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MALDI-TOF MS evidence for the linking of flax bast fibre galactan to rhamnogalacturonan backbone

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Abstract

Fibre-specific (1 → 4)-β-galactan extracted from bast fibre peels of developing flax (*Linum usitatissimum* L.) stem has been studied to elucidate its structural details. The polysaccharide was characterized by NMR and subjected to partial degradation protocols, including chemical and enzymatic approaches. The oligosaccharide fragments obtained were fractionated by gel permeation chromatography and analyzed for their molecular mass with MALDI-TOF MS. The obtained data show that this flax galactan is a complex RG-I polysaccharide with variable side chain structures. The backbone is composed of the common GalA-Rha repeats with a high degree of branching. These side chains are mainly composed of β-1,4-linked Gal oligomers: (1) short branches of only one or two Gal residue(s); (2) long (linear) branches of up to 26 Gal residues; (3) mixed branches of between 3 and 12 Gal residues (possibly derived from longer linear side chains), that are resistant to galactanase cleavage; (4) side chains of at least 17 Gal residues, decorated with single Ara moieties. The linkage between RG backbone and galactan side chains was confirmed by the presence of fragments with $(Rha\text{-}GalA)_nHex_m$ structure type. Neither chemical, nor enzymatic hydrolysis yielded oligomeric GalA residues, indicating that RG-I blocks are not interrupted by HGA regions. The polymer can be cleaved only partially by the rhamnogalacturonan hydrolase used, while the remaining part is resistant, probably due to peculiarities of side chain structure. Novel Rha-GalA oligomers were liberated by RG-hydrolase containing two or three Gal attached to Rha near the cleavage site. The native polymer is decorated by acetyl groups, with yet unknown distribution patterns. Treatment with purified and well-characterized galactanase does not change the hydrodynamic volume of flax galactan (despite considerable cleavage of Gal moieties), suggesting a complex "secondary" structure of the polymer.

Keywords: *Linum usitatissimum* L.; Flax; Fibre; Cell wall; Galactan; Cell-specific polysaccharide; MALDI-TOF MS; Rhamnogalacturonase; Galactanase

1. Introduction

Galactose-containing polymers are the major matrix components in fibre cell walls of important crops, like flax (*Linum usitatissimum* L.), making up 40–80% of non-cellulosic polysaccharides (Girault et al., 1997; Mooney, Stolle-Smits, Schols, & de Jong, 2001; Morvan et al., 2003). Pectic β-(1 → 4)-galactans attached to RG-I backbone with molecular mass not exceeding 200 kDa were described in cell wall of mature flax fibres (Davis, Derouet, Hervé du Penhoat, & Morvan, 1990; Van Hazendonk, Reinerink, de Waard, & Van Dam, 1996). However, bast fibre peels of developing flax plants contain tissue-specific soluble galactan with a molecular mass distribution ranging between 700 and 2000 kDa showing the same sugar composition throughout the mass range (Gorshkova et al., 1996, 2004).

The polymer was shown to emerge just below the snap point – the specific spot in the stem, where fibre cell growth changes from elongation to intensive cellulose deposition

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and secondary cell wall thickening (Gorshkova et al., 2003, 2004). Given the coincidence of appearance in the fibre cells of this very soluble and high molecular mass galactan at this transition point, a significant role for this polymer in this process can be surmised.

The soluble galactan is localised in the specialized Golgi-derived vesicles, which accumulate in flax fibre cytoplasm during transition from cell elongation to cell wall thickening and later fuse with plasmalemma to give their content to the apoplast (Gorshkova, Ageeva, Chemikosova, & Salnikov, 2005). Thus, at this stage the nascent structure of the polysaccharide before it is incorporated into the cell wall can be analyzed. The overall sugar (linkage) composition of the polymer revealed the presence of β-(1 → 4)-linked Gal residues as the major sugar (Gorshkova et al., 1996). The linear 1 → 4-galactan chains could be branched through the O-2 and O-3 positions of the Gal, with Gal being the only detectable terminal sugar. Rha was present in the fraction mostly as a branching point, suggesting that the galactan was linked to a rhamnogalacturonan I structural element of pectin, which, however, has not been confirmed so far.

To further elucidate the complex structure of the galactan, the polymer was characterized by NMR and subjected to both chemical and enzymatic degradation. Released oligomeric fragments were identified using HPLC and mass spectrometry.

2. Materials and methods

2.1. Plant material

Flax plants (*Linum usitatissimum* L.) were grown in soil in a greenhouse. Plants were taken at the fast growth stage (40 days after sowing). The fibre-rich phloem strips in 15 cm stem portions below snap point (Gorshkova et al., 2003) were peeled from the xylem part. Plant material was fixed in liquid nitrogen and kept frozen.

2.2. Isolation of buffer-soluble material

Plant material (1 g) was homogenized in liquid nitrogen and left to defrost in 15 ml of 50 mM K-phosphate buffer, pH 6. After centrifugation of the homogenate (7000*g*, 5 min) the supernatant was brought to 80% (v/v) ethanol to precipitate the buffer-soluble polymers (overnight, 4 °C). The resulting pellet was washed three times with 80% ethanol and fractionated over Sepharose CL-4B (Gorshkova et al., 1996) with 10 mM Na-acetate, pH 4.5. Fractions, corresponding to the galactan with molecular mass 700–900 kDa (Gorshkova et al., 2004) were combined, desalted on Sepharose CL-4B with water, dried by evaporation under reduced pressure at 40 °C and used for analysis (Fig. 1). The polysaccharide yield is 0.15%.

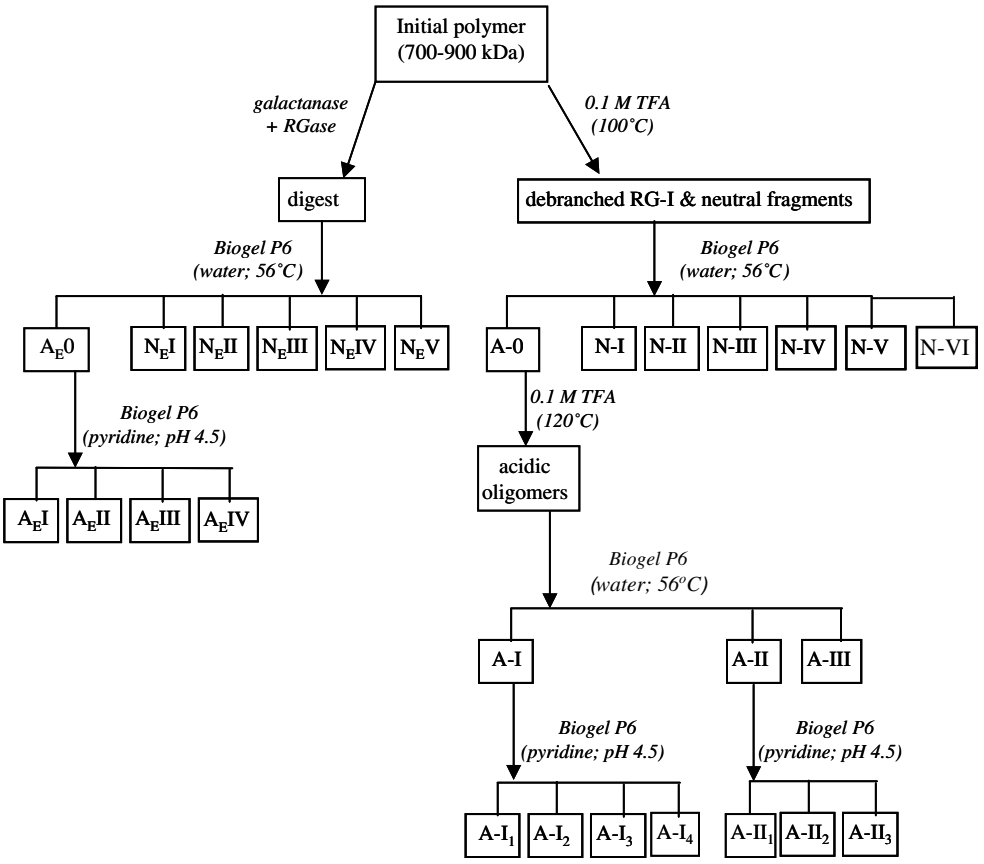

Fig. 1. Isolation scheme of the galactan fragments after 0.1 M TFA treatment.

