Carbohydrate Polymers 87 (2012) 853–861

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structural details of pectic galactan from the secondary cell walls of flax (*Linum usitatissimum* L.) phloem fibres

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ARTICLE INFO

Article history:
Received 23 May 2011
Received in revised form 19 August 2011
Accepted 24 August 2011
Available online 30 August 2011

Keywords:
Plant fibre
Secondary cell wall
Rhamnogalacturonan I
Galactan
Galactanase
Rhamnogalacturonan hydrolase

ABSTRACT

Details of the backbone and side chain structure of pectic β-(1 → 4)-galactan from the secondary cell walls of flax phloem fibres were characterised by NMR and mass spectrometry of the fragments obtained after partial hydrolysis with specific endogalactanase and rhamnogalacturonan hydrolase. The proportions of branched and linear rhamnose in the backbone of the polymer equalled 72% and 28%, respectively. Rhamnose branched with a single galactose residue comprised 47% of the total rhamnose; thus, in the bulk of the polymer backbone, rhamnose had 0–1 galactose residues. Within the backbone, residues of rhamnose branched with long galactose chains alternated with linear rhamnose and rhamnose with a single galactose. Oligomeric galactose chains averaged 14 monomers in length. Alternative glycosidic bonds of galactosyl residues were present. The established structural details of cell wall galactan are compared to those of nascent galactan before incorporation into the fibre cell wall, and galactan modifications *in muro* are discussed.

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1. Introduction

Plant cell wall polysaccharides are among the most complex carbohydrates. Their structures are characterised by a variety of details the analyses of which are important for understanding the mechanisms of the biosynthesis of these complex polymers, for the characterisation of their three-dimensional organisation, and ultimately for understanding their functional roles. Among the plant cell wall polysaccharides, there are pectic polymers grouped as rhamnogalacturonans I (RG I), which are characterised by a backbone of repeating dimers [→4)-α-D-Gal*A*p-(1 → 2)-α-L-Rha*p*(1→] in the backbone. Side chains, consisting of neutral sugars (usually individual, linear, or branched α-L-Ara*f* and β-D-Gal*p* residues) are often added to the rhamnose residues; the details of the structure are highly variable in polymers from different sources (Duan, Wang, Dong, Fang, & Li, 2003; Huisman et al., 2001; Schols, Mutter, Voragen, Niessen, & van der Hoeven, 1994).

Rhamnogalacturonans I are generally considered to be the primary cell wall polymers that are formed in growing plant cells (Goubet et al., 1995; McNeil, Darvill, & Albersheim, 1980; Ridley, O'Neill, & Mohnen, 2001). However, the presence of such polymers in the special, gelatinous-type secondary cell walls that are characteristic of many plant fibres (Gorshkova et al., 2010) was demonstrated recently (Girault et al., 1997; Gorshkova & Morvan, 2006; Gurjanov, Ibragimova, Gnezdilov, & Gorshkova, 2008). Rhamnogalacturonan I (eluting in the 100–400-kDa region) was isolated from the secondary cell walls of flax phloem fibres, where the polymer is effectively retained by cellulose microfibrils and cannot be extracted by either ammonium oxalate, commonly used to extract pectin, or alkali (4 M). A special protocol was developed to obtain cell wall rhamnogalacturonan I in its polymeric form, and RG I was characterised as having side chains built mainly of β-(1 → 4)-Gal with a Gal/Rha ratio of approximately 6.4 (Gurjanov et al., 2008).

Flax fibres, while forming secondary cell walls, have a peculiar mechanism of polysaccharide secretion. Golgi-derived vesicles first accumulate in the cytoplasm and only later fuse with the plasma membrane to give their contents to the apoplast (Salnikov, Ageeva, & Gorshkova, 2008). Such a mechanism allows the acquisition of a sufficient amount of the nascent rhamnogalacturonan I before it is incorporated into the cell wall; the polymer can be collected from tissue homogenisation buffer. This polysaccharide (eluting in the 2000-kDa region) was characterised as a very complex RG I with a high degree of branching and a varying structure of side chains, which are mainly built of β-(1 → 4)-Gal and can include: (1) only one or two Gal residues; (2) at least 26 Gal residues; (3) likely branched side chains from at least 3 to 12 Gal residues, which are not cleaved by galactanase; or (4) at least 17 Gal residues,

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doi:10.1016/j.carbpol.2011.08.068

decorated with a single pentose, most likely Ara (Gurjanov, Gorshkova, Kabel, Schols, & van Dam, 2007). The predominant monosaccharide in the polymer is galactose, which determines the polymer name as a galactan (Gorshkova et al., 1996).

Investigation of fibre cell wall formation in pulse-chase experiments (Gorshkova et al., 2010), together with data from polysaccharide composition and structure analyses (Gorshkova et al., 1996; Gurjanov et al., 2007, 2008), permit us to consider the fibre cell wall galactan as a derivative of the nascent galactan. This gives us a rare opportunity to compare the structure of galactan before and after its incorporation into the plant cell wall. The key suggested role of this polysaccharide in the realisation of a functional role for the gelatinous fibre (Gorshkova et al., 2010) makes the characterisation of the structural details of the polymer particularly relevant. The purpose of our work was to characterise the cell wall galactan structural details, including the nuances of long side chain structures and the elements of the backbone organisation, and to give insights into the mode of the *in muro* modification of the nascent polymer.

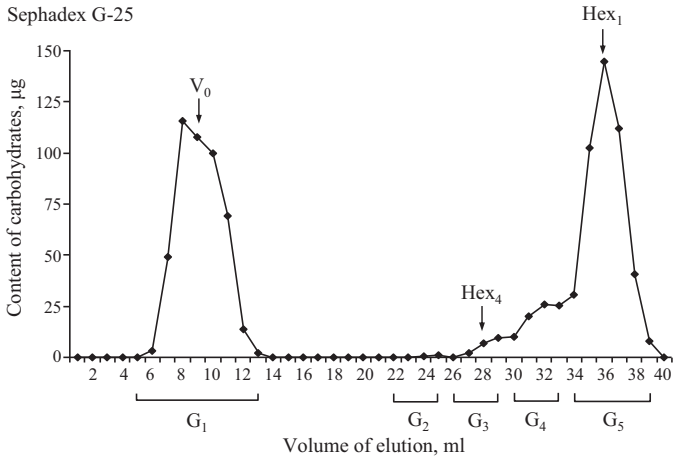


Fig. 1. Fractionation on a Sephadex G-25 column of the galactan fragments obtained after hydrolysis with galactanase. Maltotetraose (Hex_4) and galactose (Hex_1) were used as molecular weight markers.

2. Materials and methods

2.1. Plant material

Flax plants (*Linum usitatissimum* L. cv. Mogilevski) from the collection of the All-Russian Flax Research Institute (Torzhok, Russia) were grown until maturity (100 days after sowing) in boxes with a 50-cm layer of soil in the open air under natural light and with daily watering. Dried, non-retted flax stems were used for manual isolation of bast fibre bundles (Gurjanov et al., 2008).

