber with 1 L 50 mM aq. TFA at 100°C for 3 h. Subsequent steps are detailed in [10]. Enzymatic degradation of amaranth insoluble fiber was carried out twice with 10 g fiber and Driselase in water at 37°C for 48 h as detailed in [10].

2.6 Fractionation of the hydrolyzates

The water-soluble hydrolyzates from the chemical$^{(c)}$/enzymatic$^{(e)}$ degradation were applied to a column (38 × 2 cm$^{(c)}$/36 × 5 cm$^{(e)}$) of Amberlite XAD-2. Elution was carried out with 350 mL$^{(c)}$/1.5 L$^{(e)}$ H_2O, 500 mL$^{(c)}$/2 L$^{(e)}$ MeOH/H_2O 50/50 v/v and 350 mL$^{(c)}$/1.5 L$^{(e)}$ MeOH. The MeOH/H_2O fraction that contains feruloylated oligosaccharides was concentrated to 9 mL and applied to a column (85 × 2.5 cm) of Sephadex LH-20. Elution was performed with water (flow rate: 0.7 mL · min^{-1} $^{(c)}$/0.5 mL · min^{-1} $^{(e)}$), a UV-detector (325 nm) was used for the detection. Fractions were collected each 12 min and combined according to the chromatogram. Sephadex LH-20 fraction 1 from the enzymatic hydrolysis (Fig. 1) was further fractionated using semipreparative RP18-HPLC. Elution was performed isocratically with acetonitrile (ACN) and H_2O (15/85) at room temperature and a flow rate of 3 mL · min^{-1}. UV-detection was at 325 nm.

2.7 Identification of oligosaccharide hydroxycinnamates

Molecular weight was determined using RP18-HPLC-MS. Fast elution was carried out using a gradient of 1 mM ammonium formate buffer (pH 3) and ACN: initially ACN/ammonium formate 15/85 held for 2 min, linear over 8 min to ACN/ammonium formate 50/50, held isocratically for 2 min (column temperature 45°C, flow rate 1 mL · min^{-1}). Phenolic compounds were determined by RP-(phenylhexyl)-HPLC with photodiode array detection after alkaline hydrolysis. Purified fractions (0.05–0.2 mg) were saponified with NaOH (2 M, 100 µL, degassed with N_2) by stirring

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for 2 h in the dark at room temperature. The reaction was stopped by adding 2 M H_3PO_4 (150 μL). This solution was used directly for HPLC analysis, using conditions similar to those described above. Neutral carbohydrate compounds were determined as alditol acetates after hydrolysis (2 M TFA for 30 min at 120°C). Alditol acetates were prepared and separated as described above. Detailed structural identification was performed using the usual array of 1-D ans 2-D NMR experiments (^{1}H; ^{13}C; H,H-correlated spectroscopy (H,H-COSY), total correlation spectrocopy (TOCSY); heteronuclear single quantum coherence/heteronuclear multiple quantum coherence (HSQC/HMQC); heteronuclear single quantum coherence/total correlation spectrocopy (HSQC-TOCSY); heteronuclear multiple bond correlation (HMBC)). Samples were deuterium-exchanged and dissolved in 0.7/0.5 mL D_2O. Chemical shifts (δ) were referenced to internal acetone by setting the ^{1}H signal to δ 2.20 ppm and the methyl signal to δ 30.89 ppm.

2.7.1 *O*-(6-*O*-*trans*-feruloyl-β-D-galactopyranosyl)-(1→4)-D-galactopyranose (compound A, Fig. 2)