2.3. NMR spectrum

The structure of galactan was determined by ^{1}H and ^{13}C NMR spectroscopy. Sample was dissolved in D_2O (99.96 at % D). ^{1}H and ^{13}C spectra were recorded on Bruker Avance DPX300 NMR spectrometer, at room temperature. Chemical shifts are expressed in ppm relative to external aceton (^{1}H, δ 2.22; ^{13}C, δ 31.55) (Van Hazendonk et al., 1996).

2.4. High-performance size-exclusion chromatography

HPSEC was performed on three TSKgel columns (7.8 × 300 mm) in series (G4000, G3000, G2500; Tosohaas), in combination with a PW XL-guard column (Tosohaas). Elution took place at 30 °C with 0.2 sodium nitrate at 0.8 ml/min. The eluate was monitored using a refractive index (RI) detector (Shodex RI-71). Calibration was performed using pullulans (Polymer Laboratory).

2.5. Acid hydrolysis

The galactan (1–2 mg) was dissolved in 2 ml 0.1 M TFA and heated at 100 °C for 1 h (Fig. 1). The TFA solution was then evaporated with a nitrogen flush at 30–40 °C.

2.6. Size-exclusion chromatography

The dried TFA hydrolysate was solubilized in 0.5 ml water, applied to a column (2 × 60 cm) of Bio-Gel P-6 (Bio-Rad, separation range 1–6 kDa) and eluted at 56 °C with milliQ water (rate 15 ml/h, 2 ml fractions). The sugar content in each fraction was measured by the phenol–sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Fractions were combined according to the elution pattern, dried by evaporation under reduced pressure and used for monosaccharide and MALDI-TOF mass spectrometry analysis.

Fraction A-0 (void volume) was hydrolysed with 0.1 M TFA at 120 °C, 1 h and dried with nitrogen at 30–40 °C. The hydrolysate was eluted on Bio-Gel P-6 with milliQ water at 56 °C. Three fractions were prepared (A-I, A-II and A-III). The first two fractions (A-I and A-II) containing uronic acids were again separated on Bio-Gel P-6 with 0.01 M pyridine/acetic acid solution, pH 4.5 at 56 °C to avoid ionic repulsion. Dried samples were used for monosaccharide and MALDI-TOF mass spectrometry analysis (Fig. 1). They were named as A-I acid fractions (A-I_1, A-I_2, A-I_3 and A-I_4) and A-II acid fractions (A-II_1, A-II_2 and A-II_3).

2.7. Monosaccharide analysis

The samples were hydrolysed in 2 M TFA at 120 °C, 1 h, dried by evaporation in nitrogen at 30–40 °C. The sugar composition was analyzed by high-performance anion-exchange chromatography (HPAEC) on a CarboPac PA-1 column (4 × 250 mm, Dionex) using pulsed amperometric detection (PAD, Dionex). Buffers used were: A – milliQ water, B – 0.15 M NaOH, C – 0.6 M NaAc. Column was equilibrated with A – 83% and B – 17% and eluted with following linear gradient: 2–30 min A – 100%; 33–42 min A – 30%, B – 35% and C – 35%; 45–50 min B – 100%; 50.1–60 min A – 83% and B – 17%. The column was eluted with a flow rate of 1 ml/min at 30 °C. The post-column derivatisation with 0.5 M NaOH solution was performed before pulsed amperometric detector. Mannitol was used as an internal standard. Monosaccharide standards were treated by 2 M TFA at 120 °C, 1 h before they was used.

2.8. MALDI-TOF mass spectrometry analysis

MALDI-TOF MS analysis was performed using an Ultraflex instrument (Bruker Daltonics) equipped with a nitrogen laser of 337-nm. The mass spectrometer was selected for positive ions. After a delayed extraction time of 200 ns, the ions were accelerated to a kinetic energy of 12,000 V. Hereafter, the ions were detected using the reflector mode. The lowest laser power required to obtain good spectra was used and at least 100 spectra were collected. The mass spectrometer was calibrated with a mixture of maltodextrins (mass range 365–2309).

The matrix solution was prepared by dissolving 10 mg 2,5-dihydroxybenzoic acid (Bruker Daltonic's) in 700 μl water and 300 μl acetonitrile. The samples (approximately 20–40 μg sugar) were dissolved in 10 μl water. The sample desalting with resin (AG 50W-X8 Resin, H^+ form; Bio-Rad) was used if NaAc was present. A 1 μl of sample solution was placed on a MALDI-TOF-plate (Bruker Daltonic's) together with 1 μl of matrix solution and allowed to dry under a constant stream of warm air.

2.9. Treatment of galactan by galactanase and RG-hydrolase

Since the galactan contains substantial amounts of acetylated residues (see below), which may interfere with the enzyme accessibility, a saponification step with NaOH was included before incubation with enzymes. 5 mg of the isolated flax galactan was dissolved in 0.1 ml water and 0.1 ml 0.06 M NaOH. The solution was kept several hours at 4 °C and neutralised with 0.2 ml 0.03 M acetic acid. Then the volume was increased to 1 ml with 0.6 ml 0.03 M sodium acetate pH 5.0 and was incubated with 6.8 μg (protein based) endo-galactanase from *Aspergillus aculeatus* (Van de Vis, Searle-van-Leeuwen, Siliha, Komelink, & Voragen, 1991). From this galactanase treated substrate 100 μl was taken and re-incubated with an excess of RG-hydrolase from *A. aculeatus* for 24 h at 40 °C (Mutter, Renard, Beldman, Schols, & Voragen, 1998). Enzyme activities were inactivated at 100 °C for 5 min.

For a larger scale separation by Bio-Gel P-6 10 mg of saponified flax galactan was degraded by both endo-galactanase and RG-hydrolase as described. The sodium acetate was removed on column of Dowex 50 (Dowex 50 × 8, 50–100 mesh, H^+ form). The resin was prepared by washing it with several column volumes of 1 M NaOH, water, 1 M

HCl, water, 1 M acetic acid and water. Solution of the enzyme hydrolysate was dried by evaporation under reduced pressure at 40 °C. The dried digest was redissolved again and fractionated on a Bio-Gel P-6 column with milliQ water at 56 °C. Neutral fractions N_EI–N_EV were dried and used for MALDI-TOF mass spectrometry analysis. The fraction A_E0 was again separated on Bio-Gel P-6 column with 0.01 M pyridine/acetic acid buffer, pH 4.5 at 56 °C and prepared acid fractions (A_EII–A_EIV) were dried and used for MALDI-TOF mass spectrometry analysis too. Fraction A_EI was characterized by ^1H NMR.

