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Isolation and characterisation of the homogalacturonan from type II cell walls of the commelinoid monocot wheat using HF-solvolysis

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Abstract

In contrast to the typical type I cell wall of the dicot plants, the type II cell wall of the commelinoid monocot plants is known to be relatively poor in pectins. Assuming a critical role for the remaining pectins in terms of cell wall architecture and/or as a reservoir of signalling molecules, we have compared different protocols for the isolation of the main pectin polymer, homogalacturonan, from wheat leaf cell walls. Pectin was detected in these cell walls immunochemically using the monoclonal antibodies JIM5 and JIM7, and biochemically by monosaccharide analysis. The Ca^{++}-chelators CDTA and imidazole extracted a pectin rich fraction from isolated cell walls which was however contaminated with significant amounts of hemicelluloses. Pretreatment of the cell walls with anhydrous hydrogen fluoride at controlled low temperatures followed by HF/ether- and water-extraction prior to imidazole-extraction of pectins yielded a purer homogalacturonan fraction. The near absence of rhamnosyl residues proved that the isolated homogalacturonan fraction was free of rhamnogalacturonans. If HF-solvolysis was performed at $-23\,^{\circ}C$, the resulting homogalacturonan had a degree of methyl esterification identical to that of the pectins in the initial wheat cell wall. The antibodies JIM5 and JIM7 as well as PAM1 and LM5 proved that the isolated homogalacturonan had a low methyl ester content, was polymeric and free of galactan side chains. We can thus isolate native homogalacturonan from the type II wheat cell walls with the original in muro pattern of methyl esterification still intact, to further investigate e.g., its degradability by plant or microbial pectic enzymes.

Keywords: HF-solvolysis; Homogalacturonan; Cell wall; Wheat; Pectin-antibodies

1. Introduction

The architecture of primary cell walls is similar in all higher plants: cellulose microfibrils are interconnected with hemicellulose molecules to form a stress bearing net embedded in an amorphous, pressure bearing matrix.[1] In the typical (type I) primary cell wall of dicotyledonous plants, the networking hemicellulose is xyloglucan, and the matrix is made up of pectins.[2] In contrast, in the (type II) primary cell walls of the commelinoid monocotyledonous plants, most of the xyloglucan and pectins are substituted by glucuronoarabinoxylan.[3] Due to its linear backbone of (1→4)-β-xylan, this hemicellulose can bind non-covalently to the surface of cellulose microfibrils—thus replacing the xyloglucan with its linear backbone of (1→4)-β-glucan. The negative charge of the glucuronic acid makes this hemicellulose hydrophilic and enables the binding of water in the matrix—thus replacing the negative charge of the galacturonic acid in pectins. However, the type II cell wall has been reported to still contain residual amounts of both xyloglucan and pectins. It might be speculated that these represent the minimum amounts of both types of polysaccharides

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necessary to build a fully functional primary cell wall. Studying the roles of xyloglucan and pectins in the type II cell wall may thus help to establish their critical roles in both types of cell wall.

One of the non-structural roles of pectins in dicot cell walls is to act as a reservoir of biologically active oligosaccharins.[4,5] Fragments of homogalacturonan (HG) with a degree of polymerisation of around 12 have been shown to act as endogenous elicitors of induced disease resistance mechanisms in many dicot plants.[6] These oligomers are not active as elicitors in monocot plants.[7,8] Instead, small HG fragments with a degree of polymerisation of around 3 have been shown to suppress elicitor induced resistance reactions in wheat.[9,10] In both cases, thus, HG fragments produced by the action of microbial pectic enzymes appear to act as signal molecules determining resistance or susceptibility of the host plant towards the microbial pathogen.

