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Characterization of diferuloylated pectic polysaccharides from quinoa (*Chenopodium quinoa* WILLD.)

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ABSTRACT

In plants belonging to the order of Caryophyllales, pectic neutral side chains can be substituted with ferulic acid. The ability of ferulic acid to form intra- and/or intermolecular polysaccharide cross-links by dimerization was shown by the isolation and characterization of diferulic acid oligosaccharides from monocotyledonous plants. In this study, two diferulic acid oligosaccharides were isolated from the enzymatic hydrolyzate of seeds of the dicotyledonous pseudocereal quinoa by gel permeation chromatography and preparative HPLC and unambiguously identified by LC–MS^2 and 1D/2D NMR spectroscopy. The isolated oligosaccharides are comprised of 5-5- and 8-*O*-4-diferulic acid linked to the *O*2-position of the nonreducing residue of two (1 → 5)-linked arabinobioses. To get insight into the structure and the degree of phenolic acid substitution of the diferuloylated polysaccharides, polymeric sugar composition, glycosidic linkages, and polysaccharide-bound monomeric phenolic acids and diferulic acids were analyzed. This study demonstrates that diferulic acids are involved into intramolecular and/or intermolecular cross-linking of arabinan chains and may have a major impact on cell wall architecture of quinoa and other dicotyledonous plants of the order of Caryophyllales.

1. Introduction

In monocotyledonous cereal grains, phenolic acids such as ferulic acid and *p*-coumaric acid can be linked to cell wall polysaccharides, especially arabinoxylans (Harris and Trethewey, 2010). In dicotyledonous plants, comparatively high amounts of polysaccharide-bound phenolic acids are found in plants of the order Caryophyllales, as demonstrated in the early 1980s by cell wall UV-fluorescence experiments (Hartley and Harris, 1981). The isolation and characterization of feruloylated oligosaccharides demonstrated that ferulic acid is attached to arabinans and galactans (Bunzel et al., 2005; Colquhoun et al., 1994; Ishii and Tobita, 1993). These polysaccharides are generally described as neutral side chains of the pectic polysaccharide rhamnogalacturonan I. Arabinans are comprised of a backbone of α-(1 → 5)-linked arabinofuranoses (Ara*f*), which can be branched with Ara*f* at *O*2 and/or *O*3. Galactans are made up of a backbone of β-(1 → 4)-linked galactopyranoses (Gal*p*), to which arabinose or galactose units can be attached at various positions (Voragen et al., 2009). Ferulic acid mainly acylates the *O*2-position of arabinans and the *O*6-position of galactans as demonstrated by the isolation of feruloylated oligosaccharides from enzymatic hydrolyzates of spinach, amaranth, and sugar beet (Bunzel et al., 2005; Colquhoun et al., 1994; Ishii and Tobita, 1993). Radical coupling of two ferulates provides dehydrodiferulates, which can be 5-5-, 8-*O*-4-, 8-5-, 8-8- or, to a lesser degree, 4-*O*-5-coupled. Dimerization may result in the formation of intra- and/or intermolecular cross-links. The (di)ferulic acid substitution may affect the physicochemical and physiological properties such as viscosity, swelling, and enzymatic degradability (Bunzel, 2010). Several diferuloylated oligosaccharides isolated from monocotyledonous plants were characterized in detail (Allerdings et al., 2005; Bunzel et al., 2008; Ishii, 1991; Saulnier et al., 1999). In dicotyledonous plants, however, diferuloylated oligosaccharides were only studied in sugar beet. Characterization of these diferuloylated oligosaccharides was basically limited to mass spectrometry (Levigne et al., 2004; Ralet et al., 2005). Based on the fragmentation pattern and HPLC analysis of the constituents after hydrolysis, it was concluded that 8-*O*-4-diferulic acid is linked to the *O*5-position of two α-(1 → 5)-linked arabinobioses. Another oligosaccharide fraction contained mainly 8-*O*-4- and 5-5-diferulic acid and showed a fragmentation pattern typical for a linkage to the *O*2-position of the arabinoses. One diferuloylated oligosaccharide contained two single arabinose residues, while another one contained an arabinose and a (1 → 5)-linked arabinobiose residue. However, using mass spectrometry, it was