3. Results

Fresh bast fibre-enriched peels of growing flax stems were extracted with KH_2PO_4 buffer solution at pH 6. The soluble polysaccharides were precipitated in 80% ethanol and redissolved in 0.01 M NaAc buffer at pH 4.5 for GPC fractionation on a Sepharose CL 4B column. The soluble polysaccharide was collected in the mass region 700–900 kDa. The sugar composition of this pool (Table 1) showed a high proportion of Gal (54 mol%) in the polysaccharide with Rha and GalA as two other major sugars. This suggested a rhamnogalacturonan RG-I type structure with high Gal branching (Morvan et al., 2003), which was confirmed by NMR analysis.

3.1. NMR analysis

In the ^{13}C NMR spectrum (Fig. 2a) of the purified soluble polysaccharide the typical features of rhamnogalacturonan type I spectrum are present. The presence of minor signals in the ^{13}C NMR spectrum can be ascribed to the sugars of the rhamnogalacturonan backbone. At 173.3 ppm typical signal of C-6 of uronic acid is present, while also anomeric carbon signals for a C-1 of α-D-Gal*p*A and α-L-Rha*p* can be discriminated at 97.4 and 94.6 ppm. At the other end of the spectrum C-6 of α-L-Rha*p* is present at 16.9 ppm. The relatively weak signals for ring carbons in the rhamnogalacturonan backbone (C-2 to C-5) are deluged by signals of the ring carbons of the more mobile β-(1 → 4)-galactan side chains (Foster, Ablett, McCann, & Gidley, 1996). The β-(1 → 4)-linked galactan (Table 2) can be easily discriminated as the major resonance peaks at 104.4 (C-1), 77.7 (substituted C-4), 74.6 (C-5), 73.4 (C3), 71.9 (C-2) and 60.8 ppm (C-6) (Bock & Pederson, 1983). The signal at 104.4 ppm confirmed the β-configuration of the Gal linkage. The presence of terminal Gal is visible in the unsubstituted C-4 signal at 68.7 ppm. The resonance at 20.28 ppm was assigned to acetyl (Van Hazendonk et al., 1996).

In the ^1H spectrum (Fig. 2b) the intensive signals in region 3.80–3.66 ppm were assigned to C-2, C-3, C-5 and C-6 of Gal; the signals at 4.64 and 4.17 ppm to C-1 and C-4 of β-1,4-linked Gal and the signal at 3.90 ppm to C-4 of t-Gal (Table 2). Signals at 1.2–1.3 and 2.1–2.2 ppm could be assigned to methyl group of the Rha and to acetyl, respectively (Van Hazendonk et al., 1996). Signals in the region 3.4 ppm could originate from C-4 of unsubstituted Rha (Schols, Voragen, & Colquhoun, 1994).

3.2. Chemical hydrolysis

Partial chemical hydrolysis of the polysaccharide was performed stepwise using 0.1 M trifluor acetic acid (TFA) at two temperatures (Fig. 1). The first hydrolysis step was performed at 100 °C. The hydrolysate was fractionated using Bio-Gel P6 with water into seven fractions (Fig. 3a). Elution with water resulted in a first separation of low molecular weight neutral sugar fragments, while all acidic fragments were eluted in the void volume. This void volume fraction is consisting of high molecular mass fragments of sizes too large for direct MALDI-TOF MS analysis and was therefore again treated with 0.1 M TFA – at 120 °C. The hydrolysate was also fractionated on Bio-Gel P6 with water as effluent (Fig. 3b). The fractions A-I and A-II were further separated into fragments with different molecular mass on Bio-Gel P-6 with pyridine/acetic acid buffer, (pH 4.5) (Fig. 3c and d). The fractions were subjected to sugar composition and to MALDI mass spectrometry analysis. Fraction A-III (Fig. 3b) contained only Gal monomers and dimers and was not subjected to MALDI-TOF MS analysis.

The oligosaccharide fractions (Fig. 3; N-I to N-VI) derived from the first hydrolysis step of the polysaccharide

Table 1
Sugar composition (mol%) of fractions and MALDI-TOF MS assignment of corresponding major fragments after 0.1 M TFA treatment

Fractions	Rha	Ara	Gal	Glc	Xyl	Man	GalA	Major fragments	Minor fragments
Initial polymer	19	3	54	1	0	0	23		
N-I	9	Trace	69	2	3	0	16	$Hex_{18–26}$	$Hex_{11–17}$; $(GalA\text{-}Rha_2)Hex_{8–14}$
N-II	3	Trace	89	1	Trace	0	6	$Hex_{13–20}$	$Hex_{16–17}Pen_1$
N-III	Trace	Trace	95	2	Trace	0	3	$Hex_{9–16}$	$Hex_{9–13}Pen_1$
N-IV	1	1	94	1	2	0	1	$Hex_{6–11}$	$Hex_{6–9}Pen_1$
N-V	Trace	3	93	1	3	0	0	$Hex_{4–7}$	$Hex_{4–5}Pen_1$
N-VI	Trace	25	72	Trace	2	0	0	$Hex_{1–4}$	
$A\text{-}I_1$	42	0	2	2	0	0	54	$(GalA\text{-}Rha)_{5–7}$	
$A\text{-}I_2$	46	0	2	Trace	0	0	52	$(GalA\text{-}Rha)_5$	
$A\text{-}I_3$	46	0	2	Trace	0	0	52	$(GalA\text{-}Rha)_4$	
$A\text{-}I_4$	45	0	2	Trace	2	0	52	$(GalA\text{-}Rha)_3$	
$A\text{-}II_3$	45	0	3	1	0	0	51	$(GalA\text{-}Rha)_2$	