In order to investigate in molecular detail the interaction between pectic enzymes and their substrates, it is essential to devise a strategy to isolate pure and native HG from the cell walls. Classical extraction schemes usually involve the solubilization of pectins by Ca^{++}-chelators such as CDTA.[11] We have proposed the use of imidazole buffer as an alternative chelator which is more readily removed from the pectic extract upon dialysis.[12] The pectic fractions obtained using these methods are usually contaminated with considerable amounts of hemicelluloses. Purer HG fractions may be obtained if the hemicelluloses are partially hydrolysed and extracted from the cell walls prior to chelator mediated extraction of pectins.[12] Partial hydrolysis of the hemicelluloses can be achieved by solvolysis in liquid anhydrous hydrogen fluoride at controlled low temperatures. HF-solvolytic cleavage of glycosidic bonds is highly specific and is based on the different lability of glycosidic linkages in HF at defined temperatures.[13] The $(1\rightarrow4)$-α-galacturonosyl linkage of HG is more stable in HF than the glycosidic linkages of most neutral sugar residues.[13–15] HF-pretreatment at -23 °C has successfully been used to depolymerise xyloglucan and rhamnogalacturonan I (RG-I) prior to imidazole extraction of HG from type I cotton cell walls.[12] As the methyl ester of galacturonic acid is stable in HF at this temperature,[13] the isolated HG still bears the original in muro pattern of methyl esterification.

While the different pectin polymers and their roles in type I cell walls have been studied extensively, comparatively little work has been done on pectin components of the type II cell walls of the commelinoid monocots.[3,16–20] We here describe the isolation and characterisation of native HG from isolated wheat cell walls using CDTA or imidazole, with or without prior HF-solvolysis at different temperatures.

2. Results and discussion

2.1. Immunocytochemical detection of pectin in wheat cell walls

The homogalacturonan (HG) portion of the pectin in wheat leaf cell walls was visualised immunocytochemically using the anti-homogalacturonan monoclonal antibodies JIM5 and JIM7, reacting with methyl ester-poor and methyl ester-rich HG epitopes, respectively.[20] Both antibodies revealed the homogeneous presence of HG epitopes in epidermal and mesophyll cell walls throughout the wheat leaves (Fig. 1).

2.2. Biochemical detection of pectin in wheat cell walls

The major monosaccharide of pectic substances is galacturonic acid, either as a linear homopolymer (HG) rarely carrying short complex side chains (RG-II) or alternating with rhamnose in the backbone of RG-I substituted with arabinan, galactan, or arabinogalactan side chains. Monosaccharide analysis revealed that about 3% of the monomeric residues making up wheat cell wall polysaccharides were GalA, and the 'pectic monosaccharides' GalA, Rha, Ara, and Gal summed up to about 10% (Table 1). Compared to typically about 15% of GalA and about 30% of GalA/Rha/Ara/Gal in dicot cell walls,[21] these values corroborate the presence, but relatively low abundance of pectins in wheat cell walls.

2.3. Pectin extraction with CDTA and imidazole

Using CDTA or imidazole as Ca^{++}-chelators, pectin-rich fractions of almost identical monosaccharide composition were extracted from isolated wheat cell walls (Table 1). Almost half of the monosaccharide residues of these pectin-rich fractions were GalA, and about two thirds were pectic monosaccharides. As reported previously for dicot cell walls,[12] CDTA was hard to remove from the extract so that imidazole was used in all further experiments. While both methods extracted pectic material from the wheat cell walls effectively, the pectic fractions obtained were contaminated with considerable amounts (about 25%) of hemicellulosic components.

2.4. HF-solvolysis prior to imidazole extraction of pectins

In order to isolate purer pectic fractions from wheat cell walls, we attempted to remove hemicellulosic components from the isolated cell walls prior to pectin extraction. We therefore treated dry cell walls with anhydrous hydrogen fluoride (HF) at defined low temperatures to specifically cleave selected glycosidic bonds. HF-solvol-