Chemical shifts (δ [ppm]) are arranged in the order of the sugar/ferulic acid nomenclature (Fig. 2); ^{1}H NMR (500 MHz, D_2O, 300 K, internal acetone), due to the complexity of the spectra multiplicities of the sugar signals are, with the exception of the anomeric signals, not given. Reducing α-D-galactopyranose: H1: 5.24 (d, ^3J (H,H) = 3.7 Hz); H2: 3.85; H3: 3.90; H4: 4.16; H5: 4.06; H6: 3.72, 3.62. Reducing β-D-galactopyranose: H1: 4.56 (d, ^3J (H,H) = 7.6 Hz); H2: 3.53; H3: 3.69; H4: 4.09; H5: not determined; H6: 3.74, 3.66. Nonreducing β-D-galactopyranose: H1: 4.57 (d, ^3J (H,H) = 7.5 Hz); H2: 3.59; H3: 3.67; H4: 3.96; H5: 3.94; H6: 4.47, 4.32. *trans*-Ferulic acid: H2: 7.23 (bs); H5: 6.78 (d, ^3J (H,H) = 7.8 Hz); H6: 7.15 (d, ^3J (H,H) = 7.8 Hz); H7: 7.68 (d, ^3J (H,H) = 15.9 Hz); H8: 6.34 (bd, ^3J (H,H) = 15.9 Hz); OMe: 3.86 (s). ^{13}C NMR (data taken from 2-D HSQC/HMBC experiments, F1: 125 MHz, F2: 500 MHz, D_2O, 300 K, internal acetone). Reducing α-D-galactopyranose: C1: 93.0; C2: 69.5; C3: 70.2; C4: 79.6; C5: 70.5; C6: 61.9. Reducing β-D-galactopyranose: C1: 97.0; C2: 72.9; C3: 73.8; C4: 78.5; C5: 75.0; C6: 61.1. Nonreducing β-D-galactopyranose: C1: 105.1; C2: 72.0; C3: 73.3; C4: 69.3; C5: 73.4; C6: 64.1. *trans*-Ferulic acid: C1: 124.4; C2: 111.8; C3: 150.2; C4: 150.2; C5: 117.6; C6: 125.3; C7: 147.9; C8: 112.5; C9: 170.3; OMe: 56.5.

2.7.2 *O*-α-L-arabinofuranosyl-(1→3)-*O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1→5)-L-arabinofuranose (compound B, Fig. 2)

^{1}H NMR (750 MHz, D_2O, 300 K, internal acetone): Reducing α-L-arabinofuranose: H1: 5.244 (s); H2: 4.02; H3: 4.06; H4: 4.23; H5: 3.87, 3.79[2]. Reducing β-L-arabinofuranose: H1: 5.28 (d, ^3J (H,H) = 4.5 Hz); H2: 4.08; H3: 4.10; H4: 3.95; H5: 3.85, 3.79. Middle, nonreducing α-L-arabinofuranose: H1: 5.32 (s)/5.30 (s)[1]; H2: 5.17/5.16[1]; H3: 4.23; H4: 4.25; H5: 3.88, 3.77. Terminal, nonreducing α-L-arabinofuranose: H1: 5.240 (s); H2: 4.15; H3: 3.93; H4: 4.02; H5: 3.81[2], 3.69. *trans*-Ferulic acid: H2: 7.19 (s); H5: 6.89 (d, ^3J (H,H) = 7.8 Hz); H6: 7.12 (d, ^3J (H,H) = 7.8 Hz); H7: 7.63 (d, ^3J (H,H) = 15.9 Hz); H8: 6.35 (d, ^3J (H,H) = 15.9 Hz); OMe: 3.85 (s). [1] – double signal due to anomers of the reducing arabinose; [2] – precise chemical shifts difficult to estimate. ^{13}C NMR (100 MHz, D_2O, 300 K, internal acetone): Reducing α-L-arabinofuranose: C1: 101.78; C2: 82.04; C3: 76.35; C4: 81.88 (!); C5: 66.79. Reducing β-L-arabinofuranose: C1: 95.98; C2: 76.71; C3: 75.00; C4: 80.02; C5: 68.41. Middle, nonreducing α-L-arabinofuranose: C1: 106.11/106.07[1]; C2: 82.20/82.15[1]; C3: 80.79/80.73[1]; C4: 84.41/84.31[1]; C5: 61.27. Terminal, nonreducing α-L-arabinofuranose: C1: 107.65/107.69[1]; C2: 81.82 (!); C3: 77.33; C4: 84.61; C5: 61.80. *trans*-Ferulic acid: C1: 127.35; C2: 112.02; C3: 148.34; C4: 148.80; C5: 116.29; C6: 124.24; C7: 147.69; C8: 114.28; C9: 168.73; OMe: 56.52. (!) – Assignment may be interchanged; [1] – double signal due to anomers of the reducing arabinose.