2.2. Isolation of fibre cell wall galactan

Isolation of fibre cell wall galactan was performed according to the procedure developed by Gurjanov et al. (2008). The residue that remained after sequential treatments of the cell walls with 1% ammonium oxalate and 4 N KOH was dissolved in 8% LiCl in N,N-dimethylacetamide. Cellulose was precipitated by water and completely degraded by cellulase (Cellusoft-L; Novo Nordisk Bioindustrie S.A., Paris, France; 750 EGU/G). Matrix polysaccharides, which remained in their polymeric forms, were purified from the low molecular weight products and salts on a Sephadex G-25 column.

2.3. Hydrolysis of galactan by endo-β-1 → 4-galactanase and rhamnogalacturonan hydrolase

Highly purified and well characterised endo-β-1 → 4-galactanase (G) from *Aspergillus aculeatus* (Novo Nordisk, A/S, Copenhagen, Denmark) (Van de Vis, Searle-van Leeuwen, Siliha, Kormelink, & Voragen, 1991) and rhamnogalacturonan hydrolase (RGH) from *A. aculeatus* (Novo Nordisk, A/S, Copenhagen, Denmark) (Mutter, Colquhoun, et al., 1998; Schols, Geraeds, & Searle-Van Leeuwen, 1990; Schols, Voragen, & Colquhoun, 1994) were kindly provided by Prof. H. Schols (Wageningen University, The Netherlands). To remove salts and low molecular weight substances, enzymes were passed through a Sephadex G-25 column (Bio-Rad, USA). Elution was performed with 0.01 M NaOAc, pH 5.0. Protein was quantified by the Bradford method (Bradford, 1976).

A saponification step was performed for removing methyl ester and acetyl groups before the incubation of polymer with enzymes. The galactan sample (5 mg) was dissolved in 100 μl water and was saponified by adding 100 μl of 0.06 M NaOH. The solution was kept for 3 h at 4 °C and neutralised with 0.2 ml of 0.03 M acetic acid. The volume was increased to 1 ml with 0.6 ml of 0.03 M NaOAc, pH 5.0, and the enzyme was then added (25 μg of protein per 5 mg of substrate). Incubation was performed at 40 °C for 48 h in an incubator shaker-thermostat IS-971R (Jeio Tech, Korea) at 120 rpm. The enzyme was inactivated at 100 °C for 5 min.

Products of the galactanase-mediated galactan hydrolysis were desalted twice on a Dowex 50w × 8 (50–100 mesh) (Sigma, USA) column (1 ml) with the addition of pyridine (10–20 μl). The column was preliminary washed sequentially with 0.5 M NaOH (25–30 ml), water to neutral pH, 1 M HCl (15–20 ml), water (20–30 ml), 1 M acetic acid (15–20 ml) and water (15–20 ml). The desalted galactan fragments were subjected to chromatography on a Sephadex G-25 column (1.2 cm × 100 cm, BioRad, USA) with volatile buffer (0.01 M pyridine/acetic acid solution, pH 4.5, at a flow rate of 0.15 ml/min, 1.0-ml fractions). The sugar content in each fraction was measured by phenol–sulphuric acid assay (Dubois, Gilles, & Hamilton, 1956). One-millilitre fractions were combined according to the peaks into five fractions (G_1 – 5–13, G_2 – 22–25, G_3 – 26–29, G_4 – 30–33, G_5 – 34–39 tubes, Fig. 1).

The high molecular weight fraction, which eluted on a Sephadex G-25 column in the void volume (Fig. 1, G_1), was treated with RGH (25 μg of protein per 5 mg of substrate). Desalting and concentration of the hydrolysis products were performed similarly to the experiments with galactanase. The obtained fragments were subjected to chromatography on a Sephadex G-50 column (2.0 cm × 80 cm, BioRad, USA) with 0.01 M pyridine/acetic acid solution at a flow rate of 0.5 ml/min, with a 1.9-ml fraction volume. The sugar content was measured by phenol–sulphuric acid assay (Dubois et al., 1956).

2.4. Monosaccharide analysis

The samples were hydrolysed with 2 M TFA (Sigma, USA) at 120 °C for 1 h and dried in a stream of air at 60 °C. Monosaccharide analysis was performed using high performance anion-exchange chromatography (HPAEC) on a CarboPac PA-1 column (4 mm × 250 mm, Dionex, USA) using pulse-amperometric detection (PAD, Dionex, USA). Buffers included A (0.015 M NaOH) and B (1 M NaOAc in 0.1 M NaOH). The column was equilibrated with 100% eluent A. The sample was eluted with the following linear gradient: 0–20 min A – 100%; 20–21 min A – 90%, B – 10%; and 21–31 min A – 70%, B – 30%; the flow rate was 1 ml/min at 30 °C. Monosaccharide standards were treated with 2 M TFA at 120 °C for 1 h before they were used for calibration. Mannitol was used as the internal standard.

Table 1
Monosaccharide composition (mol%) of galactan and its fragments obtained after hydrolysis by galactanase,[a] and API ESI assignment of corresponding fragments.

Fractions	Monosaccharide composition (mol%)					Fraction proportion (%)	Fragments composition according to mass spectrum
	Rha	Ara	Gal	Glc	GalA		
Initial polymer	17	3	64	2	15		
G_1	25	5	41	2	27	45	
G_2	Trace	11	87	1	Trace	1	Hex_{2-5}; $Hex_{2-4}Pen$; $Hex_{5-8}+18$; $Hex_{5-6}Pen+18$
G_3	Trace	8	91	Trace	Trace	4	Hex_{2-4}; $Hex_{2-3}Pen$; $Hex_{4-6}+18$; $Hex_{5-6}Pen+18$
G_4	Trace	3	95	1	Trace	10	Hex_{2-4}; Hex_2Pen; $Hex_{4-6}+18$; $Hex_3Pen+18$
G_5	–	–	99	Trace	–	40	Hex_{1-2}

[a] Designation of fractions in Fig. 1.

2.5. NMR spectroscopy

Samples – fraction G1 (3.5 mg), which was obtained after hydrolysis by galactanase, and galactan (20 mg) – were dissolved in D_2O (99.996%). NMR spectra were recorded on a Bruker Avance III 600 MHz (Bruker, Germany) at 303 K. Two-dimensional homonuclear COSY and NOESY experiments were recorded and processed using standard Bruker protocols.

2.6. Mass spectrometry

Low molecular weight fragments obtained after the treatment of galactan by galactanase (G_2–G_4, Fig. 1) and the low molecular weight fraction obtained after RGH hydrolysis (26–43 tubes, Fig. 6) of the high molecular weight fraction (G_1) after galactanase treatment were dried in a stream of air at 60 °C and used for analysis by mass spectrometry.

Galactanase-treated fractions were dissolved in water and purified on a Dowex 1 × 8 (100–200 mesh) column (1 ml) in the Ac-form. The resin was prepared with several column volume washes as follows: 1 M NaOH; water; 1 M acetic acid; water; and a small amount of methanol/water solution (50:50, v/v).