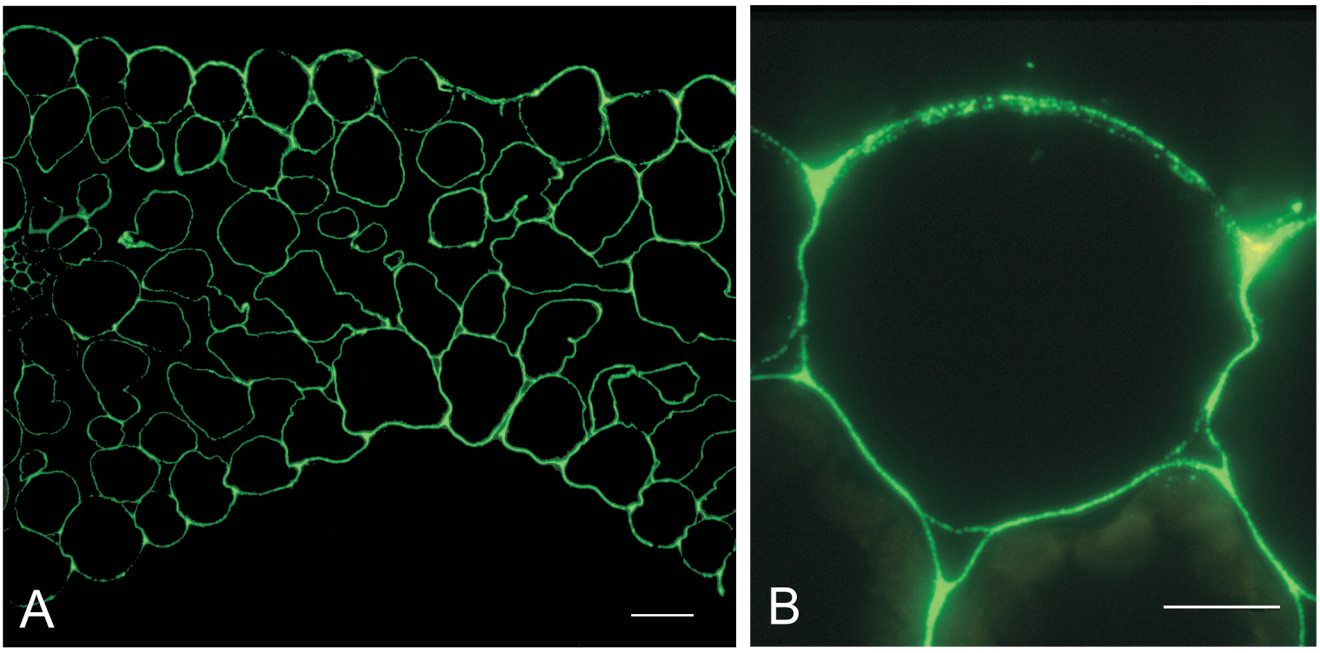

Fig. 1. Cytochemical staining of homogalacturonan by the monoclonal antibody JIM7 directed against a highly methyl esterified epitope of HG (visualised by FITC-coupled secondary anti-rat antibodies) in cross sections of primary leaves of the wheat cultivar Prelude. Similar staining was achieved when the antibody JIM5 directed against a weakly methyl esterified HG epitope was used. No staining was seen with the secondary antibody alone. Scale Bar A = 40 μm, B = 10 μm.

Table 1
Relative monosaccharide compositions of wheat leaf cell walls and of pectic fractions extracted from them using CDTA or imidazole as Ca^{++}-chelators

sample	weight	relative monosaccharide composition [weight %]							
	[mg]	GalA	Rha	Gal	Ara	GlcA	Xyl	Glc	Man
		(pectin)			(hemicellulose)			(cellulose)	(glycoprotein)
total cell wall[a]	100	3	1	2	5	1	27	61	1
imidazole extract	10[b]	47	5	9	4	6	18	11	0
CDTA extract	30[c]	45	7	7	4	4	21	12	0

Horizontal lines indicate the most likely polymeric sources of the individual monosaccharides.
[a] After 0 °C HF pretreatment.
[b] Residue after imidazole extraction: 82 mg.
[c] Residue after CDTA extraction: 83 mg.

ysis was terminated by the addition of cold anhydrous ether, and the HF/ether-insoluble residue obtained after filtration was first washed with water to remove oligomeric materials prior to imidazole extraction of pectins (Fig. 2). With increasing solvolysis temperatures, the HF/ether-soluble fraction increased from 5 to

almost 50% of the initial dry weight of the cell walls, and the water-soluble fraction doubled from 15 to almost 30%. In contrast, the dry weight of the imidazole-soluble fraction decreased slightly from 3 to about 2%. Consequently, the insoluble residue decreased from about 70% to about 20% with increasing temperature of the HF-solvolysis (68.5, 60.9, 50.4, and 21.1%, at −73, −43, −23, and 0 °C, respectively).