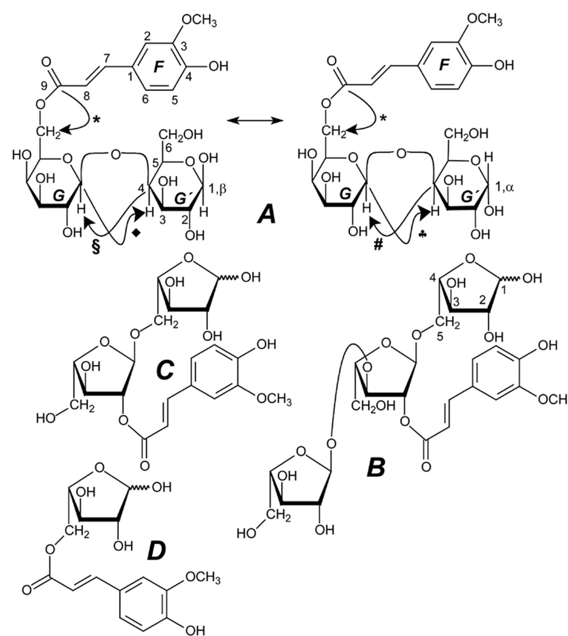

Figure 2. Structures of isolated feruloylated mono/oligosaccharides from amaranth insoluble dietary fiber. Correlations used for the identification of glycosidic and ester bonds by NMR are indicated by arrows (see also Fig. 3). Compound A: *O*-(6-*O*-*trans*-feruloyl-β-D-galactopyranosyl)-(1→4)-D-galactopyranose. Compound B: *O*-α-L-arabinofuranosyl-(1→3)-*O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1→5)-L-arabinofuranose. Compound C: *O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1→5)-L-arabinofuranose. Compound D: 5-*O*-*trans*-feruloyl-L-arabinofuranose. F, ferulate; G, nonreducing galactose; G′, reducing galactose.

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2.7.3 *O*-(2-*O*-*trans*-Feruloyl-α-L-arabinofuranosyl)-(1→5)-L-arabinofuranose (compound C, Fig. 2)

^{1}H NMR (500 MHz, D_2O, 300 K, internal acetone): Reducing α-L-arabinofuranose: H1: 5.25 (d, ^3J (H,H) = 2.2 Hz); H2: 4.02; H3: 4.03; H4: 4.24; H5: 3.87, 3.77. Reducing β-L-arabinofuranose: H1: 5.29 (d, ^3J (H,H) = 3.2 Hz); H2: 4.09; H3: 4.09; H4: 3.96; H5: 3.86, 3.77. Nonreducing α-L-arabinofuranose: H1: 5.23 (bs)/5.24 (bs)$^{(1)}$; H2: 5.04; H3: 4.17; H4: 4.17; H5: 3.86, 3.74. *trans*-Ferulic acid: H2: 7.11 (s); H5: 6.86 (d, ^3J (H,H) = 7.9 Hz); H6: 7.06 (d, ^3J (H,H) = 7.9 Hz); H7: 7.54 (d, ^3J (H,H) = 16.0 Hz); H8: 6.28 (d, ^3J (H,H) = 16.0 Hz); OMe: 3.83 (s). $^{(1)}$ – Double signal due to anomers of the reducing arabinose. ^{13}C NMR (100 MHz, D_2O, 300 K, internal acetone): Reducing α-L-arabinofuranose: C1: 101.82; C2: 82.01; C3: 76.53 (!); C4: 82.01; C5: 67.31. Reducing β-L-arabinofuranose: C1: 95.99; C2: 76.69 (!); C3: 75.09; C4: 80.04; C5: 68.75. Nonreducing α-L-arabinofuranose: C1: 106.19/106.12$^{(1)}$; C2: 84.20/84.13$^{(1)}$; C3: 75.85/75.81$^{(1)}$; C4: 85.15/85.10$^{(1)}$; C5: 61.64/61.60$^{(1)}$. *trans*-Ferulic acid: C1: 127.32; C2: 111.88; C3: 148.26; C4: 148.68; C5: 116.18; C6: 124.17; C7: 147.54; C8: 114.18; C9: 168.82; OMe: 56.43. (!) – Assignment may be interchanged; $^{(1)}$ – double signal due to anomers of the reducing arabinose.