The low molecular weight fraction following RGH treatment of G_1 was purified without pretreatment on a Dowex 1 × 8 column.

The samples (10^{-6}–10^{-4} M) were dissolved in water and were prepared for mass spectrometry with the addition of an equal volume of methanol. They were then injected into a Bruker Daltonics MicrOTOF Q mass spectrometer (Bruker, Germany) by electrospray at atmospheric pressure (API-ESI). Detection was set in a negative mode, with a rate of injection of 3 μl/min. Collision energy in MS/MS analysis was from −5 to −20 eV.

3. Results

Three main approaches were used to study the structure of the galactan: (1) various types of NMR spectroscopy to analyse the whole polysaccharide and its backbone that remained after treatment with galactanase; (2) mass spectrometry of low molecular weight fragments obtained after hydrolysis of side chains by galactanase; and (3) mass spectrometry of low molecular weight fragments obtained after hydrolysis of the backbone by RGH.

3.1. Characterisation of hydrolysates obtained after treatment of galactan by galactanase

Products of galactan hydrolysis were subjected to chromatography on a Sephadex G-25 column (Fig. 1). Fraction G_1 eluted in the void volume and contained the fragments with molecular masses higher than 5000 Da. Galactose and digalactose, representing the end products of galactanase treatment (Van de Vis et al., 1991), eluted in fraction G_5. The neutral oligosaccharides with a small degree of polymerisation were concentrated in fractions G_2–G_4. The monosaccharide compositions of the oligosaccharides in these fractions were not significantly different: galactose prevailed in all fractions, but its proportions decreased significantly with the increasing molecular masses of the fragments, while the proportions of arabinose increased (Table 1).

All of the rhamnose and galacturonic acid were concentrated in fraction G_1. Thus, fraction G_1 contained the rhamnogalacturonan backbone of galactan. Galactanase treatment did not remove all the galactose from the polymer backbone; the Gal percentage in fraction G_1 was approximately 40% (Table 1). Arabinose was present in minor amounts in fraction G_1. This monosaccharide was also found in the low molecular weight fractions (G_2 and G_3); thus, before enzymatic treatment, some arabinose was within the structures of the β-(1 → 4)-galactose chains, which were removed *in vitro* by galactanase.

3.2. Evaluation of backbone branching degree, side chain length and composition by NMR spectroscopy

NMR spectra of the flax cell wall galactan confirmed that the polymer is built as a rhamnogalacturonan I (Gurjanov et al., 2008) and allowed the description of some details of its structure (Table 2). We used a one-dimensional proton NMR and two-dimensional COSY (COrrelation SpectroscopY) and NOESY (Nuclear Overhauser Effect SpectroscopY).

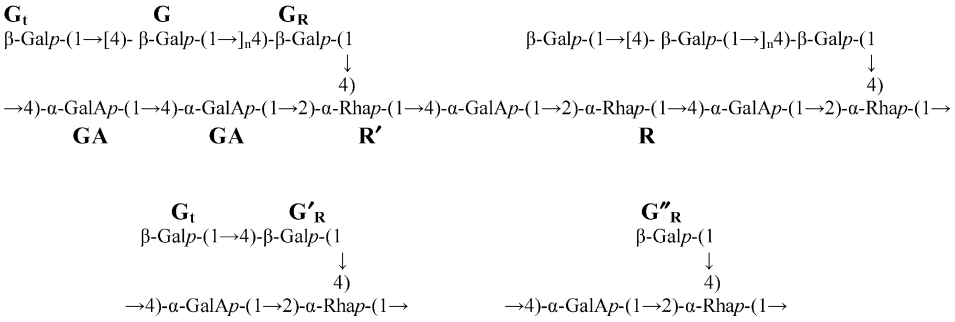
From the ratio of H-6,6′ signal intensity of rhamnose residues on the one-dimensional proton spectrum of galactan, it followed that the proportions of branched (2,4-Rha, the protons at C-6, 1.31 ppm) and linear rhamnose (2-Rha, the protons at C-6, 1.24 ppm) were, respectively, 72% and 28%. A similar ratio of branched and linear rhamnose was observed in the G_1 fraction.

The signal at 3.53 ppm correlated with H-1 galactose on the two-dimensional correlation spectrum (COSY) of galactan (Fig. 2a); therefore, it belonged to H-2 galactose residues, with chemical shifts of H-3 at 3.66 ppm (Table 2). On a similar spectrum of the galactan fragment obtained after the removal of the bulk of the galactose by galactanase (fraction G_1, Fig. 1), the chemical shift of H-4 for this molecular system was clearly seen at 3.90 ppm (Fig. 2b), thus identifying the residue as a terminal galactose. Judging by the NOESY spectrum (Fig. 3), the H-1 (4.64 ppm), H-4 (3.90 ppm) and H-2 (3.53 ppm) of this residue correlated with the methyl protons of the branched rhamnose; hence, this residue corresponded to a terminal galactose linked to rhamnose (Fig. 3).

The proportion of rhamnose branched with terminal galactose from the total branched rhamnose residues could be estimated from the ratio of the signal intensities at 3.53 ppm and 1.31 ppm on the ^1H spectrum of the polysaccharide. The COSY spectrum showed that the signal intensity in the area of 3.53 ppm was dependent on two molecular systems, one of which included the H-2 of the terminal galactose; the other correlated with a chemical shift of 4.44 ppm and was not related to galactose (Fig. 2 a). Considering the intensities of these signals, we can assume that at least 2/3 of the signal intensity of 3.53 ppm originated from the H-2 of the terminal galactose. Based on the comparison of this value with the intensity of

Table 2
Chemical shifts of ^{13}C and ^{1}H (*italics*) in the carbohydrate residues of the galactan and fraction G_1 obtained after the galactan hydrolysis with galactanase. Designation of the residues are given on the schemes below the table.