2.5. Degrees of polymerisation of the HF/ether-, water-, and imidazole-soluble extracts

Only glycosyl fluorides and, with rare exceptions, only monomers are to some extent soluble in HF/ether.[22] High-pH anion exchange chromatography of the HF/ether-soluble extracts followed by pulsed amperometric detection in all cases revealed the presence of the major monosaccharides also detected by GLC (see below), and very little small oligomers. In contrast, the water-soluble extracts always contained a complex mixture of oligosaccharides, in some cases dominated by a series of homooligomers (oligoxylans after −73 °C HF, oligoglucans after 0 °C HF). No monomers or oligomers were detected in the imidazole-soluble extract, indicating their polymeric nature.

2.6. Monosaccharide composition of the HF/ether-, water-, and imidazole-soluble extracts

Monosaccharide composition of all HF/ether-, water-, and imidazole-soluble extracts were analysed using gas–liquid chromatography (GLC) of the trimethylsilylated methyl glycosides (Table 2). The monosaccharide composition of all fractions closely reflect the temperature dependent cleavage of specific glycosidic bonds at the different temperatures of the HF-solvolyses. The mostly monomeric HF/ether-soluble fraction is dominated by arabinosyl-residues after solvolysis at −73 °C, and by xylosyl-residues after solvolysis at −43 °C, but its composition is more complex after the solvolyses at higher temperatures until, at 0 °C, it mainly contains about equal amounts of xylosyl- and glucosyl-residues. Similarly, the predominantly oligomeric water-soluble fraction is dominated by xylosyl-residues after solvolysis at −73 °C, and its composition becomes increasingly complex at higher temperatures until, at 0 °C, it is dominated by glucosyl-residues. In contrast, the imidazole-soluble fraction is always dominated by galacturonosyl-residues, and increasingly so at increasing temperature of the HF-solvolysis.

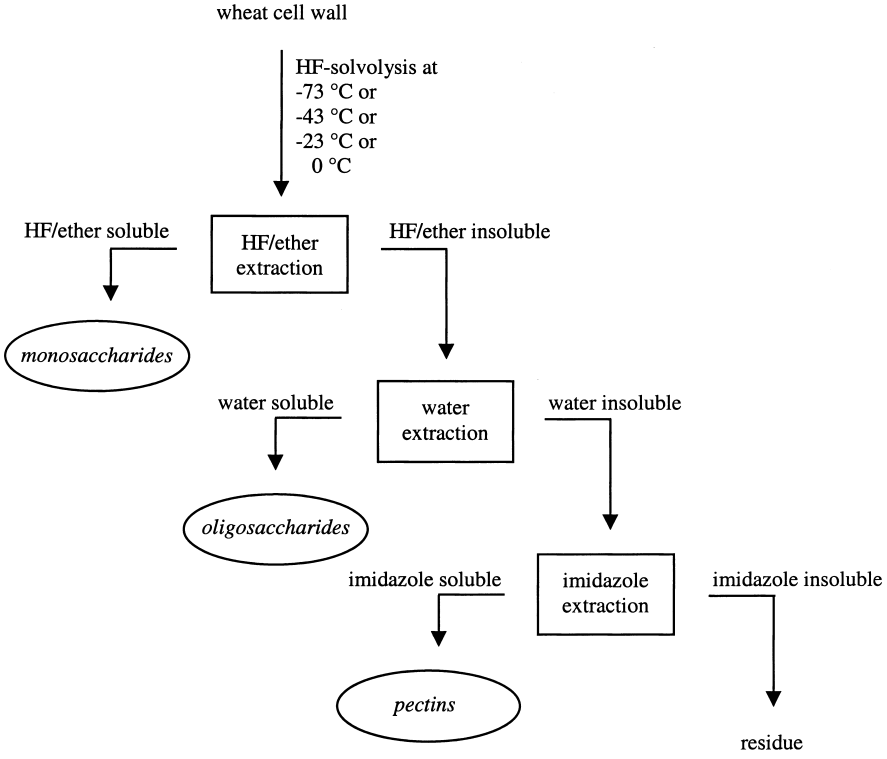

Fig. 2. Isolation of pectin by HF-solvolysis of cell walls prior to imidazole extraction, as outlined by Ref. 13. Cell walls are treated with HF at the temperatures indicated, and the reaction is stopped by the addition of ether. Most of the monosaccharides produced are soluble in the HF/ether mixture. After filtration, the HF/ether-insoluble cell wall residue is further extracted with water. Most of the oligosaccharides produced during the initial HF-solvolysis are soluble in water. The water-insoluble cell wall residue is finally extracted with imidazole to solubilise pectins.

