Phenolic Compounds as Cross-Links of Plant Derived Polysaccharides

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Abstract: Plant cell wall polysaccharides are partially cross-linked via phenolic compounds. As shown in the past, the most important phenolic compounds to cross-link plant cell-wall polysaccharides are ester-linked ferulic acid dimers, but *p*-coumarate dimers were also shown to be potential cross-linking compounds. Recently, ferulic acid dimers were identified and quantified in a range of cereal grains. The isolation of 8-*O*-4-dehydrodiferulic acid-di-arabinoside from maize bran shows that diferulic acids are able to form intermolecular cross-links between arabinoxylans. The more recently identified sinapic acid dehydrodimers and ferulic acid dehydrotrimers provide additional contributions to building up a strong network of plant cell wall polysaccharides.

Keywords: plant cell walls; arabinoxylans; ferulic acid; ferulate dimers and trimers; sinapate dimers

INTRODUCTION

Cross-linking of plant cell wall polymers, especially of wall polysaccharides, is of considerable interest in food chemistry, food technology and nutritional sciences but also in neighbouring disciplines like agricultural chemistry and plant physiology [1]. Regarding the fields of food chemistry and food technology it is worth mentioning the influence of phenolic cross-links on the thermal stability of cell adhesion and maintenance of crispness of plant based food [2], on the gelling properties of sugar beet pectins [3] and other food compounds as well as on the solubility properties of cereal dietary fibres derived form plant cell walls. Phenolic cross-links decrease enzymatic degradation of plant cell walls [4]. In this way cross-links may influence microbial degradation of dietary fibre polysaccharides in the human gut thus controlling, for example, e.g. the formation of short chain fatty acids and the bulk properties of these fibres.

Hydroxycinnamic acids, especially ferulic acid, are the most important phenolic compounds to form cross-links in plant cell walls. Ferulic acid and *p*-coumaric acid are ester-linked to arabinoxylans in cereals and other grasses and ferulic acid is linked to pectins in some dicots [5]. Similarly, sinapic acid is thought to be bound to polysaccharides via ester-linkages [6].

Here, we briefly summarise our recent work to identify and quantify phenolic cross-links in cereal grains.

EXPERIMENTAL

Isolation of fibre material. Insoluble and soluble cereal dietary fibres were prepared according to an upscaled enzymatic procedure using a sequence of heat-stable α-amylase, protease and amyloglucosidase [7].

Alkaline hydrolysis of fibres and extraction of phenolic acids. Saponification was generally performed using 2M NaOH for 18 h under nitrogen and protected from light [7]. Following acidification extraction was carried out with diethyl ether. Additional clean-up steps using liquid-liquid extraction with $NaHCO_3$ were performed in the isolation procedure of standard compounds [8, 9].

Synthesis of standard compounds. Diferulic acids were synthesised as previously described [10, 11]. Disinapic acids were synthesised from methyl or

ethyl sinapate using two different single-electron metal oxidant systems [12].

Isolation of standard compounds. Isolation of diferulic and triferulic acids from alkaline hydrolysates of maize bran is possible using a procedure combining Sephadex LH-20 chromatography with organic solvents and RP-HPLC as recently described [8, 9].

Identification of new compounds. New phenolic compounds were identified by high and low resolution ESI-MS and application of the standard set of one- and two dimensional NMR-experiments.

Identification and quantification of ferulate and sinapate dimers. Monomeric phenolic acids as well as diferulic and disinapic acids were identified as their trimethylsilylated derivatives by GC-MS [7, 12, 13]. Mass spectra and relative retention times against monomethylated 5-5-diferulic acid as internal standard [1, 13] were compared with those of authenticated standard compounds. Quantification was carried out using GC-FID.

Isolation and identification of 8-O-4-dehydrodiferulic acid-di-arabinoside. 8-*O*-4-dehydrodiferulic acid-di-arabinoside was isolated from the acidic hydrolysate of insoluble maize bran fibres. Separation was performed using Amberlite XAD-2 pre-separation, Sephadex LH-20 chromatography and RP-HPLC. Identification was carried out by ESI-MS and one-, two- and three-dimensional NMR experiments [14].

RESULTS AND DISCUSSION

Dehydrodiferulates as polysaccharide cross-links

Acylation of polysaccharides with hydroxycinnamic acids in planta [5] leads to suitable conditions for cross-coupling these polysaccharides by formation of hydroxycinnamate dimers. Dimerisation is possible by either photochemical (leading to cyclobutan-dimers) [15] or radical coupling (leading to dehydrodimers) [11]. Although underestimated for a long time the radical mechanism is the predominant mechanism to cross-link feruloylated polysaccharides. Ferulate esters dimerise via their phenoxy radicals that couple at their 4-*O*-, C5- or C8-positions to give rise to 8-5-, 8-*O*-4-, 5-5-, 8-8- and 4-*O*-5-coupled dehydrodiferulic acid esters. Saponification releases the nine dehydrodiferulic acids (DFA) shown in Figure 1.