Residue		C-1 *H-1*	C-2 *H-2*	C-3 *H-3*	C-4 *H-4*	C-5 *H-5*	C-6 *H-6,6′*
→4)-α-GalA*p*-(1→2	**GA**	99.4, 99.7 *5.03, 5.05*	69.6 *3.91*	71.6 *4.11*	79.0 *4.43*	72.4 *4.85*	175.0
→4)-α-GalA*p*-(1→4	**GA**	100.8, 100.9 *5.11, 5.09*	70.0 *4.02*	71.0 *4.13*	79.2 *4.44*	72.4 *4.85*	175.0
→2)-α-Rha*p*-(1→	**R**	100.6 *5.23*	78.2 *4.11*	70.2 *3.87*	73.8 *3.41*	71.0 *3.70*	18.3 *1.24*
↓ 4)		100.6	78.6	71.1	82.2	69.4	18.5
→2)-α-Rha*p*-(1→	**R′**	*5.23*	*4.12*	*4.07*	*3.68*	*3.78*	*1.31*
→4)-β-Gal*p*-(1→	**G**	106.1 *4.62*	73.6 *3.68*	75.1 *3.76*	79.4 *4.16*	76.2 *3.70*	62.6 *3.82, 3.79*
β-Gal*p*-(1→	$\mathbf{G_t}$	106.1 *4.59*	73.2 *3.60*	74.1 *3.65*	70.4 *3.91*	76.9 *3.67*	62.3 *3.80, 3.74*
→4)-β-Gal*p*-(1→	$\mathbf{G_R}$	105.2 *4.60*	73.5 *3.52*	75.1 *3.78*	79.4 *4.16*	76.2 *3.70*	62.6 *3.82, 3.79*
→4)-β-Gal*p*-(1→	$\mathbf{G'_R}$	105.9 *4.64*	72.8 *3.60*	74.9 *3.77*	78.0 *4.12*	75.9 *3.67*	62.6 *3.80, 3.75*
β-Gal*p*-(1→	$\mathbf{G''_R}$	104.9 *4.59*	72.8 *3.53*	74.2 *3.66*	70.2 *3.90*	76.8 *3.68*	62.3 *3.80, 3.73*



the three protons of the methyl group, the proportion of rhamnose branched with a single galactose residue from all branched rhamnose residues is 91%; from total backbone rhamnose, it is 66%.

This value may be somewhat overestimated, as the specific intensity (per 1 mol) of the galactose signals was higher than that of monosaccharides of the backbone. This comes, for example, from comparing the Gal/Rha ratio obtained by monosaccharide analysis (64/17 = 3.8, Table 1) and the ratio of integral values for the H-1 of galactose (4.60 ppm) and the H-1 of rhamnose (5.23 ppm) on the one-dimensional proton spectrum (5.37/1.00). This indicates that the backbone signals were underestimated 1.4 times (5.37/3.8) compared to the side chains. After appropriate correction, it followed that rhamnose branched with terminal galactose equalled 47% of the total backbone rhamnose. During the analysis of the G_1 fraction spectra, in which considerable part of the side chains were removed and in which there were no differences between the molar ratio of galactose to rhamnose and the ratio of the NMR integral values for the H-1 of galactose and the H-1 of rhamnose, similar calculations returned the same value (47%). In summary, the proportion of rhamnose, which includes linear rhamnose and rhamnose branched with a terminal galactose, was 75% (28 + 47%, respectively); that is, the bulk of the backbone had no side chains longer than a single galactose residue. The proportions of rhamnose with a terminal galactose and galactose attached as a single residue accounted for 8% each (17 × 0.47) of all monosaccharides in the polymer.

Based on the above data and the overall ratios of galactose and rhamnose in the polymer, we calculated the average length of the oligomeric galactose chains. The proportion of rhamnose to which they were attached in a molar calculation equalled approximately 4% of all monosaccharides (17 × 0.25); the proportion of attached side chain galactose equalled 56% (64–8%). Consequently, the average length of the oligomeric galactose chains was approximately 14 monomers (56%/4%).

In addition to the main signals on the COSY spectrum of the galactan, many low-intensity signals, which could originate from the H-2 of galactose with minor types of glycosidic linkages (3-Gal, 6-Gal, 2,4-Gal, 3,4-Gal), were observed in the correlations of H-1 and H-2 galactose (Fig. 2a). For example, the signals at 3.7–3.8 ppm did not belong to the H-2 of 1 → 4-linked galactose, but could be attributed to the H-2 of 1 → 3-Gal (Ovodov, 1998) or 1 → 2-Gal. Galactanase removed galactose with minor types of glycosidic linkages; only four major signals could be traced on the COSY spectrum of the G_1 fraction (Fig. 2b). Thus, NMR spectroscopy suggested the presence of minor types of glycosidic linkages of galactose in the side chains of the galactan.

3.3. Mass-spectrometry analysis of low molecular weight fragments obtained after hydrolysis of side chains by galactanase

Mass spectra of G_2–G_4-fraction neutral oligosaccharides (Fig. 4a) contained intense signals (*m*/*z* 341, 503, 665 and 827), which were produced by deprotonated Hex_{2-5} (Table 1). These signals were accompanied by less intense peaks of deprotonated $Hex_{2-4}Pen_1$ (*m*/*z* 473, 635 and 797) and adducts of hexoses with chlorine, sulphate and acetate ions. Galactose prevailed in the

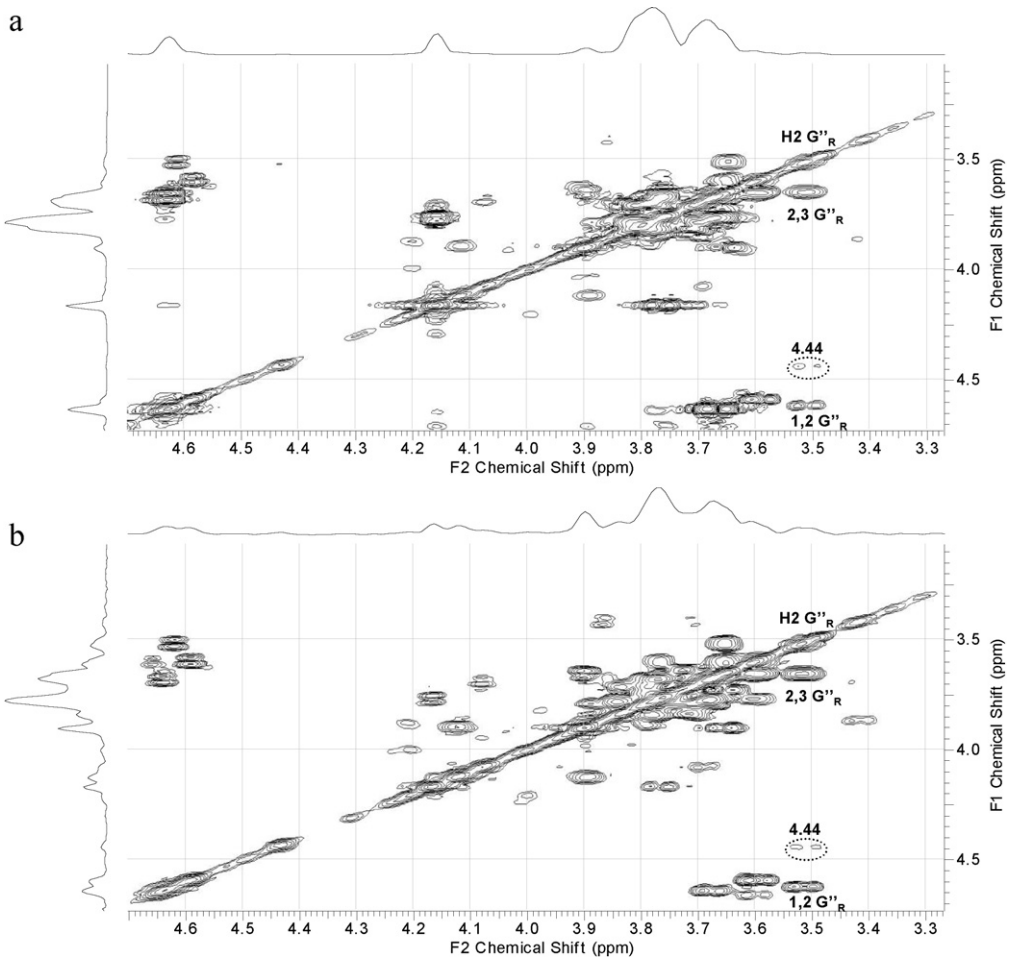


Fig. 2. COSY spectra fragments of galactan (a) and fraction G_1, obtained after hydrolysis of galactan by galactanase (b).