Table 2
Relative monosaccharide composition of the different extracts of wheat leaf cell walls upon solvolysis in anhydrous hydrogen fluoride at different temperatures (see Fig. 2)

temperature of solvolysis	sample	weight	relative monosaccharide composition [weight %]							
		[mg]	GalA	Rha	Gal	Ara	GlcA	Xyl	Glc	Man
			(pectin)[a]			(hemicellulose)[a]			(cellulose)[a]	(glycoprotein)[a]
-73 °C	HF/ether extract	4.6	0	0.6	0.2	88.5	0	9.5	1.3	0.0
	H_2O extract	15.2	1.5	0.5	4.0	7.2	3.0	79.3	4.2	0.0
	imidazole extract	2.9	46.4	7.1	9.5	2.4	4.7	20.8	7.1	0.8
-43 °C	HF/ether extract	21.0	0	0	0	23.6	0	78.4	0	0.0
	H_2O extract	15.4	6.2	3.3	12.2	3.2	7.1	55.8	12.1	1.2
	imidazole extract	2.6	68.0	6.8	7.0	3.9	3.9	2.7	6.6	0.9
-23 °C	HF/ether extract	23.4	8.6	5.2	20.3	6.3	4.6	35.0	12.4	7.4
	H_2O extract	24.5	11.2	4.3	6.9	5.1	4.4	29.1	36.1	2.3
	imidazole extract	1.7	69.0	1.0	1.2	1.0	2.9	4.2	19.5	1.0
0 °C	HF/ether extract	47.6	0	0.9	2.4	6.0	1.4	42.3	44.0	0.0
	H_2O extract	27.9	2.9	0.3	2.4	2.6	0.6	8.1	82.7	0.3
	imidazole extract	1.8	79.4	0	1.2	2.2	3.0	2.2	11.6	0.0

[a] Horizontal lines indicate the most likely polymeric sources of the individual monosaccharides.

2.7. Behaviour of pectic polysaccharides during the HF-solvolyses

The different pectic polysaccharides, thus, are differently affected by the HF-solvolyses. Arabinosyl-linkages are cleaved at temperatures as low as −73 °C so that the arabinan and arabinogalactan side chains of RG-I are degraded at all temperatures,[13] and arabinosyl- and galactosyl-residues end up mostly in the HF/ether- and water-soluble fractions. The rhamnosyl-linkage starts to become labile at −43 °C, and it is quantitatively cleaved at −23 °C.[13] Consequently, the RG-I backbone is degraded at these temperatures, and the resulting oligomers can be extracted from the cell wall with water. In contrast, the galacturonosyl-linkage is completely stable at temperatures as high as −23 °C, and it is only partially cleaved even at 0 °C.[13] HG, therefore, remains in the water-insoluble residues of all HF-solvolyses, and can be extracted with imidazole. As galacturonosyl-linkages start to become labile in HF at 0 °C, and as the methyl esters of galacturonic acid also start to become labile in HF at that temperature,[15,23] the −23 °C HF-pretreatment was chosen for the isolation of polymeric, native HG from the wheat cell walls.

2.8. Immunochemical characterisation of the imidazole-soluble fraction after −23 °C HF-pretreatment

In order to further characterise the imidazole-soluble, pectin-rich fraction obtained after HF-solvolysis at −23 °C, a range of monoclonal antibodies directed against HG (JIM5, JIM7, PAM1) and (1→4)-β-galactan (LM5) was used. JIM5 recognises low ester/unesterified epitopes of HG, binding to some extent to fully de-esterified pectate and increasingly to pectins with increasing degrees of methyl esterification (DM) up to 40%; higher DMs strongly decrease binding of JIM5.[20] In contrast, JIM7 recognises a more highly methyl esterified epitope of HG, binding to pectins with DMs ranging from 15 to 80%, with an absolute requirement of at least some methyl groups for binding.[20] As illustrated in Fig. 3, both JIM5 and JIM7 bind to the isolated wheat pectin, the stronger labelling by JIM5 indicating a rather low DM. Chemical analysis of the isolated HG revealed a DM of 19%, a value identical to that of the total cell walls prior to HF-solvolysis (18%).