We recently surveyed soluble (SDF) and insoluble (IDF) dietary fibres from whole grains of maize, wheat, spelt, rice, wild rice, barley, rye, oat and

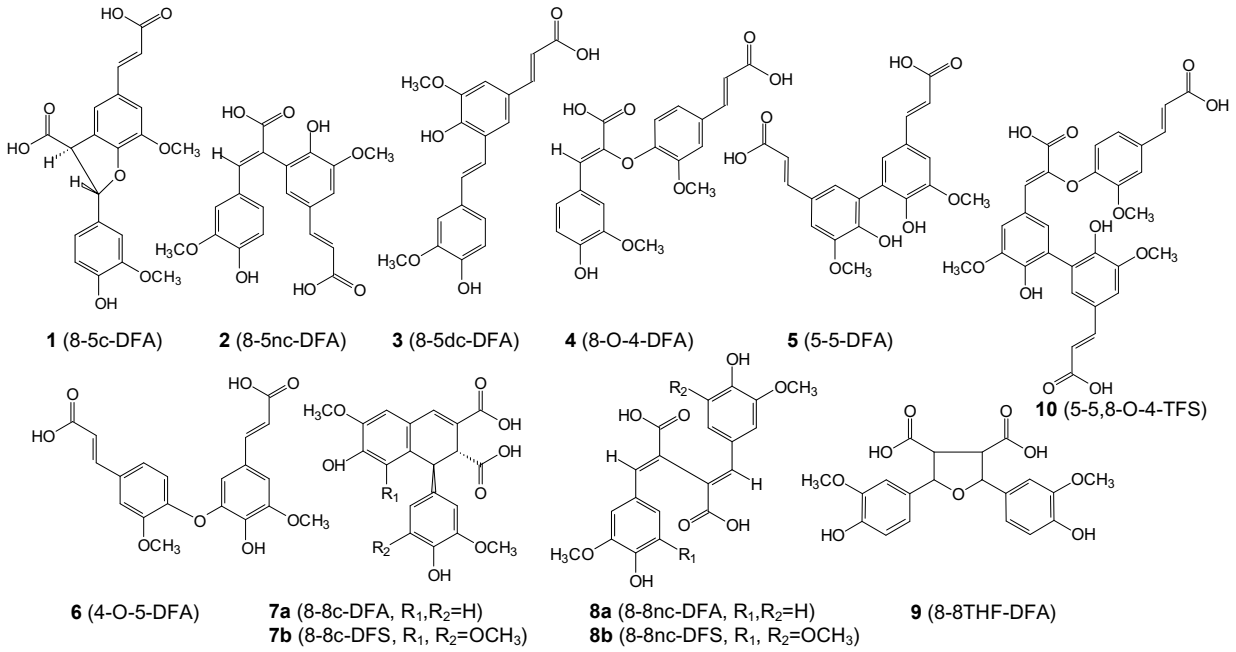

Figure 1. Structures of phenolic cross-link compounds; DFA-diferulic acid, DSA – disinapic acid, TFA – triferulic acid, c – cyclic, nc – non-cyclic, dc – decarboxylated, THF – tetrahydrofuran

millet for their DFA levels (with the exception of the later identified DFA **9**) [7]. From most cereal IDF the whole spectrum of DFAs (compounds **1–6**, **7a**, **8a**, Figure 1) could be identified. From cereal SDF we detected DFAs **1–5**, **7a** and **8a**; the 4-*O*-5-coupled DFA 6 was missing in all investigated cereal SDF. In cereal IDF the absolute contents of total DFAs ranged between 2.4 and 12.6 mg/g IDF, from cereal SDF much lower contents were detected (40–230 μg/g). In cereal IDF 8-5-coupled dimers dominated, whereas in cereal SDF 8-8-coupled dimers were relatively enhanced and they often became the major dimers. It was estimated that arabinoxylans of cereal IDF contain 8 to 39 times more diferulates than arabinoxylans of cereal SDF. From these investigations we suggested that diferulates may be partly responsible for producing IDF, e.g. by increasing the arabinoxylan molecular weight. The above mentioned quantification did not include the *cis*-isomers of some DFAs that can be formed by light-induced isomerisation in planta but also during the analytical procedure. *cis*-Isomers of DFAs **1**, **2** and **5** were identified. A careful estimation of the *cis*-DFA levels based on peak areas in the GC-FID chromatograms showed that the *cis*-isomers may contribute up to 3% to the total level of DFAs in cereal IDF and up to 5% in cereal SDF. However, in most cases this percentage is lower (< 1% in cereal IDF and < 3% in cereal SDF).