compositions of the G_2–G_4 fractions; arabinose was the only pentose present (Table 1). Thus, analysed hydrolysates contained small oligogalactans (DP 2-8), some of which contained one arabinose. A series of non-intensive signals, the *m*/*z* of which were lower than of those from Hex_{2-5} by 18, 60, 78, 90 and 120 amu (Fig. 4a), were identified as degradation products of oligosaccharides by carbohydrate ring fragmentation; the formation of these signals indicated low stabilities of $[Hex_n–H]^{1-}$ and $[Hex_nPen–H]^{1-}$ ions. Series of molecular ions $[Hex_{4-8}–H+18]^{1-}$ (*m*/*z* 683, 845, 1007, 1169 and 1331) and $[Hex_{3-6}Pen_1–H+18]^{1-}$ (*m*/*z* 653, 815, 977 and 1139), differing from hexoses by only 18 amu (H_2O), were observed for fragments containing more than four monomers (Fig. 4a).

Intense molecular ions (*m*/*z* 341, 503, 665, 653 and 473) were analysed using tandem mass spectrometry (MS/MS) to confirm the compositions, the carbohydrate sequences, and the types of glycosidic bonds of the fragments (Fig. 4b). The formation of

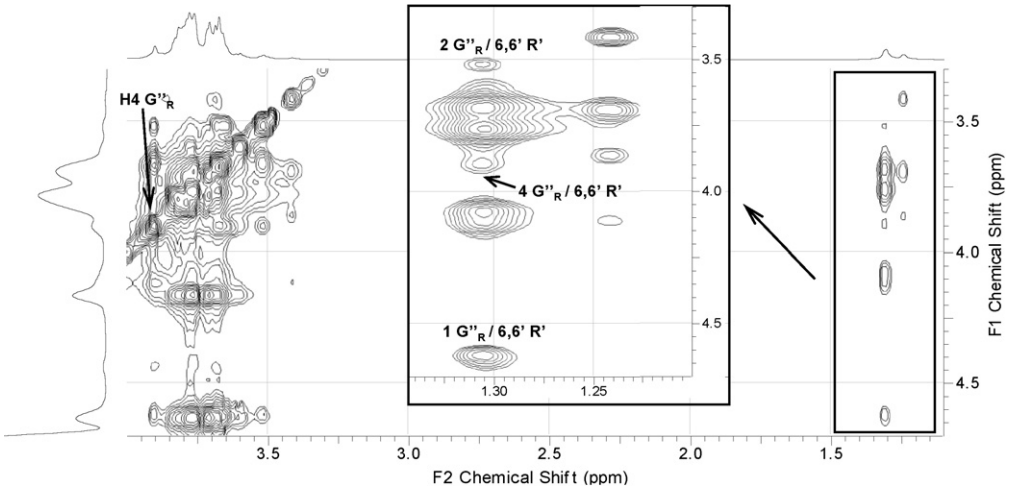


Fig. 3. The fragment of the NOESY spectrum of galactan.

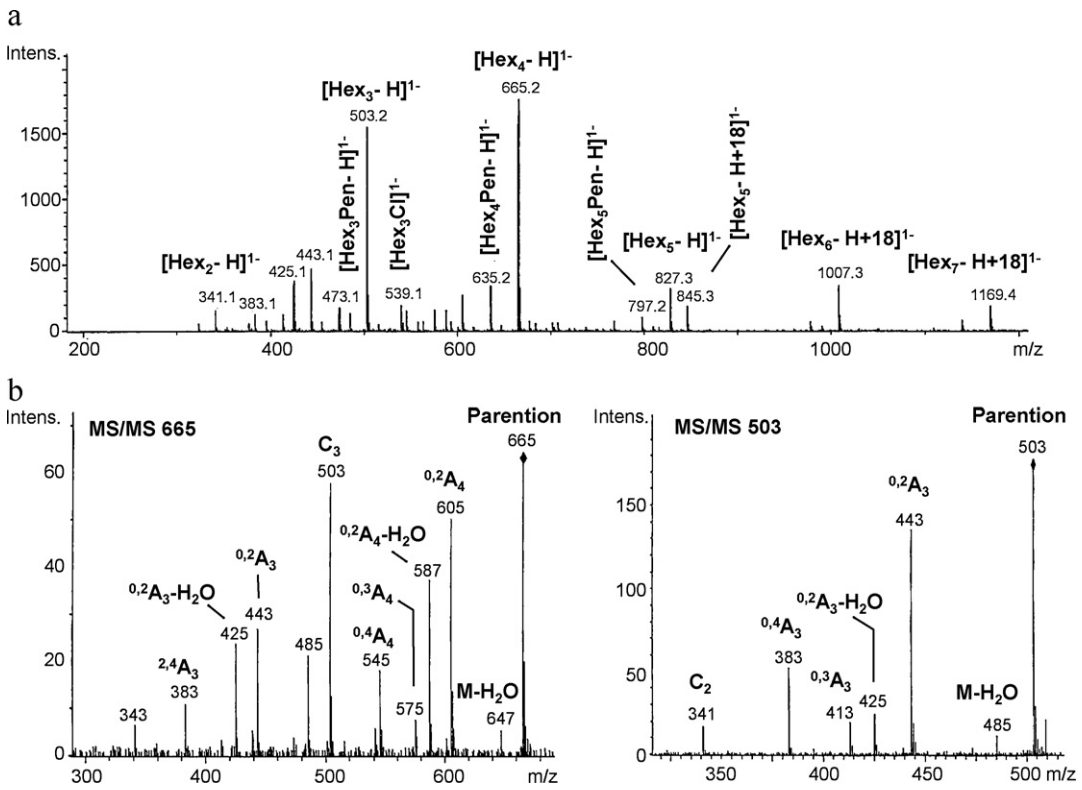


Fig. 4. The mass spectrum of low molecular weight fragments (fraction G_2), obtained after hydrolysis of galactan by galactanase (a). MS/MS spectra of ions 503^{1-} and 665^{1-} from low molecular weight fragments obtained after hydrolysis of galactan by galactanase (b).

fragments that differed from the initial ion by 162 and 132 amu (indicating the decoupling of dehydrohexose and dehydropentose) confirmed the compositions of the molecular ions as $[Hex_n–H]^{1-}$ and $[Hex_nPen_1–H]^{1-}$.