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not possible to unambiguously assign the dimers to the oligosaccharides (Ralet et al., 2005). In an earlier study, an 8-*O*-4-diferulic acid was also found to be linked to two arabinobioses, but it was not possible to locate the linkage position by using mass spectrometry (Levigne et al., 2004). Thus, although most useful as a secondary method, mass spectrometry data need to be supported by NMR data for unambiguous structure elucidation. Just like sugar beet, the pseudocereal quinoa belongs to the family of *Amaranthaceae*, and it was already demonstrated that various tissues of the quinoa plant contain diferulic acids (Renard et al., 1999). It was also demonstrated that quinoa seeds contain significant amounts of arabinans (Cordeiro et al., 2012; Lamothe et al., 2015). These polysaccharides are a potential target for ferulic acid substitution and diferulic acid cross-linking.

Thus, the aim of this study was the isolation of diferuloylated oligosaccharides from an enzymatic quinoa seed hydrolyzate and the unambiguous characterization of the individual compounds based on NMR spectroscopy and supported by MS data. Because the seeds are the most important organ of the quinoa plant for human nutrition, they were in the focus of this study. In addition, the main structural characteristics of the diferuloylated polysaccharides were evaluated.

2. Results and discussion

2.1. Isolation of diferuloylated oligosaccharides

To study compositional details of insoluble non-starch polysaccharides, insoluble dietary fiber is often isolated on a preparative scale from starch-rich materials, which is then hydrolyzed by the fungal enzyme mixture Driselase (Bunzel et al., 2005). Because fiber isolation is a time- and cost-intensive procedure, a modified digestion was used to prepare large amounts of defatted, destarched quinoa meal for subsequent studies of the non-starch polysaccharides. Compared to standard fiber isolation procedures, incubation with amyloglucosidase, and filtration and drying steps were skipped in this method. To hydrolyze quinoa non-starch polysaccharides, Viscozyme L was used as a cost-efficient alternative to Driselase. Viscozyme L also showed minimal cleavage of ester-bound ferulic acid but good hydrolysis rates on pectic side chains (data not shown). (Di)feruloylated oligosaccharides were separated from non-phenolic compounds (mostly mono- and oligosaccharides) by using Amberlite XAD-2. Sephadex LH-20 chromatography was used to separate (di)feruloylated oligosaccharides as described earlier (Bunzel et al., 2005; Ishii and Tobita, 1993; Ralet et al., 1994). Elution with water yielded several feruloylated oligosaccharides, with *O*-(2-*O*-*trans*-feruloyl-α-L-arabinofuranosyl)-(1 → 5)-L-arabinofuranose being the main compound. The following elution steps using water/methanol mixtures (70:30 (v/v) and 30:70 (v/v), elution step with water/methanol 30:70 is shown in Fig. 1) yielded two peaks, containing compounds with *m*/*z* 913 as the main UV-active compounds. This quasimolecular ion corresponds to a diferulic acid ester-linked to four pentoses. To remove minor impurities, these fractions were further purified by preparative HPLC prior to structure characterization.

2.2. Characterization of the diferuloylated oligosaccharides

Monosaccharide analysis and determination of D/L-configuration identified L-arabinose as the only monosaccharide present in both compounds. The MS^2 analysis of the quasimolecular ion at *m*/*z* 913 yielded a similar mass spectrum for both compounds (Fig. 2). Besides weak fragment peaks representing mass losses of water (−18 Da, *m*/*z* 895), one anhydro-arabinose (−132 Da, *m*/*z* 781), and one arabinose (−150 Da, *m*/*z* 763), the dominant