PAM1 has been characterised to bind to long stretches of HG, requiring a minimum of 30 contiguous

galacturonosyl-residues.[24] The binding of PAM1 to the imidazole-soluble extract from wheat cell walls after HF-solvolysis at −23 °C further underlines the presence of polymeric HG in this fraction. LM5 recognises an epitope of four galactosyl-residues as it appears in RG-I side chains, thus indirectly detecting the RG-I molecule.[25] LM5 did not bind to the isolated wheat HG fraction, underlining the efficient removal of RG-I from the HG preparation as already indicated by the near absence of rhamnosyl-residues in this fraction (Table 2).

3. Conclusion

Biochemical and immunochemical evidence support the existence of low but significant amounts of pectic substances in the type II cell walls of the commelinoid monocot wheat. In spite of the very different composition of type I and type II cell walls, the homogalacturonan fraction of both can be extracted using CDTA or, preferably, imidazole. HF-solvolysis at −23 °C followed by HF/ether- and water-extractions is an ideal pretreatment prior to imidazole extraction of homogalacturonan, yielding a homogalacturonan fraction virtually devoid of rhamnogalacturonan and hemicellulose contaminants. The HG isolated from wheat leaf cell walls is polymeric and retains its native methyl esterification with a rather low DM of around 20%. The HG isolated can now be analysed for its pattern of methyl esterification and for its enzymatic degradability e.g., by pectic isoenzymes isolated from plant pathogenic micro-organisms. The resulting oligomers can then be analysed for their biological activities, e.g., as endogenous elicitors or suppressors of induced disease resistance mechanisms.[10,26,27]

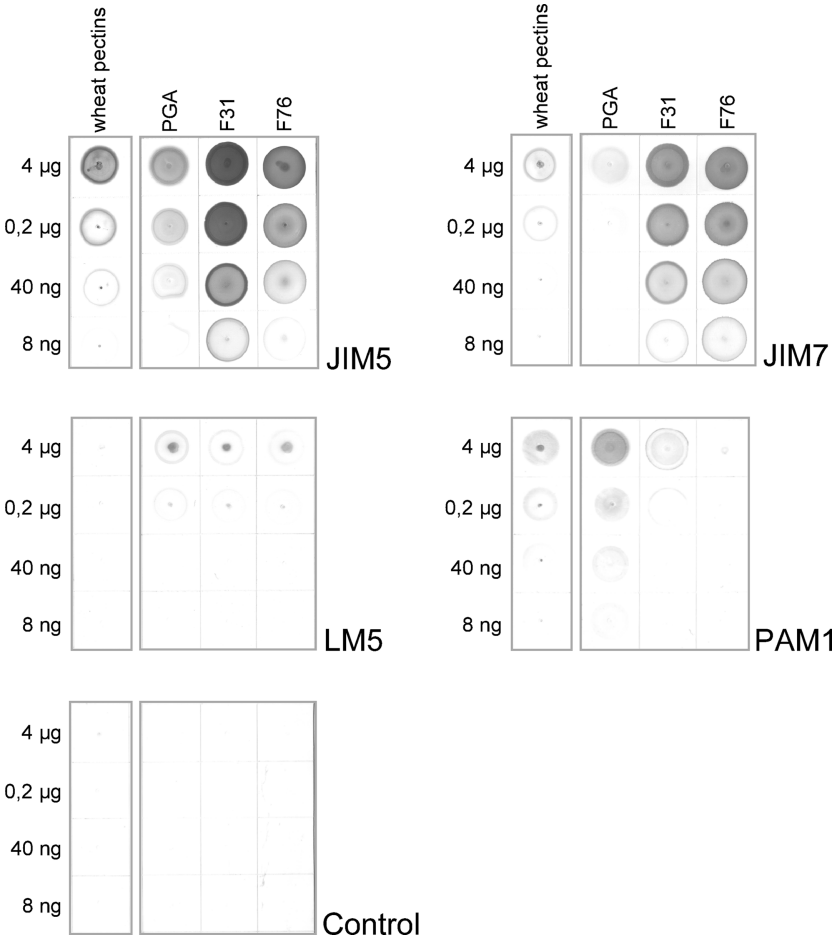

Fig. 3. Immunochemical characterization of isolated wheat leaf pectins using the anti-homogalacturonan monoclonal antibodies JIM5, JIM7, and PAM1, and the anti-(1→4)-β-galactan monoclonal antibody LM5. The pectins were extracted from wheat cell walls using imidazole, after pretreatment with HF at −23 °C. The standard pectins were generated from a highly methyl esterified lime pectin by incubation with a fungal pectin methyl esterase generating a random distribution of methyl esters. Wheat and standard pectins were applied to nitrocellulose in dilution series, and binding of the antibodies was visualised using FITC-coupled secondary anti-rat antibodies. No staining was seen with the secondary antibody alone.