Fragments resulting from the carbohydrate ring fragmentation contained information about the types of glycosidic linkage. It is known that fragmentation of the 1 → 4 glycosidic bond removes neutral fragments of 60 and 78 amu; fragmentation of a 1 → 2 glycosidic bond – 18, 78, 120 amu; and fragmentation of 1 → 6 glycosidic bond – 60, 90 and 120 amu; fragments containing 1 → 3 glycosidic linkages barely break (Carroll, Ngoka, Beggs, & Lebrilla, 1993). At the fragmentation of a molecular ion with *m*/*z* 665 (fraction G_2), the decay of the carbohydrate ring from the reducing end led to the formation of ions with *m*/*z* 605 (665–60) and 587 (665–78) amu, characterising a 1 → 4 glycosidic bond. Ions with *m*/*z* 575 (665–90) and 545 (665–120) amu indicated a 1 → 6 glycosidic bond, and ions with *m*/*z* 647 (665–18), 587 and 545 amu indicated a 1 → 2 glycosidic bond. Series of ions observed during fragmentation of molecular ion *m*/*z* 665 (−60, −120, −90, −162, −18) suggested that a 1 → 6 glycosidic bond from the reducing end was the most likely (Spengler, Dolce, & Cotter, 1990). Upon fragmentation of the following carbohydrate ring, ions of *m*/*z* 443 (503–60) and 425 (503–78) amu characterised a 1 → 4 glycosidic bond, and *m*/*z* 485 (503–18), 425 and 383 (503–120) amu indicated the presence of a 1 → 2 glycosidic bond.

Upon fragmentation of a molecular ion with *m*/*z* 503 (fraction G_5), ions with *m*/*z* 443 (503–60), 413 (503–90) and 383 (503–120) amu characterised a 1 → 6 glycosidic bond. Fragments with *m*/*z* 443 and 425 (503–78) amu suggested a 1 → 4 glycosidic bond, and ions with *m*/*z* 485 together with ions with *m*/*z* 425 and 383 amu suggested a 1 → 2 glycosidic bond (Carroll et al., 1993).

The simultaneous presences of fragments characteristic for several types of glycosidic linkages is most likely due to a mixture of neutral oligosaccharides with different types of glycosidic bonds in the G_2–G_4 fractions. Thus, in addition to a 1 → 4-Gal glycosidic linkage, the galactan contained minor amounts of other glycosidic linkages: 1 → 2-Gal, 1 → 6-Gal and Ara–Gal.

3.4. Analysis of low molecular weight fragments obtained after hydrolysis of the backbone by RGH

RGH degraded fibre cell wall galactan mainly to polymeric fragments eluting in the inclusion volume of the Sepharose CL-4B column (molecular mass above 30 kDa) (Fig. 5a). Additional sequential treatment of the sample with galactanase resulted in decreases in molecular mass; however, a considerable portion of fragments still eluted in the inclusion volume of the column. When the enzymatic treatment was performed in the opposite sequence, so that the polymer was first digested with galactanase and then by RGH, the elution profile of the obtained fragments on the Sepharose CL-4B column did not change significantly, indicating that long galactose chains were not the only obstacle for hydrolysis of the initial polymer with RGH (Fig. 5a).

To obtain low molecular weight fragments of the polymer backbone for mass spectrometry, fraction G_1, collected after the removal of considerable part of the side chains by galactanase (Fig. 1), was hydrolysed by RGH (Fig. 5b). The Gal-Rha linkage cannot be cleaved by galactanase; this was confirmed by the similar ratios of signals from branched and unbranched Rha in one-dimensional proton NMR spectra of the initial polymer and the G_1 fraction – 72% and 28%, respectively. Galactanase was also not able to cleave galactose dimers (Van de Vis et al., 1991); thus, the second galactose in the branch also remained attached to the backbone. The hydrolysis of fraction G_1 by RGH was not complete: some fragments still eluted in the void volume on the Sephadex G-50 column (molecular mass 10 kDa and above), but approximately half of the sugars eluted as low molecular weight fragments (Fig. 5b, tubes 28–43).

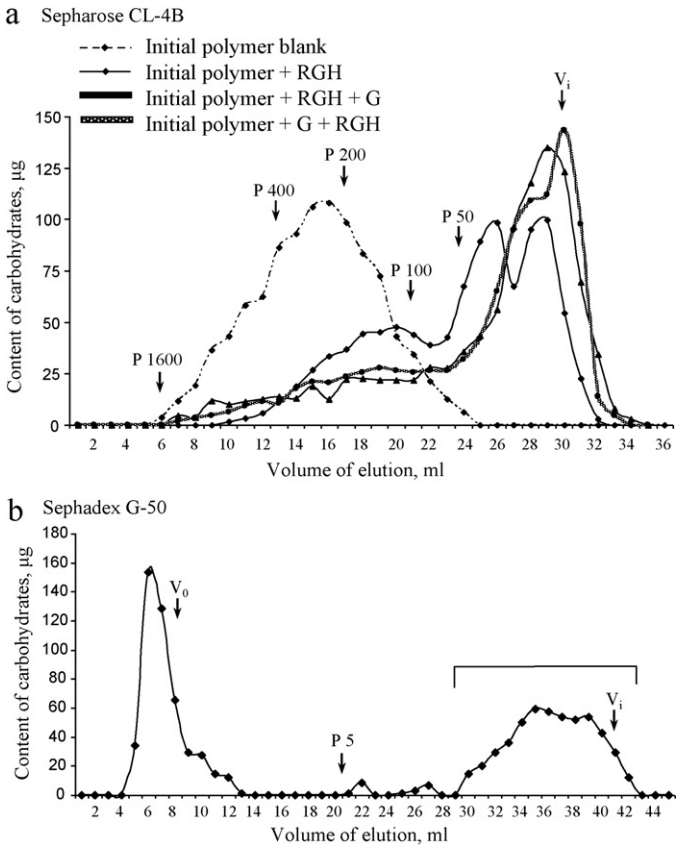


Fig. 5. Fractionation of fragments after hydrolysis with rhamnogalacturonan hydrolase of galactan (a) and of fraction G_1 obtained after treatment with galactanase (b). (a) Sepharose CL-4B column; a different treatment sequence with galactanase and rhamnogalacturonan hydrolase was used. (b) Sephadex G-50 column. The fraction collected for mass spectrometric analysis is designated by a bracket in the low molecular weight area.