2.7.4 5-*O*-*trans*-Feruloyl-L-arabinofuranose (compound D, Fig. 2)

^{1}H NMR (500 MHz, D_2O, 300 K): α-L-Arabinofuranose: H1: 5.28 (d, ^3J (H,H) = 2.5 Hz); H2: 4.06; H3: 4.05; H4: 4.32; H5: 4.42, 4.27. β-L-Arabinofuranose: H1: 5.31 (d, ^3J (H,H) = 4.5 Hz); H2: 4.11; H3: 4.14; H4: 4.02; H5: 4.43, 4.24. *trans*-Ferulic acid: H2: 7.10 (s)/7.09 (s)$^{(1)}$; H5: 6.84 (d, ^3J (H,H) = 8.0 Hz); H6: 7.04 (d, ^3J (H,H) = 8.0 Hz); H7: 7.54 (d, ^3J (H,H) = 16.0 Hz)/7.53 (d, ^3J (H,H) = 16.0 Hz)$^{(1)}$; H8: 6.29 (d, ^3J (H,H) = 16.0 Hz)/6.27 (d, ^3J (H,H) = 16.0 Hz)$^{(1)}$; OMe: 3.81 (s). $^{(1)}$ – Double signal due to anomers of the reducing arabinose. ^{13}C NMR (100 MHz, D_2O, 300 K): α-L-Arabinofuranose: C1: 101.64; C2: 81.66; C3: 76.32; C4: 81.08; C5: 64.35. β-L-Arabinofuranose: C1: 95.72; C2: 76.42; C3: 74.72; C4: 79.04; C5: 65.48. *trans*-Ferulic acid: C1: 127.19/127.15$^{(1)}$; C2: 111.62/111.59$^{(1)}$; C3: 148.25; C4: 147.95; C5: 115.90; C6: 123.77; C7: 146.84/146.78$^{(1)}$; C8: 114.37/114.25$^{(1)}$; C9: 169.58/169.51$^{(1)}$; OMe: 56.18. $^{(1)}$ – Double signal due to anomers of the reducing arabinose.

3 Results and discussion

3.1 Amaranth insoluble fiber composition

Amaranth insoluble fiber fractions that were used for the release of feruloylated oligosaccharides were investigated for their ferulic acid content and carbohydrate composition. Alkaline hydrolysis at room temperature released *trans*- and *cis*-ferulic acid. A *trans*-ferulic acid content of 0.620 ± 0.010 mg · g^{-1} ($n = 3$) was determined. The analyzed *cis*-ferulic acid content was surprisingly high (0.203 ± 0.006 mg · g^{-1}; $n = 3$), revealing that about 25% of ferulic acid was in the *cis*-configuration. The ferulic acid content is comparable to that of quinoa, another pseudo cereal that, however, belongs to the family of Chenopodiaceae (635 µg total ferulic acid g^{-1} insoluble fiber) [22].

The relative neutral sugar composition of amaranth insoluble fiber was determined ($n = 3$): 57.35% glucose, 21.77% arabinose, 8.71% xylose, 6.07% galactose, 3.95% rhamnose, 1.59% mannose, and 0.55% fucose. Pedersen *et al.* [3] also determined the neutral sugar composition of fiber fractions of different *Amaranthus caudatus* varieties. As in our investigation, they found high arabinose/xylose ratios and deduced an arabinoxylan composition different from cereals, with consequences for the dietary fiber properties. However, as amaranth is a dicotyledonous plant, we assume that arabinoxylans are not the dominant non-starch polysaccharides but, next to cellulose, pectins, and xyloglucans are the major polysaccharides. Preliminary results from methylation analysis support this assumption: most arabinose units are (1→5)-linked. This is in agreement with the structure of pectic arabinans that are built of a backbone of (1→5)-linked α-L-arabinofuranosyl residues with other α-L-arabinofuranosyl residues attached to the backbone residues by (1→3)- and/or (1→2)-linkages. We also identified small amounts of (1→4)-linked xylose units that indicate xylans, but their amounts are minor in comparison to terminal xylose units. The dominance of terminal xylose units indicates the presence of xyloglucans. The neutral sugar composition implies that pectins play a major role in the investigated fiber fractions.