4. Experimental

4.1. Plant material and chemicals

Wheat plants (*Triticum aestivum* L cv. Prelude) were grown in automatically regulated growth chambers at 20 °C with 60% relative humidity and a photoperiod of 16 h/day in pre-fertilized soil. Chemicals were purchased from Sigma (Taufkirchen, Germany) and Fluka (Taufkirchen, Germany) unless otherwise stated.

4.2. Isolation of cell walls

Cell walls were prepared from the middle section of first to fourth leaves from 5 to 7-week old wheat plants after discarding the senescing tips and the growing bases of the leaves using standard procedures.[28] Briefly, leaves were ground in liquid nitrogen, extracted sequentially with K-phosphate buffer (500 mM, pH 7.0), distilled water, 2:1 MeOH–$CHCl_3$, acetone, EtOH, and distilled water, and finally freeze-dried.

4.3. Chemical extraction of cell wall polymers

Pectins were extracted from 500 g of isolated wheat cell walls by incubation (3×6 h, 4 °C) with 300 mL of a CDTA-soln (50 mM, pH 6.5) or imidazole (500 mM, pH 7.0). Insoluble residues were removed by filtration (sintered glass funnel, pore diameter 0.45 μm) and the combined filtrates were dialyzed extensively and then lyophilized.

4.4. HF-solvolysis followed by water and imidazole extraction of insoluble residues

Solvolysis in anhyd hydrogen fluoride (HF) at low temperatures (controlled in a bath of stirred EtOH with external cooling and regulated heating) was performed in a closed Teflon line.[29] Briefly, 500 mg of dried wheat cell walls (pre-cooled to 10 °C below the reaction temperature) were dissolved in 20 mL of HF (pre-cooled to 10 °C below the reaction temperature), and the EtOH bath was quickly brought to the reaction temperature. Thirty minutes later, the reaction was stopped by immersing the reaction vessel in liquid nitrogen. Dry ice-cooled anhyd diethyl-ether (200 mL) was added to the frozen HF, and the HF/ether-mixture was stirred for 30 min after having reached room temperature (rt). The HF/ether was removed by filtration through a Teflon filter, the residue was washed twice with 200 mL of ether, then extracted with three changes of distilled water (6 h, 300 mL each). The water-soluble fraction was removed by filtration, pooled, dialyzed and freeze-dried. The water-insoluble residue was extracted three times with imidazole (0.5 M, pH 7.0) for 6 h and 300 mL each. Filtration and dialysis were carried out as described above.

4.5. Analytical methods

HF/ether-, water-, and imidazole-soluble fractions were subjected to HPLC-analysis by anion exchange chromatography at high pH with pulsed amperometric detection of carbohydrates using a Dionex Bio LC and a CarboPac PA 1 (4×250 mm) column. Mono- and disaccharides were separated isocratically using 50 mM Na-acetate in 100 mM NaOH as an eluant, the separation of oligosaccharides including oligogalacturonides required a linear salt gradient to 200 mM Na-acetate in 40 min.

Monosaccharide compositions were analyzed by capillary gas–liquid chromatography of the trimethyl-silylated methyl-glycosides.[30] Briefly, carefully dried samples (approx 100 μg) were methanolyzed (200 μL 1.5 M methanolic HCl, 50 μL methyl acetate, 16 h, 80 °C) and derivatized using a commercial silylating agent (Silyl 2110, Chromatographie Service, Langerwehe, Germany) containing hexamethyldisilizane, trimethylchlorosilane and pyridine (2:1:10). Prior to injection, samples were dried under a gentle stream of nitrogen and dissolved in 30 μL isooctane. One μL samples were split-injected (1:10, injection port temperature 230 °C) into the gas–liquid chromatograph (Hewlett-Packard model HP 5840A GC equipped with a flame ionization detector maintained at 260 °C) equipped with an OV-1 column (25 m × 0.32 mm i.d.; 0.2 μm film thickness; Macherey-Nagel, Düren, Germany). Carrier gas was nitrogen at 1 mL/min. The temperature was held at 140 °C for 4 min, then increasing at 2 °C/min to 200 °C, followed by a 6 °C/min rise to 250 °C. The degree of methyl esterification was examined according to Ref. 23.