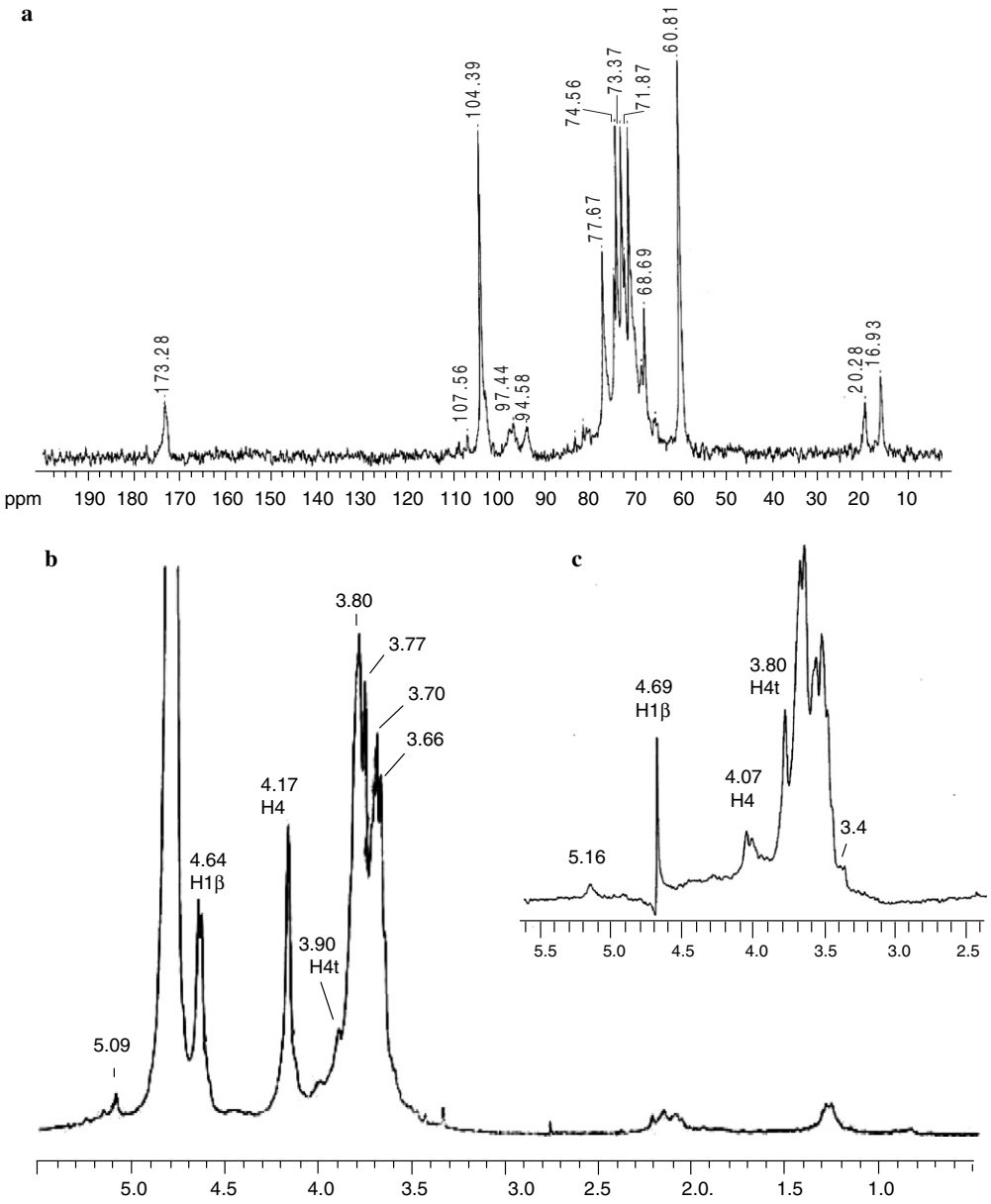

Fig. 2. (a) ^{13}C NMR spectrum of galactan in native form; ^{1}H NMR spectrum of the galactan before (b) and after (c) enzyme treatment (fraction A_E-I).

Table 2
Assignment of ^{13}C and ^{1}H chemical shifts of galactan, together with reference data

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	C-4/H-4[b]
→4)-β-D-Gal$_p$-(1→	^{13}C	104.39	71.87	73.37	77.67	74.56	60.81	68.69
	^{1}H	4.64	3.66	3.80	4.17	3.70	3,77	3.90
→4)-β-D-Gal$_p$-(1→[a]	^{13}C	105.66	73.12	74.61	78.96	75.81	62,05	69.94
	^{1}H	4.60	3.67	3.81	4.14	3.75	3,78	3.86

[a] Reference data (Van Hazendonk et al., 1996).
[b] Terminal Gal.

contained neutral sugars – almost only pure Gal residues (Table 1). The fractions A-I and A-II obtained by additional hydrolysis of the void volume were predominantly acidic, with close to equal proportion of Rha and GalA (Table 1).

MALDI-TOF MS revealed that the first eluted neutral fraction N-I had the highest peak intensity at m/z 3282, corresponding to 20 Hex units $[Hex_{20}Na]^+$, but also other significant peaks were present in this fraction ranging between m/z 2958 and 4254 corresponding to, respectively,

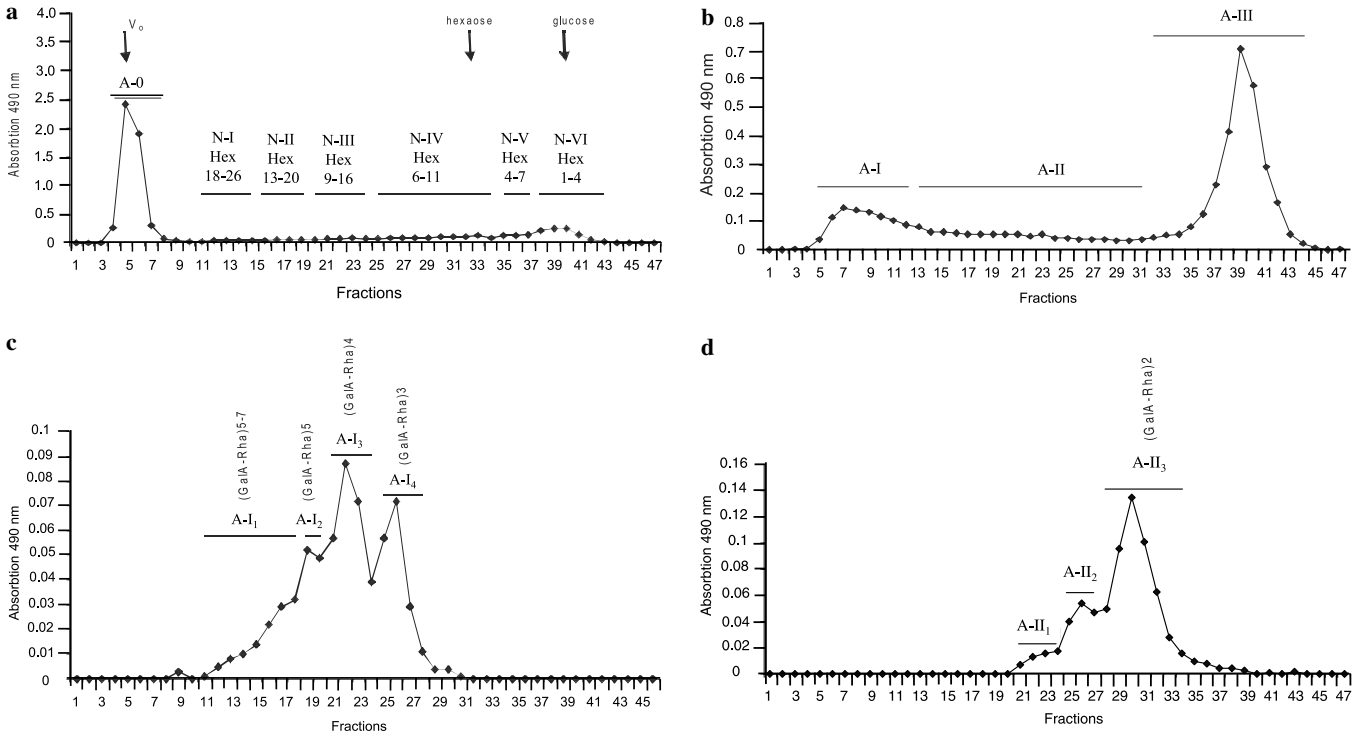

Fig. 3. Fractionation of the galactan fragments after TFA hydrolysis. (a) Chromatography of the galactan fragments after 0.1 M TFA treatment at 100 °C. Bio-Gel P-6, elution with water. (b) Chromatography of fraction A-0 (void volume) after additional 0.1 M TFA treatment at 120 °C. Bio-Gel P-6, elution with water. (c) Chromatography of A-I. Bio-Gel P-6, elution with 0.01 M pyridine/acetic acid, pH 4.5. (d) Chromatography of A-I_1. Bio-Gel P-6, elution with 0.01 M pyridine/acetic acid, pH 4.5.

18 and 26 Hex units (Fig. 4a). Even higher mass peaks can be distinguished. The signals were also present of lower intensity at 162 mass units intervals with declining mass from m/z 2796 to 1337, corresponding to 17–8 Hex units. A parallel series of minor mass peaks at m/z −18 could be observed for oligosaccharides corresponding to sequence of GalA-Rha_2-Hex_n starting at $n = 5$ (m/z 1319), to $n = 15$ (m/z 2940). These minor fragments are evidence for the RG-I type structure of the polymer, having Gal branches directly linked to the RG backbone.