3.2 Isolation of feruloylated oligosaccharides from enzymatically hydrolyzed amaranth insoluble fiber

Driselase is an enzyme mixture that contains several carbohydrate hydrolases, *e.g.*, cellulase, xylanase, galactanase, arabinanase, and polygalacturonase, but is conveniently devoid of feruloyl esterases. Using this mixture of carbohydrases, 51.3% of insoluble amaranth fiber was degraded to water-soluble fragments. The enzymatic hydrolyzate was prefractionated using Amberlite XAD-2. Whereas the water fraction should contain carbohydrates and high-molecular-weight feruloylated oligosaccharides, the MeOH/H_2O fraction should predominantly contain low molecular weight feruloylated oligosaccharides. Fractionation using Sephadex LH-20 chromatography gave three main peaks as shown in Fig. 1. RP-HPLC of the Sephadex fractions showed that fraction 1 (eluting between 1840 – 2100 min)

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contained four different fractions F1.1 – F1.4 (Fig. 1) and that fractions 2 and 3 were fairly clean.

3.3 Structural elucidation of feruloylated oligosaccharides in F1.1/F1.2, F1.3, and F1.4

^{1}H NMR of F1.1 and F1.2 showed that both fractions contained the same compound. This effect is well known from the RP-HPLC isolation of feruloylated oligosaccharides that contain a reducing xylose residue: It is feasible to separate α- and β-anomers of the reducing xylose moiety by HPLC, but equilibration during the separation, and after isolation, does not allow purification of the anomers. Less separation of the anomers is achieved by RP-HPLC with feruloylated oligosaccharides having a reducing arabinose-moiety. Further structural elucidation experiments were applied only to one of these fractions (F1.1/F1.2).

HPLC-MS of F1.2 showed adduct ions with *m/z* 557 $[M + K]^+$, *m/z* 541 $[M + Na]^+$, and *m/z* 536 $[M + NH_4]^+$ indicating a molecular weight of 518, corresponding to one ferulic acid and two hexoses. Indeed, following hydrolysis the phenolic moiety was proven to release *trans*-ferulic acid and the analysis of the neutral sugars showed galactose to be the sole monosaccharide. NMR analysis confirmed the constituents of compound A (Fig. 2) to be *trans*-ferulate and galactose and showed the β-anomeric configuration of the nonreducing galactopyranose. 2-D NMR experiments revealed the linkages between the constituents. Slices of the HMBC spectrum showing diagnostic correlation peaks are presented in Fig. 3. Linkage of ferulic acid to the 6-position of the nonreducing galactose (G, Fig. 2) is indicated by the marked correlation peaks showing scalar coupling between the 6-protons of G and the carbon 9 of ferulate *via* three bonds (Fig. 3, top). The (1→4)-linkage of the galactose compounds was proven by the correlation peaks shown in Fig. 3, bottom; the upper slice shows signals resulting from