The molecular weights and compositions of the low molecular weight fragments were assessed by mass spectrometry (API-ESI MS), with detection in the negative mode (Fig. 6). A series of fragments – GalA-Rha (m/z 339), $(GalA\text{-}Rha)_2Gal_{1-3}$ (m/z 823, 985, 1147 and a doubly charged ion with m/z 492) and $(GalA\text{-}Rha)_3Gal_{2-3}$ (doubly charged ions with m/z 653 and 734) with proportional ratios of GalA and Rha – were detected. The ions $GalA_2Rha_1Gal_{0-1}$ (m/z 515 and 677) and $GalA_1Rha_2Gal_2$ (m/z 809) contained galacturonic acid and rhamnose in disproportionate amounts. It is likely that the enzyme used performed a partial hydrolysis of the glycoside bond -α-L-Rha*p*(1 → 4)-α-D-GalA*p*-, as was observed in the work of Macquet et al. (2007). The fragments $GalA_2Rha_1Gal_{0-1}$ and $GalA_1Rha_2Gal_2$ together result in a normal fragment $(GalA\text{-}Rha)_3Gal_{2-3}$. The fragments $(GalA\text{-}Rha)_2Gal_1$ (m/z 823) and $(GalA\text{-}Rha)_3Gal_2$ (a doubly charged ion with m/z 653) (Fig. 6) indicated that within the backbone of the initial polymer, there were fragments with unbranched and branched rhamnose with terminal galactose residues located side-by-side. The highest m/z of the doubly charged ions were 653 and 734, suggesting that the maximum length of the backbone fragments released by RGH was 6 monosaccharide residues ($(GalA\text{-}Rha)_3$).

To confirm the compositions of the intensive ions and the distribution of the galactose residues along the backbone fragment, tandem mass spectrometry was employed. Fragmentation of an ion with m/z 985 ($(GalA\text{-}Rha)_2Gal_2$) led to the removal of a galacturonic acid residue (−176 amu) from the reducing end of the fragment and the formation of an ion with m/z 809 (Fig. 6). Fragmentation of a galacturonic residue in the ring, along with the formation of ions with m/z 925 (−60), 907 (−78) and 851 (−134) indicated the presence of a glycosidic bond in position 4 of the galacturonic residue. Formation of an ion with m/z 659 (985, −162, −146, −18; C–Z-cleavage) indicated linkage at the non-reducing end between rhamnose and one galactose (Fig. 6). Additional removal of a galacturonic acid residue led to the formation of an ion with m/z 483 (985, −162, −146, −176, −18 amu). Presence of ions 543 and 483 suggested linkage of one galactose to rhamnose at the non-reducing end. Fragments indicating linear rhamnose or digalactose within

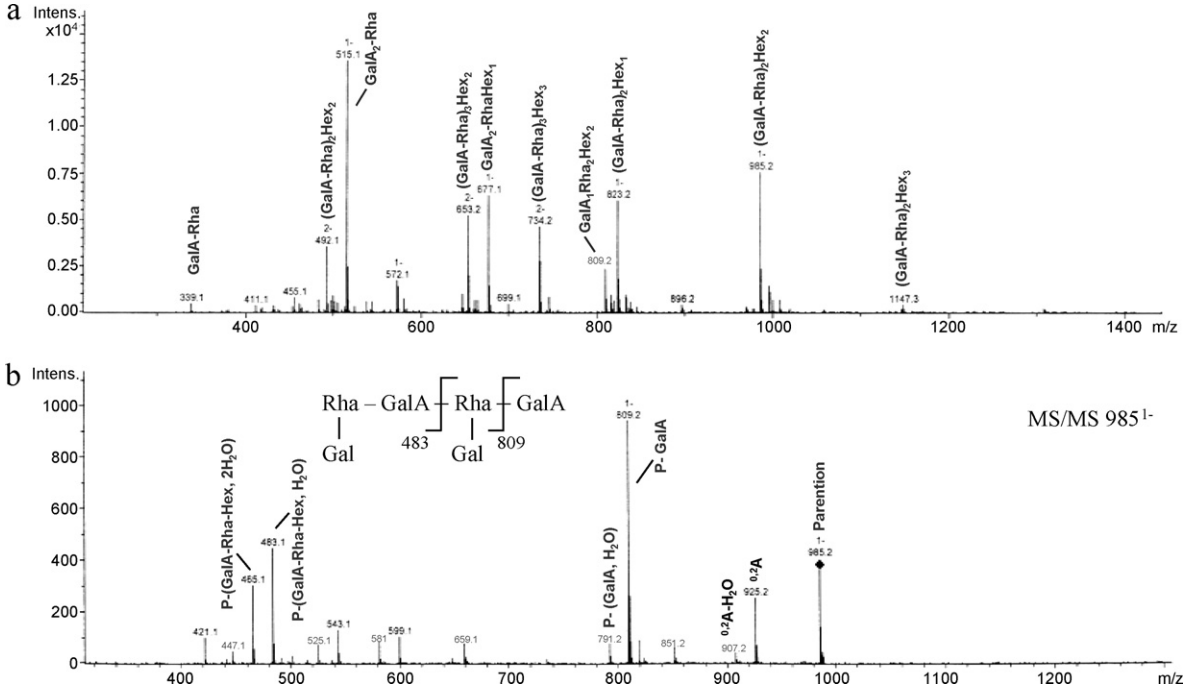


Fig. 6. Mass spectrum of the low molecular weight fractions[a] obtained after G_1 fraction hydrolysis with rhamnogalacturonan hydrolase (a) and the MS/MS spectrum of ion 985^{1-} (b). [a]Designation of fractions indicated in Fig. 5.

the molecular ion 985 were absent. The above data suggest that within the backbone of the initial polymers, there were regions with at least two consecutive residues of rhamnose branched with a single galactose.

4. Discussion

4.1. Elements of the galactan backbone structure

NMR analysis of pectic galactan of flax phloem fibres revealed that 75% of the polymer backbone contained linear rhamnose or rhamnose branched with single galactose. The remaining 25% of the backbone had longer side chains. To study the distribution of various side chains within the backbone, the polymer was treated with specific RGH. This RGH is capable of cleaving RG-oligomers, which contain five Rha units or more. The preferential cleavage site is located four monosaccharides from the Rha closest to the non-reducing end; the major products are α-L-Rha*p*-(1 → 4)-α-D-GalA*p*-(1 → 2)-α-L-Rha*p*(1 → 4)-D-GalA*p* and α-D-GalA*p*-(1 → 2)-α-L-Rha*p*-(1 → 4)-α-D-GalA*p*-(1 → 2)-α-L-Rha*p*-(1 → 4)-D-GalA*p* (Mutter, Renard, Beldman, Schols, & Voragen, 1998).

RGH degrades the flax fibre cell wall galactan; the polysaccharide contains sections of backbone that are available for cutting, but hydrolysis mainly produces fragments of large molecular weight (Fig. 5a). It is known that short chains of galactose (DP 1–3) do not inhibit the activity of RGH (Gurjanov et al., 2007), but the presence of long side chains inhibits the enzyme (Sengkhamparn et al., 2009). Thus, the absence of low molecular weight fragments after hydrolysis by RGH can be explained by the following: (1) the inability of RGH to split the RG I backbone in the case of Rha substitution with long galactose side chains; (2) the unavailability of the polymer backbone to RGH for other reasons (i.e., conformational reasons); and (3) the high molecular masses of the cleaved fragments due to long galactose chains. The absence of low molecular mass fragments among the products of the initial polymer hydrolysis with RGH means that in the backbone, there were no long sequences with Rha containing 0–1 Gal residues, even though they comprise 75% of the total rhamnose. Such sequences must have been interrupted by long side chains at least at every fifth rhamnose residue. RGH treatment of the G_1 fraction, in which a significant portion of galactose chains were removed, led to the formation of backbone oligosaccharides (Fig. 5b), indicating that long chains were indeed the obstacles for the enzyme.