The eluted fractions N-II to N-VI showed the complete range of oligosaccharides of declining chain length from 13–20, 9–16, 6–11 to 4–7 and 4–1 Hex units (Table 1). Most of those peaks were accompanied by minor peaks of m/z −30 corresponding to Hex_nPen_1. Only trace amounts of Ara or Xyl could be determined in the sugar analysis of these fractions, except N-VI that contained a significant proportion of Ara (25%) (Table 1). In the MALDI-TOF spectrum this high pentose content was not observed in the oligosaccharides fragments. This can probably be ascribed to unstable glycosidic bonds of Ara and the presence of monosaccharides in this fraction.

The acidic fractions (Fig. 3c and d) showed the typical features of the pectic rhamnogalacturonan backbone with $(GalA\text{-}Rha)_n$ disaccharide units. In the first fraction (A-I_1) peaks in the high mass ranged from five to seven disaccharide units at m/z 1651–2295 for $[(GalA\text{-}Rha)_{5-7}Na]^+$ (Fig. 4b). In the following fractions (A-I_2 to A-I_4 and A-II_3) other peaks at regular mass distances were present for oligosaccharides with declining number of disaccharide units (m/z 1651, 1329, 1007 and 685). Fractions A-II_1 and A-II_2 are the same as fractions A-I_3 and A-I_4 and were not used for further analysis (Fig. 3c and d). The major signal, corresponding to $[(GalA\text{-}Rha)_nNa]^+$ was accompanied by the signals of declining intensities (differing by 16, 22, 38, 54, 60 and 76), which corresponded to the disaccharides units with various combinations of Na and K counter-ions. (Fig. 4c). However, accompanying peaks with 60 mass units difference can be also ascribed to replacement of two Rha by GalA. Mass peaks at m/z 1711, 2033 and 2355 then would correspond to $[GalA_2\text{-}(GalA\text{-}Rha)_nNa]^+$ in which $n = 3$, 4 and 5, respectively.

3.3. Enzymatic hydrolysis of the galactan with galactanase and RG-hydrolase

To further elucidate the polymer structure, the galactan was treated with well characterized galactanase (Van de Vis et al., 1991) and rhamnogalacturonan hydrolase (Mutter et al., 1998). Galactanase treatment of flax galactan released a considerable portion of Gal: out of 1565 μg/ml of (anhydro)-Gal in the starting galactan sample, 330 μg/ml of (anhydro)-Gal was released as monomer and as dimer in similar amounts. Besides, the presence of Hex oligomers (up to at least 7 U) could be demonstrated in

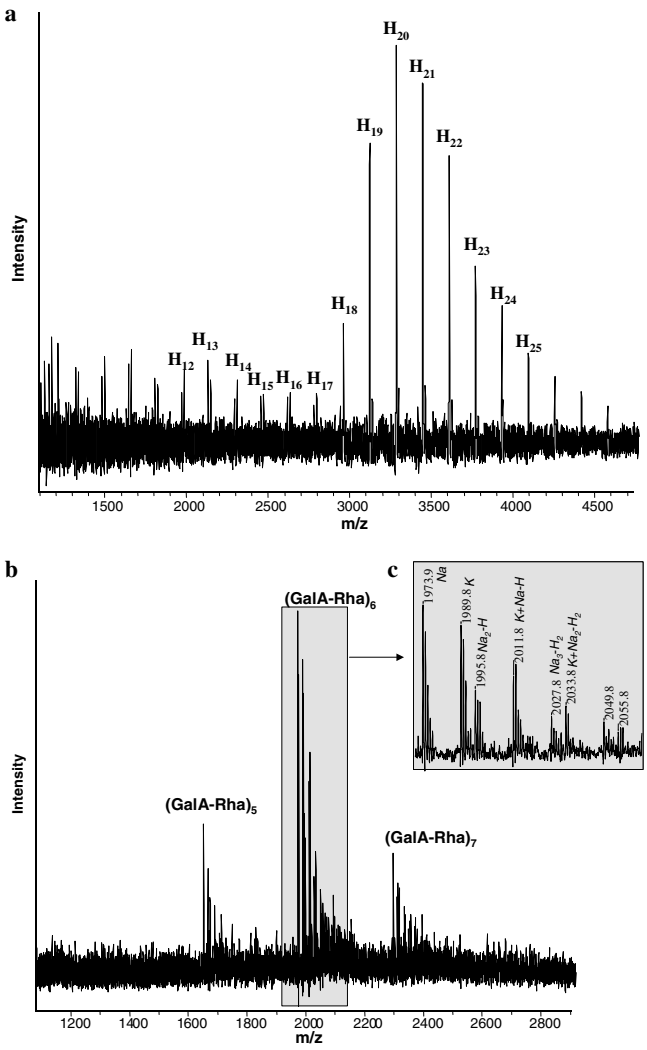

Fig. 4. MALDI-TOF mass spectrum of fractions after 0.1 M TFA treatment. (a) N-I; (b) A-I_1; (c) A-I_1 – part of (b). Designation of fractions on Fig. 3.

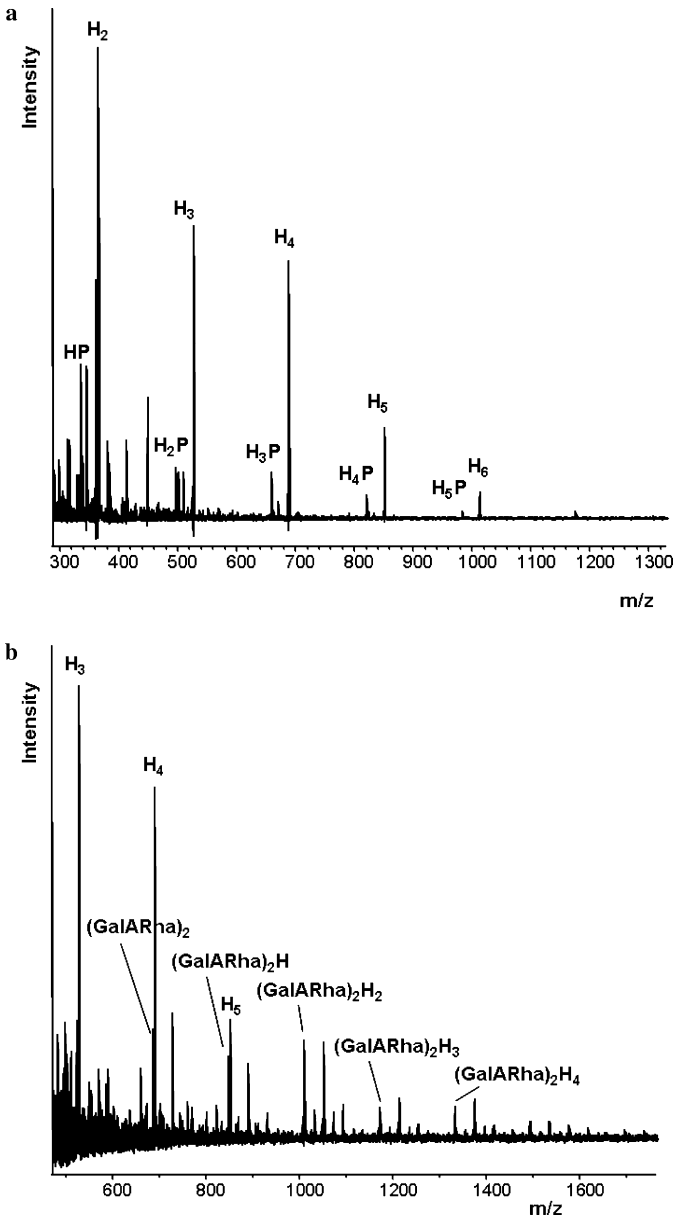

Fig. 5. MALDI-TOF mass spectrum of total hydrolysate after enzyme treatment of the galactan (a) galactanase treatment; (b) combined galactanase and RG-hydrolase treatment.

the sample by MALDI-TOF MS (Fig. 5a). The reference samples without enzyme added, or enzyme without galactan contained no mono- and oligosaccharides. Thus, at least 45% of Gal present in the polymer was cleaved off. The results were similar with saponified or non-saponified polymer, so no effect of acetyl ester groups on the action of the enzyme on the polysaccharide was observed.