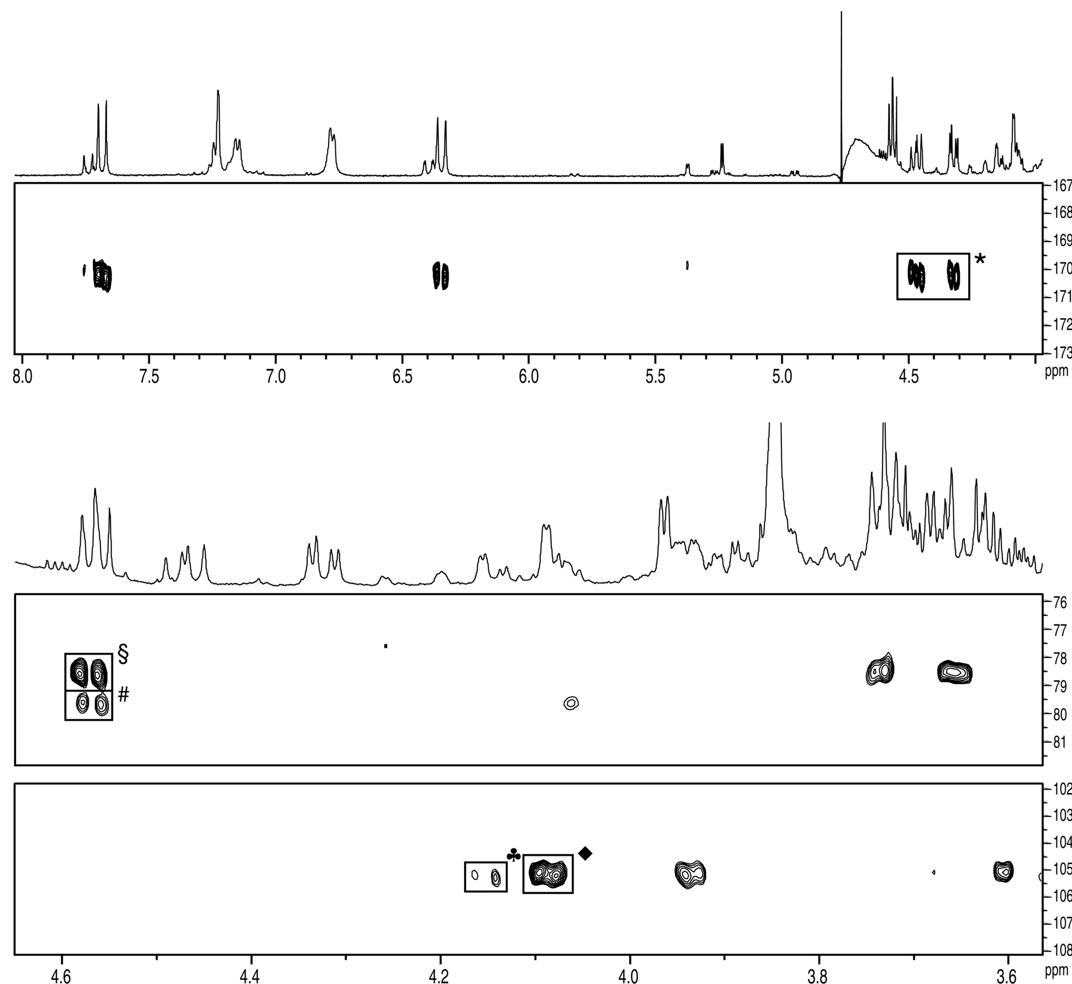

Figure 3. Slices of the HMBC-spectrum of *O*-(6-*O*-*trans*-feruloyl-β-D-galactopyranosyl)-(1→4)-D-galactopyranose (compound B) used to identify linkages between carbohydrate compounds and ferulate. Correlation peaks correspond to the correlations indicated by arrows in Fig. 2.

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scalar coupling between the carbon 4 (from reducing α- or β-galactose, G′) and the β-anomeric proton of the nonreducing galactose G, whereas the lower slice shows signals resulting from the anomeric carbon of the nonreducing galactose to the 4-protons of the reducing α/β-galactose. These data identify compound A as *O*-(6-*O*-*trans*-feruloyl-β-D-galactopyranosyl)-(1→4)-D-galactopyranose. This compound has previously been isolated from sugar beet [16, 23] and spinach [23–25].

Structural characterization of compounds from fractions 1.3 and 1.4 was not fully achieved. Fraction 1.3 did not contain a single compound. From NMR data we propose a mixture of feruloylated oligosaccharides containing arabinose and galactose. Fraction 1.4 was difficult to characterize due to a change in the NMR over the time. We assume that this change is due to the isomerisation of a *cis*-ferulolylated oligosaccharide to the preferred *trans*-feruloylated oligosaccharide as described below.

3.4 Structural elucidation of feruloylated oligosaccharides in F2 and F3

The molecular masses of compounds B (Fig. 2) (fraction 2) and C (Fig. 2) (fraction 3) were determined to be 590 (adduct ions: *m/z* 629 $[M+K]^+$, *m/z* 613 $[M+Na]^+$, *m/z* 608 $[M+NH_4]^+$) and 458 (adduct ions: *m/z* 497 $[M+K]^+$, *m/z* 481 $[M+Na]^+$, *m/z* 476 $[M+NH_4]^+$), respectively, corresponding to molecules consisting of one ferulic acid and two or three pentoses. Neutral sugar analysis revealed arabinose as the sole sugar in both fractions. Following saponification *trans*-ferulic acid was detected in both fractions. In addition small amounts of the light-isomerization product *cis*-ferulic acid was found in fraction 2. NMR analysis confirmed ferulate and arabinose as constituents and gave information about the anomeric configurations of the sugar constituents and the linkages.