4.6. Antibodies and standard-pectins

Production and characterisation of the anti-homogalacturonan monoclonal antibodies JIM5, JIM7, and PAM1 as well as the anti-(1→4)-β-galactan monoclonal antibody LM5 have been described elsewhere.[20,24,25,31,32] The standard pectins PGA (degree of methyl esterification DM = 1.4%, average degree of polymerisation DP = 220), F31 (av. DM = 31%, av. DP = 380) and F76 (av. DM = 76%, av. DP = 500) are described in Ref. 33 and were kindly provided by Danisco Biotechnology, Langebrogade, Denmark.

4.7. Immunolabelling of wheat pectins

Primary wheat leaves were infiltrated under reduced pressure with paraformaldehyde (4%, w/v) in 100 mM Pipes-buffer (1,4-piperazine-diethanesulfonic acid) pH 6.9 and further incubated in this soln for 1 h.[34] After washing three times with Pipes-buffer (100 mM, pH 6.9) leaves were dehydrated with an increasing gradient

of EtOH, incubated in 70% EtOH overnight, followed by final washing steps in 90%, 100% and anhyd EtOH. After dehydration, the leaf tissue was embedded in LR-white acrylic resin (London Resin Co., UK). Leaf sections (0.5 μm thick) were applied to multi-well slides (ICN Biomedicals, USA) coated with Vector bond soln (Vector Laboratories, UK). Before labelling with monoclonal anti-homogalacturonan antibodies, leaf sections were blocked with 5% MPBS (Phospate-Buffered-Saline containing 5% fat free milk powder, PBS = 0.14 M NaCl, 2.7 mM KCl, 7.8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) for 1 h. Primary antibodies (hybridoma supernatants of JIM5 and JIM7, diluted 1:10 in MPBS) were applied onto the leaf sections for 1 h and washed five times with PBS. Leaf tissue was then incubated with secondary antibody (anti-rat conjugated to fluorescein isothiocyanate, diluted 1:100 in MPBS, Sigma, UK) for 1 h prior to washing five times with PBS. All incubation steps were carried out at rt in darkness. To avoid fading of the labelling, a drop of anti-fade soln (Citifluor AF1, Agar Scientific, UK) was applied and sections were covered with a cover slide. Binding of JIM5 and JIM7 to wheat leaf material was examined on a Olympus BH-2 microscope equipped with epifluorescence (Olympus instruments, UK) and photographs were taken on colour slide films (400 ASA, Kodak). Leaf sections treated only with secondary antibody were used as controls.

4.8. Immuno-dot-blots

Pectins (1 mg/mL) were dissolved by rocking in deionized water overnight and diluted 1:5 in water. Four μL each were spotted by hand onto nitrocellulose (Schleicher und Schuell, Einbeck, Germany). The dot blots were air dried at rt overnight, and binding of anti-homogalacturonan antibodies were tested as follows: Hybridoma supernatants of the monoclonal antibodies JIM5, JIM7, and LM5 were diluted 1:5 in MPBS, PAM1 was used at a concn of 10^{11} phage/mL (1:100 dilution in MPBS of phage prepared by polyethylene glycol precipitation.[34] Membranes were incubated with the primary antibodies for 1.5 h and washed extensively with tap water on both sides. As secondary antibodies, anti-rat horse radish peroxidase conjugate (Sigma, UK) was used for JIM5, JIM7 and LM5, and anti-M13 horse radish peroxidase for PAM1 (Pharmacia, UK). Secondary antibodies were diluted 1:1000 in MPBS, and membranes were incubated for 1.5 h prior to washing them with tap water as described above. Visualization of antibody binding was carried out by adding substrate soln (25 mL deionised water, 5 mL of 4-chloro-1-naphthol (5 mg/mL) in MeOH, 30 μL 6% v/v H_2O_2). The reaction was stopped by replacing the substrate soln with tap water.

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