The Gal content in initial flax galactan is 54% (Table 1), so approximately 25% of the polymer was cleaved by galactanase. However, the HPSEC profile of the galactan peak did not change noticeably (Fig. 6a). The same galactanase treatment of potato galactan (containing similar proportions of Gal), changed the HPSEC profile significantly by shifting the peak to the low molecular mass region (data not shown).

Besides the pure Hex fragments, the galactanase treatment released oligosaccharides with mass peaks corresponding to $[Hex_n + Pen_1Na]^+$ as was shown by MALDI-TOF MS (Fig. 5a).

The residual flax galactan after galactanase treatment was still too large to be analyzed by MALDI-TOF MS and was additionally treated with RG-hydrolase. The combined treatment further split the polymer and led to formation of new peaks in the HPSEC profile, eluting mainly at 27–28 and 29–32 min (Fig. 6b). In addition to neutral oligosaccharides the mixture obtained by combined galactanase and RG-hydrolase treatment (Fig. 5b) contained the acidic fragments. The oligosaccharide mixture was rather complex and was again fractionated on Bio-Gel P-6 with water elution as mentioned above (Fig. 7a): the void volume peak was refractionated with pyridine/acetic acid buffer (pH 4.5) (Fig. 7b).

The low molecular mass peak N_EV represents the major neutral fraction (Fig. 7a) that contained Hex chains with

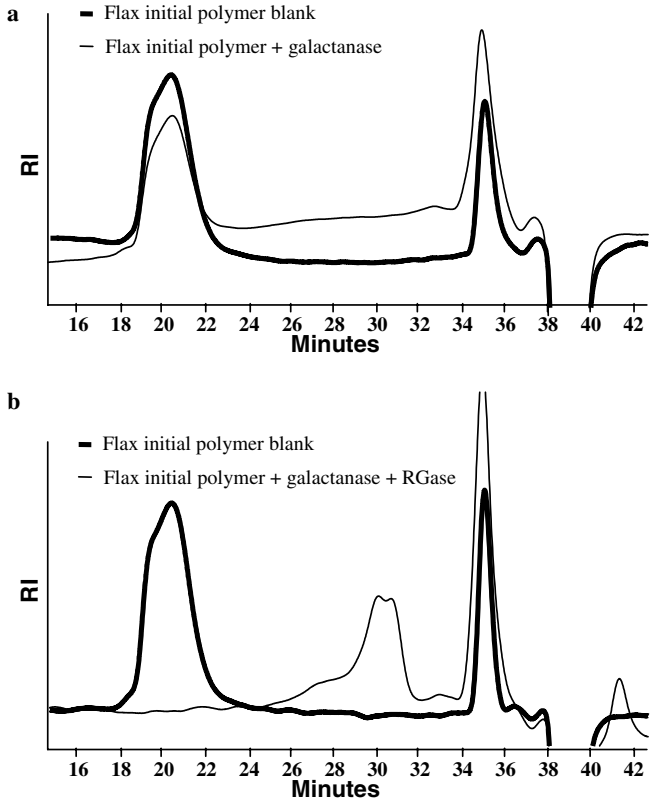

Fig. 6. HPSEC of the galactan after enzyme treatment. (a) After galactanase treatment (24 h); (b) after combined galactanase and RG-hydrolase treatment (24 h).

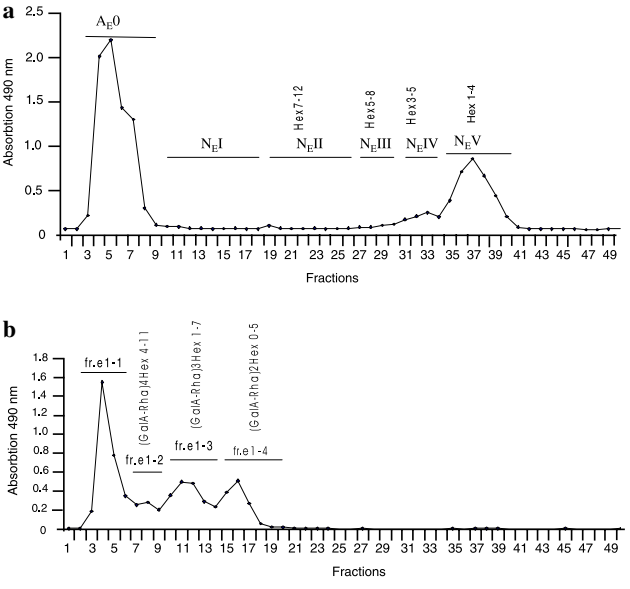

Fig. 7. Fractionation of the galactan fragments after combined galactanase and RG-hydrolase treatment. (a) Chromatography of oligosaccharides after enzyme treatment on Bio-Gel P-6, elution with water. (b) Chromatography of fraction A_E0 (void volume) on Bio-Gel P-6, elution with 0.01 M pyridine/acetic acid, pH 4.5. Sugar content was determined according to Dubois et al. (1956).

Table 3
MALDI-TOF mass spectra of neutral and acid fragments after enzyme treatment

Fractions	Major fragments	Minor fragments
N_EI		$(Rha\text{-}GalA)_1Hex_1$
		$(Rha\text{-}GalA)_2Hex_{1–5}$
N_EII	$Hex_{7–12}$	$Hex_{6–11}Pen_1$
N_EIII	$Hex_{5–8}$	$Hex_{4–7}Pen_1$
N_EIV	$Hex_{3–5}$	$Hex_{2–4}Pen_1$
N_EV	$Hex_{1–4}$	Hex_2Pen_1
A_EII	$(Rha\text{-}GalA)_4Hex_{4–11}$	$(Rha\text{-}GalA)_3Hex_{6–7}$
A_EIII	$(Rha\text{-}GalA)_3Hex_{1–7}$	*m/z* 665
A_EIV	$(Rha\text{-}GalA)_2Hex_{0–5}$	*m/z* 701