The anomeric configuration of both nonreducing arabinofuranoses of compound B is α. Considering the chemical shifts, the arabinose constituents were assumed to be (1→3)- and (1→5)-linked, respectively. These glycosidic linkages were confirmed by diagnostic correlation-peaks in the HMBC experiment (not shown). The HMBC experiment also revealed ferulate to be bound at the C2-position of the middle arabinose unit. From these data compound B was unambiguously identified as *O*-α-L-arabinofuranosyl-(1→3)-*O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1→5)-L-arabinofuranose.

The nonreducing arabinose of compound C has an α-configuration. The HMBC experiment unambiguously showed that the arabinofuranose units are linked *via* a (1→5)-linkage and that ferulate acylates the C2-position of the nonreducing arabinose (Fig. 2). Compound C was therefore identified as *O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1→5)-L-arabinofuranose. To date, compounds B and C have only been isolated from spinach and sugar beet [16, 23, 25].

As already mentioned we assume that fraction 1.4 originally contained the *cis*-isomer of compound C, *i. e.*, *O*-(2-*O*-*cis*-feruloyl-α-L-arabinofuranosyl)-(1→5)-L-arabinofuranose. In the first acquired ^{1}H NMR spectrum the phenolic region showed *trans*-ferulate and *cis*-ferulate in approximately equal amounts as determined by integration (Fig. 4). However, when we started 2-D experiments, the proton spectrum showed that the *cis/trans*-ratio had decreased considerably, only showing about 15% *cis*-ferulate. In the 2-D HSQC-TOCSY we identified all carbon signals of compound C (the *trans*-isomer) starting from the anomeric proton signals and the diagnostic H2 signal of the nonreducing

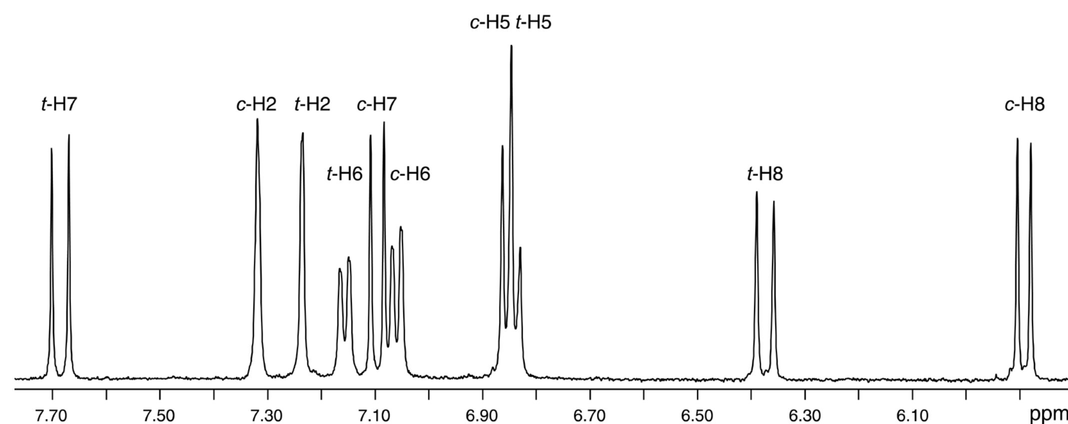

Figure 4. Phenolic region of the ^{1}H NMR spectrum of fraction 1.4 showing *trans (t)*- and *cis (c)*-ferulate in almost equal amounts. *cis*-Ferulate signals were considerably decreased in a second ^{1}H NMR experiment carried out later, indicating isomerization over the time.