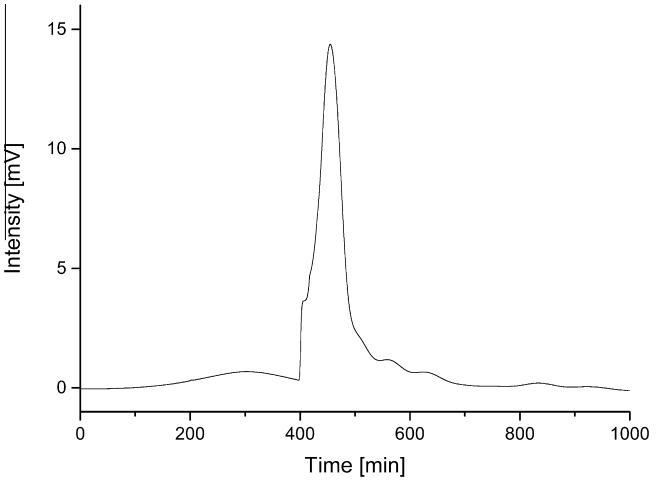

Fig. 1. Sephadex LH-20 chromatogram of quinoa hydrolyzate (1 mL/min, water/methanol 30:70); detection at 325 nm.

fragments were *m*/*z* 853 and *m*/*z* 823. This corresponds to mass losses of 60 Da and 90 Da, which are typical for a cross ring cleavage through a reducing 5-linked Ara*f* unit (Quemener and Ralet, 2004). The occurrence of these fragments indicated that the diferulic acid is not linked to the reducing arabinoses. The fragment with *m*/*z* 631 (mass loss of 282 Da) corresponds to the loss of an anhydro-arabinobiose unit. All obtained fragments were also described by Levigne et al. (2004) for a diferulic acid coupled to four arabinoses, but different intensities were observed, and additional fragments were reported. Either the structure of the analyzed oligosaccharide or the MS^2 conditions were different, resulting in a modified fragmentation pattern. MS^3 experiments of *m*/*z* 781 or *m*/*z* 631 were not possible due to the low intensities of these fragments.

NMR spectroscopic analysis was performed for unambiguous structural characterization. Compound 1 showed a relatively simple spectrum with well-resolved signals (Fig. 3). Due to the low number of signals it was concluded that the structure of the diferuloylated oligosaccharide was symmetric. Two doublets in the phenolic region (6.28 ppm and 7.67 ppm) with coupling constants of 15.5 Hz were assigned to H8 and H7 of the diferulic acid, respectively (Table 1 and Fig. 4). This was confirmed by the corresponding cross peaks in the COSY spectrum. The two broad singlets at 7.10 and 7.27 ppm only showed very weak cross peaks in the COSY spectrum and were assigned as H2 and H6 of the diferulic acid. Since the phenolic region of this spectrum did not show signals for H5, the occurrence of 5-5-diferulic acid was assumed. This was confirmed by the ^{13}C chemical shifts (Table 1), which are in good agreement with previously published data for oligosaccharide-bound 5-5-diferulic acid (Ishii, 1991; Saulnier et al., 1999). The obtained ^{1}H chemical shifts of H6 and H2 of the 5-5-diferulic acid were, however, different from those previously published (the proton shifts for H2 and H6 were interchanged), probably due to different solvents used. However, the ^{13}C shifts as well as an HMBC cross peak between H2 and C3 confirmed the assignments made (coupling of H6 and C3 would correspond to a fairly uncommon four bond correlation). The anomeric sugar region of the proton spectrum showed two doublets with coupling constants of 1.7 Hz and 4.5 Hz, which are typical for a reducing Ara*f*. The signal at 5.05/5.06 ppm representing H1 of the non-reducing arabinose units was split, demonstrating the far reaching influence of the α/β-anomers of the reducing arabinose unit. The peak at 5.01 ppm, assigned to H2 of the nonreducing arabinose, was shifted downfield because of the attached diferulic acid. The remaining sugar protons were identified by using the 2D spectra. The assignments were confirmed by the

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