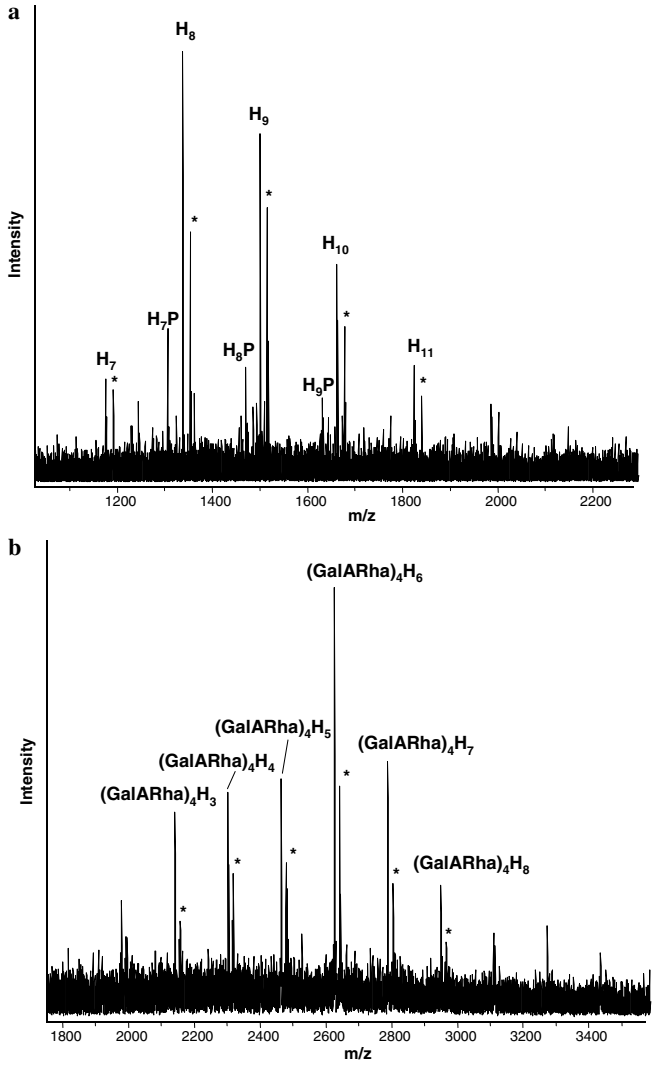

Fig. 8. MALDI-TOF mass spectrum of fractions after combined galactanase and RG-hydrolase treatment of the galactan. (a) N_EII; (b) A_EII. Designation of fractions on Fig. 6. *Oligomers including potassium ions instead of sodium ones.

dp 1–4 (Table 3). The fractions N_EII to N_EIV contained higher mass Hex oligomers of dp up to 12 (Fig. 8a). The fraction N_EI contained the acidic fragments, including $[GalA\text{-}Rha\text{-}Hex\ Na]^+$(*m/z* 525) (Table 3).

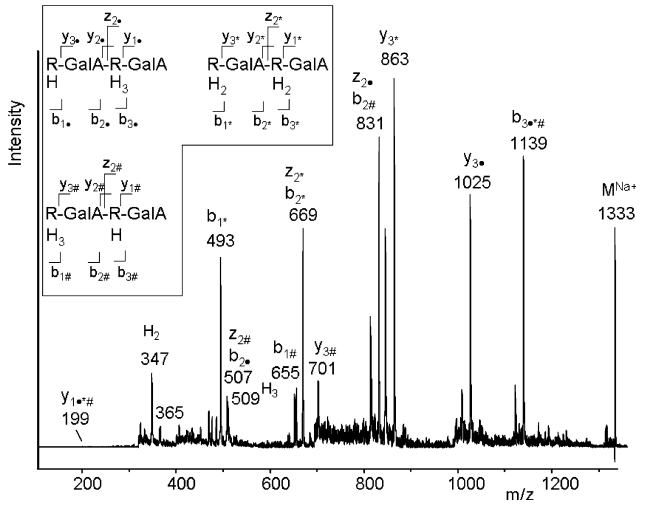

Fig. 9. PSD MALDI-TOF mass spectrum of the fragments with (Rha-GalA)$_2$Hex$_4$ structure.

The major fragments found in the acidic fraction A_EII (Fig. 7b) were $[(GalA\text{-}Rha)_4Hex_nNa]^+$ with $n = 4$–11 (Fig. 8b); in A_EIII – $[(GalA\text{-}Rha)_3Hex_nNa]^+$ $n = 1$–7, and A_EIV $[(GalA\text{-}Rha)_2Hex_nNa]^+$ $n = 0$–5. The structure of (GalA-Rha)$_2$Hex$_4$ fragment was characterized with PSD (post-source decay) MALDI-TOF MS technique. The four Gal residues were distributed between the two Rha units in different ways 1:3, 2:2, 3:1 (Fig. 9). Thus, the RG-hydrolase treatment of the polymer released oligomers of Rha and GalA with two and three Gal moieties attached to one Rha, which were not reported before. Fraction A_EI contained still too high molecular mass fragments to be analyzed by MALDI-TOF MS. The ^{1}H NMR spectrum was recorded and compared to the original polysaccharide spectrum (Fig. 2c). The major signals in the fragment A_EI still were derived from the β-linked Gal (4.69 ppm). However, the resonance at 4.07 ppm assigned to C-4 of 4-linked Gal was considerably decreased and was overshadowed by the signal at 3.80 ppm, assigned to C-4 of terminally linked Gal. Thus, the proportion between terminal and in-chain Gal was considerably changed in the fraction A_E-I as compared to the undigested galactan. The signal at 3.4 ppm, which showed up in the ^{1}H NMR spectrum of the fraction A_EI and was undetectable in the undigested polymer (Fig. 2b and c), can be assigned to the linear Rha (Schols et al., 1994).

4. Discussion

4.1. RG-I type of structure

Fibre-specific soluble galactan is accumulated in the specialized Golgi-derived vesicles, which later fuse with plasmalemma and give their content to the apoplast (Gorshkova et al., 2005). Thus, it represents the nascent polysaccharide synthesized in Golgi substructures. The molecular mass of the galactan is higher than is usually reported for cell wall extracted polymers – between 700 and 2000 kDa. Such high molecular mass (several million dalton) were also reported for the Golgi vesicles glucuronoxylan, xyloglucan and xylan (Crosthwaite, MacDonald, Baydoun, & Brett, 1994; Kerr & Fry, 2003).

NMR studies and specific enzymatic hydrolysis using purified and well-characterized enzymes (e.g., galactanase and RG-hydrolase) in combination with MALDI-TOF MS were used to study structural features of the polymer. The degradability of the polymer by RG-hydrolase and the presence of the GalA-Rha disaccharide chains in all acidic fragments obtained by both chemical and enzymatic hydrolysis proves the presence of a RG-I backbone linked to the galactan. The linkage between RG-backbone and galactan side chains was demonstrated by the presence of fragments with hexose units attached (Rha-GalA)$_n$Hex$_m$ (Tables 1 and 3). Neither chemical, nor enzymatic hydrolysis yielded oligomeric GalA residues, indicating that RG-I blocks are not accompanied by HGA regions. The general structure of the flax galactan was confirmed by NMR studies to be of rhamnogalacturonan I type with β-Gal as a major sugar, with partial substitution of acetyl groups. In general, the NMR spectrum is similar to the ones obtained by Davis et al. (1990) and Van Hazendonk et al. (1996) for the mature fiber cell wall fractions.

4.2. Galactanase treatment

The unusual character of the fibre-specific galactan was revealed when it was subjected to enzymatic treatment. The absence of a shift of retention time of the peaks in HPSEC to lower Mw shows that the hydrodynamic volume of flax galactan is not changed by galactanase treatment (Fig. 6a), despite the release of considerable amounts of Gal moieties (45%). The flax polymer differed in this respect from potato galactan, which also contained low amounts of the RG-I type of structure. The HPSEC profile of potato galactan changed considerably after galactanase treatment and the major peak shifted to the lower molecular mass region. The absence of similar effects with flax galactan was not due to the void volume effect, since the polymer fraction of 700–900 kDa was used, separated from the 1000–2000 kDa fraction on the same column. The retention of the hydrodynamic volume after removal of 45% of Gal may be indicative for the inaccessibility of the "secondary" structure of the galactan branches, that are kept intact during galactanase digestion. This highly relevant observation should be clarified in further studies and may lead to novel ideas in polysaccharide structure.