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arabinose (moved to higher chemical shifts due to its feruloylation) and the HMBC clearly showed the linkage of ferulate at the C2 position of the nonreducing arabinose. However, the carbohydrate signal assignment of the minor *cis*-isomer of compound C was not unambiguously possible due to its low abundance in the mixture. From these data we suppose that it is possible to separate the *cis*-isomer from the *trans*-isomer by GPC on Sepahdex LH-20. The *trans*-isomer of this feruloylated diarabinoside seems to be the preferred one in aqueous solution thus the *trans*-isomer is reformed during storage in aqueous solution. Another hint for this assumption is that NMR data of the mixed fraction 1.3 indicate that the *trans*-isomer, compound C, is part of this fraction, showing the reformation of the *trans*-isomer following Sephadex LH-20 chromatography.

3.5 Isolation of a feruloylated monosaccharide from partially acid hydrolyzed amaranth insoluble fiber

The sole compound isolated in pure form by Sephadex LH-20 chromatography from the acid hydrolyzate was compound D, 5-*O*-*trans*-feruloyl-L-arabinofuranose (Fig. 2). Coupling of ferulic acid in C5-position of arabinofuranose was clearly detected by HMBC. Coupling of ferulate to C5 of arabinose is well-known in monocotyledonous plants, *e.g.*, in the grasses and cereals, where ferulate is principally bound to arabinoxylans [10, 13, 14].

3.6 Ferulic acid-polysaccharide association in amaranth insoluble dietary fiber

The feruloylated oligosaccharides isolated from the enzymatic hydrolyzate show that in amaranth insoluble fiber ferulate predominantly acylates pectic arabinans and galactans. Arabinans and galactans are side chains of rhamnogalacturonan I, a pectic polymer with a backbone of alternating α-(1→2)-linked L-rhamnosyl and α-(1→4)-linked D-galactosyluronic acid residues and side chains attached to about one-half of the rhamnose units. These side chains are characterized as arabinans, galactans, and arabinogalactans. As already mentioned, arabinans are built of a backbone of (1→5)-linked α-L-arabinofuranosyl residues with other, mostly single α-L-arabinofuranosyl residues attached to the backbone residues by (1→3)- and/or (1→2)-linkages. The isolation of compounds B and C indicates the association of ferulate with arabinose residues of the arabinan main chain whereas compound B also shows the localization of ferulate at the branching point of the arabinan main chain. Compound A logically indicates the association of ferulate with galactans or arabinogalactans that are built from a backbone of (1→4)-linked β-D-galactopyranosyl residues. Compound D isolated from the acid hydrolyzate is a hint that ferulate may also be bound to arabinoxylans in amaranth insoluble fiber. However, we did not succeed in the isolation of feruloylated oligosaccharides that still contain xylose residues from the enzymatic hydrolyzate. Results from a recent paper may help to clarify this contradiction. From sugar beet cell walls, Levigne *et al.* [26] isolated an arabinotriose and an arabinotetraose both of them associated with two ferulates. In both compounds ferulates acylate the C5-position of arabinose in addition to the C2-position of a neighboring arabinose. Although the linkage of ferulate to arabinoxylans remains a possibility, the linkage of ferulate to the C5-position of an arabinose that is part of a pectic arabinan should also be considered for amaranth insoluble fiber.

Amaranth belongs to the family of Amaranthaceae. To date, in discotyledonous plants the association of ferulate and polysaccharides was only shown in sugar beet and spinach. Until recently, both of these species were placed in the family Chenopodiaceae. As described in the introduction section, the incorporation of the family Chenopodiaceae in the broadly circumscribed family Amaranthaceae is matter of a current discussion. However, spinach, sugar beet and amaranth belong to the same order (Caryophyllales). Further screening for the association of ferulate and polysaccharides in dicotyledonous plants that do not belong to the order of Caryophyllales would be useful for a better understanding of the chemical structures of dietary fibers and the physicochemical background of their physiological effects. Such a screening would also help to strengthen the assumption that diferulate cross-links of pectic polymers influence the texture and crispness after cooking of fruits and vegetables [27]. Although such a screening has already done by Hartley and Harris [28] several years ago, some questions still remain. Hartley and Harris screened 251 species in 150 families of dicotyledons and found that the presence of ferulate is restricted to the families of the order Caryophyllales. However, Waldron's group [29] recently found alkali-extractable ferulates and diferulates in cell walls of carrots (*Daucus carota* L.).

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