Another DFA was detected very recently from alkaline hydrolysates of grass cell walls. From its mass spectra the structure shown in Figure 1, compound **9**, was proposed. This DFA contains an additional oxygen resulting from water incorporation following radical coupling [1]. DFA 9 has been synthesised (Schatz, unpubl.) confirming our preliminary structure suggestion. Contrary to the *cis*-DFAs the amounts of DFA **9** should not be neglected. Complete structural elucidation, synthesis and levels of DFA **9** in different fibres will soon be reported.

Intermolecular or intramolecular polysaccharide cross-linking by dehydrodiferulates?

Evidence for polysaccharide cross-linking via DFAs is possible by isolation and identification of diferuloylated oligosaccharides following enzymatic or acidic hydrolysis of the polymer. The isolation of a DFA linked to arabinoxylan fragments from bamboo shoot and maize bran has been reported in the past [16, 17]. In both cases, 5-5-diferuloyl saccharides have been isolated (Xyl-Xyl-Ara-FA-5-5-FA-Ara-Xyl-Xyl, Ara-FA-5-5-FA-Ara, Xyl-Ara-FA-5-5-FA-Ara, Ara – arabinose, Xyl – xylose, FA – ferulic acid). This provided the first structural evidence that DFAs may act as polysaccharide cross-links in the cell walls. However, the isolation of 5–5-diferuloyl oligosaccharides does not prove intermolecular cross-linking between two different polysaccharide chains. This assumption is based on molecular modelling experiments that demonstrated the 5-5-linked diferulate is unique in that it could be formed intramolecularly [18]. We therefore prospected for DFA-oligosaccharides incorporating DFAs other than the 5-5-DFA. As a result, we recently succeeded in isolation and identification of a di-arabinosyl 8-*O*-4-DFA (Ara-FA-8-*O*-4-FA-Ara) from maize bran [14]. The isolation of this compound is a strong indication of intermolecular coupling of two different arabinoxylans by DFAs.

Dehydrodisinapates and ferulate-sinapate cross-products

Sinapic acid can be released in small quantities by saponification from different grasses including cereals [6, 19]. There are indications that sinapic acid is bound via ester-linkages to plant cell wall polysaccharides [6]. However, the isolation of defined sinapic acid-oligosaccharides following hydrolysis, which would be unambiguous proof for the attachment of sinapic acid to polysaccharides, has not yet been successful. Theoretically, sinapates should be capable radical dehydrodimerisation as their ferulate analogues. Indeed we succeeded in the identification of dehydrodisinapic acids (DSA) from alkaline hydrolysates of wild rice fibres and other cereal fibres. It was possible to authenticate two 8-8-coupled DSAs (Figure 1, compounds **7b** and **8b**) by comparison of their relative GC retention times and mass spectra with synthesised standard compounds [12]. Due to the additional methoxyl in 5-position 5-5-, 8-5- and 4-*O*-5-coupling is not possible. Nevertheless, 8-*O*-4-coupling is still an alternative. However, screening GC-MS-chromatograms for 8-*O*-4-coupled DSA was not successful. This is in accordance with the fact that sinapates are known to predominately couple in 8-8-positions [20]. The highest levels of DSAs were found in wild rice IDF (481 μg/g). DSAs were also identified in IDF from wheat, spelt, rye, barley, corn and rice

and in SDF from wheat, spelt, rye, barley, rice and wild rice but not from IDF from oat and millet and from SDF from oat, millet and corn [12].

Although homo-coupling should be the major radical reaction between ferulates on the one hand and sinapates on the other hand we were able to detect very small amounts of heterodimers of sinapic and ferulic acid. Tentatively identification was carried out by their mass spectra and their fragmentation similarity with their diferulate and disinapate analogues. The tentatively identified heterodimers were 8-8-, 8-5- and 8-*O*-4-coupled [12].

Triferulates as polysaccharide cross-links

The polysaccharide network can be strengthened by further hydroxycinnamate oligomers, e.g. ferulic acid trimers or tetramers. In 2000, Fry's group presented evidence for higher oligomers in maize suspensions cultures but did not present defined structures [21]. Recently we isolated and identified a ferulic acid dehydrotrimer (Figure 1, **10**) [9]. This compound that was discovered at essentially the same time by another group [22] involves 8-*O*-4- and 5-5-coupling. The involvement of the 5-5-coupling in this trimer prevents unambiguous assertions as to whether this compound is able to cross-link three different polysaccharides or not. As already mentioned above, the 5-5-diferulate unit is the only one that can be formed intramolecularly. Very recently we succeeded in isolating two more ferulic acid trimers that do not contain a 5-5-unit [23]. The finding of these 8-*O*-4/8-O-4- and 8-*O*-4-/8-8-coupled trimers shows that coupling of three polysaccharide chains via higher oligomers is still a possibility. However, "back-crossing", as more detailed in [1], should still be considered.

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