Oligosaccharides obtained after hydrolysis of the G_1 fraction with RGH had the general formula $(GalA\text{-}Rha)_{2\text{-}3}Gal_n$ (Table 1, Fig. 5b), which were the final products of enzymatic hydrolysis (Mutter, Renard, et al., 1998; Schols et al., 1994b). The low molecular weight fraction contained: (1) the fragments $(GalA\text{-}Rha)_2Gal_1$ and $(GalA\text{-}Rha)_3Gal_2$, with both linear rhamnose and rhamnose with a single galactose and (2) the fragment $(GalA\text{-}Rha)_2Gal_2$, containing two Rha with a terminal Gal, separated by GalA. The oligosaccharides containing only linear rhamnose (except for a low intense signal with m/z 339) were absent. Hence, the linear Rha and the Rha with a terminal Gal were not separated from each other, but alternated in sequence.

Treatment of the G_1 fraction with RGH left part of the backbone as polymeric fragments, which eluted on a Sephadex G-50 column in the void volume (molecular mass above 10 kDa, Fig. 5b). The existence of part of the backbone that was not completely degraded to low molecular weight fragments by RGH, even after the removal of a large portion of Gal by galactanase (Fig. 5b), can be explained by the presence of long side chains (more than three galactose residues) in the galactan structure. Such galactose chains may remain after hydrolysis by galactanase due to the presence of alternative-type linkages (1 → 2-Gal, 1 → 6-Gal and Ara-Gal). The Gal/Rha ratio in the fragment, which remained polymeric, was less than two (Table 1); however, that did not exclude the presence (in combination with unbranched rhamnose) of side-chains longer than three Gal residues. To prevent the action of RGH, the long side chains must have substituted at least every fifth Rha residue. Thus, there was a special part of the polymer backbone (consisting of about half of it) (Fig. 5b) that remained in polymeric form after RGH treatment.

4.2. Structural features of long side chains of galactan

The branched Rha that was substituted with more than one Gal residue accounted for a quarter of the polymer backbone rhamnose. Hydrolysis of galactose side chains by galactanase generated small oligosaccharides besides galactose and digalactose (Gurjanov et al., 2007). The analysis of these yielded information about the nature of the minor glycosidic bonds in the side chains.

Both NMR and mass spectrometry indicated the presence of 2-Hex, 3-Hex, 6-Hex and linear arabinose in the sequence of neutral oligosaccharides. Because some of the carbohydrate residues with minor glycosidic bonds were cleaved off by galactanase, they did not form independent chains and were included within the main chain of the 1 → 4-linked galactose residues.

It is known that negatively charged molecular ions are cleaved mainly to C_n-type fragments, but Y_n- and Z_n-type fragments are also formed in smaller proportions (Carroll et al., 1993; Chai, Piskarev, & Lawson, 2001, 2002). Thus, it is impossible to clearly understand the localisation of pentose along the oligosaccharide sequence. Nevertheless, removal of 132 amu from the molecular ion indicated that some of the pentose was at the ends of the oligosaccharides released by galactanase. This pentose should be located at the non-reducing ends of the galactose chains. Otherwise, the hydrolysis of the bond between galactose and arabinose by galactanase would have been allowed.

It is interesting to note the presence of ions with masses increasing by 18 amu on the mass spectra. This was observed only for fragments with DP > 4 and can be explained by the formation of very stable complexes between oligosaccharides and water, which did not decay in vacuum at high temperature during ionisation. The region of the signals from the fragments $[Hex_n\text{–}H + 18]^{1-}$ had only low-intensity ions, which were characterised by weak cleavage of the glycosidic bond and the ring ($[Hex_n\text{–}H + 18\text{–}60]^{1-}$ was the only observed ion). The low intensity of cleavage fragments on rings may have several explanations: (1) the presence of hexose 1 → 3-binding residues, (2) the formation of stable complexes of oligosaccharide with water and (3) the presence of the Hex1 → 1Hex glycosidic bond. Another part of the molecule, which was adjoined to the non-reducing end, did not form a complex with water and fragmented as described above for Hex_n.

4.3. The modifications of galactan after incorporation into the cell wall

Plant cell wall matrix polysaccharides are synthesised in and secreted by the Golgi apparatus. In flax fibres, the secretion of cell wall polysaccharides is organised in a special way, involving accumulation of large amounts of Golgi derivatives within the cytoplasm (Salnikov et al., 2008). This permits the accumulation of the fibre-specific galactan before it is incorporated into cell wall. The structure of the nascent galactan has been analysed using a number of approaches (Gorshkova et al., 1996; Gurjanov et al., 2007). Together with the data presented in this paper, we have a rare opportunity to compare the structure of a polysaccharide before and after incorporation into the cell wall and to characterise any accompanying structural modifications.

It is known that the molecular weight of galactan (as measured by the time of elution during gel-filtration) decreases by approximately an order of magnitude after incorporation into the cell wall, from 2000 kDa to 100–400 kDa (Gurjanov et al., 2008). There is also a decrease of galactose content (from 90% and above to 60–70%) in the polysaccharides, which is probably due to the activity of the identified fibre-specific cell wall galactosidase (Mikshina, Chemikosova, Mokshina, Ibragimova, & Gorshkova, 2009). The trimming of galactose leads to an increase in the proportion of linear rhamnose from 6% in the nascent galactan (Gorshkova et al., 1996) to 28% in the cell wall galactan. Altering the ratio of branched and unbranched rhamnose indicates that approximately every fifth galactan side chain is fully hydrolysed *in vivo*. The removed chains may consist exclusively of 1 → 4-linked galactose.

At the same time, other types of galactose chains are not degraded (or are only partially degraded) during modification of the galactans in the cell wall. Such side chains include alternative glycosidic bonds. Minor amounts of 3-Gal (4.6%), 6-Gal (2.2%), 2,4-Gal (2.0%), 3,4-Gal (3.5%) and 5-Ara (0.7%), along with the main 4-Gal, were reported in the nascent galactan (Gorshkova et al., 1996). According to the data presented here, all these types of linkages remain in the cell wall galactans. Minor glycosidic bonds can block the galactosidase activity and can prevent total destruction of galactose chains and influence the spatial configuration of the side chains. Thus, the incorporation of galactan into the fibre cell wall is accompanied by a decrease in the amount and length of the polymer side chains; removal of the side chains occurs selectively.

Acknowledgements

This work was partially supported by a grant from the Russian Foundation for Basic Research (11-04-01602). We thank Dr. H.A. Schols (Wageningen University, The Netherlands) for providing highly purified enzymes.

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