4.3. Endo-galactanase action

The galactanase used cleaves the linear Gal chains into dimers and monomers with a minimal chain length requirement of three residues (Van de Vis et al., 1991). The Gal-Rha linkage can not be cleaved by galactanase. Since galac-

tanase is not able to cleave Gal dimers, the second Gal in the branch also remains attached to the backbone (Van de Vis et al., 1991). This could also be true for the third Gal residue, since the Gal attached to the backbone differs from the free galactotriose. Indeed, oligosaccharides with three Hex units attached to one Rha were observed after combined galactanase and RG-hydrolase treatment by PSD MALDI-TOF MS technique among the $(Rha\text{-}GalA)_2Hex_4$ fragments (Fig. 9). No fragments with Hex_4 chains at one Rha residue were found, which confirms that enzyme activity needs at least three linear Gal residues linked to the RG backbone to perform its activity.

4.4. RG-hydrolase action and the structure of the fraction A_EI

The major fragments obtained from the RG-backbone after RG-hydrolase treatment of the galactanase hydrolysate had the basic structure $(Rha\text{-}GalA)_{2\text{–}4}$, corresponding to the degree of polymerization from 4 to 8 (Table 3). This is explained by the properties of the enzyme used, which degrades the linkage between GalA and Rha, and requires at least nine sugars in a chain for cleaving fragments not shorter than four sugar residues (Mutter et al., 1998). The enzyme is not sensitive to the branching of Rha with single Gal (Mutter et al., 1998) and, according to our data, also not to branching with three Gal-units since such side chains have been shown to be present near the cleavage site Rha (Fig. 9).

Further insight into the mode of RG-hydrolase action is derived from study of fraction A_EI – the more or less enzyme resistant part of the polymer treated with combined galactanase and RG-hydrolase and collected in the void volume of Bio-Gel P6. The peak area of this fraction was close to the half of the total area of acidic fragments (Fig. 7b). Re-injection to the calibrated column (data not shown) demonstrated that the retention time of the peak during HPSEC was 27–28 min (Fig. 6b), indicating the molecular mass in the region of 10 kDa and thus, the dp around 60. It was too large to be analyzed by MALDI-TOF MS and was characterized by ^1H NMR (Fig. 2c). Compared to the original RG-1 type of structure a lower proportion of 1,4-Gal was characteristic for the fraction A_EI. This can be ascribed to the partial removal of linear chains by galactanase.

This fraction was either formed because of the inability of RG-hydrolase to degrade the RG-backbone or of galactanase to split off resistant parts of the side chains. If the RG-hydrolase is indeed insensitive to hindrance by the attached side chains, the major backbone fragments would still have dp 4, 6 or 8. As the total dp is around 60, the ratio between Gal and Rha would vary from (60–4):(4:2) = 28:1 to (60–8):(8:2) = 13:1.

If in addition the RG-hydrolase has also other restrictions to degrade the linkage of certain Rha, the minimal RG-fragment would contain more than eight sugars, i.e., a decasaccharide with at position 5 the enzyme resistant Rha residue. In this case the ratio between Gal and Rha would be (60–10):5 = 10:1 or lower, if the length of RG-backbone is longer than 10. Since the Gal:Rha ratio in the A_EI fraction is even lower than in the original polymer (3:1) the latter option seems likely. Thus, it can be concluded that RG-hydrolase action has to be restricted by the structure of side chains. With present data it is not possible to distinguish if this is the effect of the side chain structure at the cleavage site or at neighbouring branching Rha(s). However, since the side chain is longer than three residues and non-cleavable by galactanase, this must be due to the type of branching or other structural features, such as different type of Gal linkage.

4.5. Fine structure of the polymer

The partial hydrolysis of the galactan helped to describe the backbone and the side chains of the polymer in more detail. The $(Rha\text{-}GalA)_n$ chains with $n > 2$ without Gal were never observed after enzymatic hydrolysis, indicating that most of Rha residues are branched. Similar results were previously obtained by linkage analysis (Gorshkova et al., 1996).

There are several types of side chains. The Hex_2 and Hex_1 chains attached to a single Rha residue after enzymatic treatment (Fig. 9) indicate that such side chains are present in the original polymer, since it is unlikely that they are a result of the galactanase treatment. On the other hand, the presence of these short chains can be concluded from the presence of very long Gal chains and the Gal:Rha ratio of 3 in the initial polymer. Chains of up to 26 Hex units are seen after mild chemical hydrolysis (Fig. 4a), and may be even longer in the original polysaccharide. From these data it cannot be claimed that the galactan side chains cover the whole range from 3 to at least 26. TFA treatment trims off sugar chains, leading to a set of oligomers with a certain length distribution. But the presence of galactanase resistant pure Hex oligosaccharide fragments of dp 3–12 (Table 3) demonstrates that there are at least 10 different side chains released that also may be part of a larger chain. An oligosaccharide structure of 12 Gal residues, resistant to galactanase, must be branched at least twice (if the galactanase requires more than three linear Gal residues for cleavage).

The galactanase treatment cleaved off the neutral fragments, corresponding to $Hex_n + Pen_1$ with n ranging from 2 to 11. This is giving evidence that galactanase is cleaving between galactose units from the third glycosidic bond, indifferently from the sugar residue at the terminal position. The longer chains released most probably are of branched nature.

The RG-backbone is uniform in sugar sequence, but has parts with different properties. Approximately half of it is depolymerised by RG-hydrolase into 4, 6 and 8 sugar fragments (according to the enzyme properties), while the other half has resistant sites to enzyme action

– probably by the number and size of the Gal-side chains – and is cleaved only into larger fragments. The length of the RG-backbone in these fragments cannot be very high. In A_EI the Gal:Rha is 4:1 when the RG-backbone contains 20 sugars. Then the Rha residues from at least position 5 to 15 are not accessible for the enzyme. It should be noted that in these fragments unsubstituted Rha is more pronounced, than in initial polymer (Fig. 2b and c). Combining the high proportion of the terminal Gal and the smaller proportion of the branched Rha, we can assume that the Gal side chains, providing resistance to RG-hydrolase have branching points.

Additional complexity to the polymer could be given by the distribution of acetyl groups. Their presence is revealed by ^{1}H and ^{13}C NMR. The integration of the spectrum estimates the amount of acetyl groups as 1 per 4–5 Gal residues. However, the exact distribution of acetyl groups is not known. Acetylation of RG-I from mature flax fibre cell wall was described but without definite assignment of its position (Van Hazendonk et al., 1996). Galactanase has cleaved the Gal chains of both saponified and non-saponified polymer (data not shown). This could mean that either the enzyme is not sensitive to acetylation, or all acetyl groups are distributed along the backbone and linked to GalA as has been reported to be common in RG-I (Schols and Voragen, 2004).

In summary the fibre-specific galactan from flax stem is shown to be a very complex polysaccharide with varying structure of side chains. The backbone is composed of the GalA-Rha repeats with high degree of branching. The side chains are built mainly from β-1,4-linked Gal and are of several types: (1) side chains of only one or two Gal residues; (2) side chains of at least 26 Gal residues; (3) probably branched side chains from at least 3 to 12 Gal residues (could be originating from the longer side chains with linear portions), which are not cleaved by galactanase; (4) side chains of at least 17 Gal residues, decorated with single pentose, most likely Ara. Half of the polymer can be cleaved by the RG-hydrolase used, while the other has resistant sites, due to the special structure of branched side chains. The polymer is decorated by the acetyl groups, the distribution of which along the RG-backbone is unknown.

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