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Carbohydrate Polymers 48 (2002) 263–269

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Pectic substances from red beet (*Beta vulgaris* L. var. *conditiva*). Part II. Structural characterisation of rhamnogalacturonan II

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Received 12 July 2000; revised 27 September 2000; accepted 26 April 2001

Abstract

Cell wall material from ripe red beet (*Beta vulgaris* L. var. *conditiva*) was isolated as alcohol-insoluble residue (AIR). The cyclohexane-*trans*-1,2-diaminotetraacetate (CDTA) soluble extract (CDTA-soluble pectin) was fractionated by anion exchange and gel filtration chromatography. The main fraction was degraded with an *endo*-α-(1 → 4)-polygalacturonase and further fractionated by gel filtration chromatography. Methylation analysis of the intermediate-molecular weight fractions was performed after carbodiimide-activated reduction of the pectic polysaccharides with $NaBD_4$. Besides galacturonic acid and rhamnose, the fraction contained rare sugar residues, such as apiose, 2-*O*-methyl-xylose, 2-*O*-methyl-fucose, 3,4-linked fucose, 2,3,4-linked rhamnose, 2-linked glucuronic acid, aceric acid, 3-deoxy-D-manno-2-octulosonic acid (Kdo), and 3-deoxy-D-lyxo-2-heptulosonic acid (Dha) which are characteristic for rhamnogalacturonan II (RG-II). The constituents were present in amounts which approximately corresponded to the structural model for RG-II. The molecular weight of RG-II was determined by MALDI-TOF-MS and showed to contain two populations with molecular weights of about 4100 and 4300, respectively.

Arabinopyranose residues were shown to occur only terminally linked. This result suggests that the aceric acid containing side-chain of red beet RG-II is shorter than the one from other plants. 1,3,4-linked galactose and 1,2,3,4-linked galacturonic acid were detected as well, their positions within the RG-II molecule remain to be established.

Keywords: Red beet pectic substances; Rhamnogalacturonan II; Characterisation

1. Introduction

Pectins are a group of polysaccharides from the primary cell wall and the middle lamella of higher plants. They have been investigated extensively for their structure and functions within the plant cell wall using chemical analysis and enzymatic degradation (Pellerin, Doco, Vidal, Williams, Brillouet & O'Neill, 1996; Shin, Kiyohara, Matsumoto & Yamada, 1997). Different types of pectic substances, homogalacturonan, rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) have been isolated and characterised from different sources. RG-II is released from CDTA-soluble pectin by treatment with *endo*-α-(1 → 4)-polygalacturonase or with pectinases. It is described to be a low molecular-weight (5–10 kDa), structurally complex pectic polysaccharide (Vidal et al., 2000; Whitcombe, O'Neill, Steffan, Albersheim & Darvill, 1995). RG-II has been isolated from the walls of suspension-cultured sycamore cells (Darvill, McNeil & Albersheim, 1978), Douglas fir (Thomas, McNeil, Darvill & Albersheim, 1987), rice (Thomas, Darvill & Albersheim, 1989), onion (Ishii, 1982), kiwi fruits (Redgwell, Melton & Brasch, 1988; Redgwell, Melton, Brasch & Coddington, 1992), and red wine (Doco & Brillouet, 1993; Pellerin et al., 1996). Doco, Williams, Vidal & Pellerin (1997) isolated RG-II from apple, tomato and carrot juices treated with two commerical liquefying enzyme preparations. Moreover, Matoh, Kawaguchi and Kobayashi (1996) showed RG-II to be present in several plant species belonging to the families of Brassicaceae, Cucurbitaceae, Leguminosae, Apiaceae, Liliaceae, Araceae, Amaryllidaceae, and Gramineae. The structure of RG-II has been shown to be virtually the same in every plant analysed hitherto, what is most unusual. It contains a homogalacturonan backbone composed of at least eight 1 → 4 linked α-D-galactosyluronic acid residues. Four different complex oligoglycosyl side-chains are attached to this backbone. The preferred conformations of the four side-chains of RG-II have been determined by Mazeau and Pérez (1998) by computational conformational analysis. The general basic structure of RG-II as published by Vidal et al. (2000) is shown in Fig. 1. Variations in the molecular weight of this highly conserved polysaccharide structure could result from differences in the

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PII: S0144-8617(01)00257-0

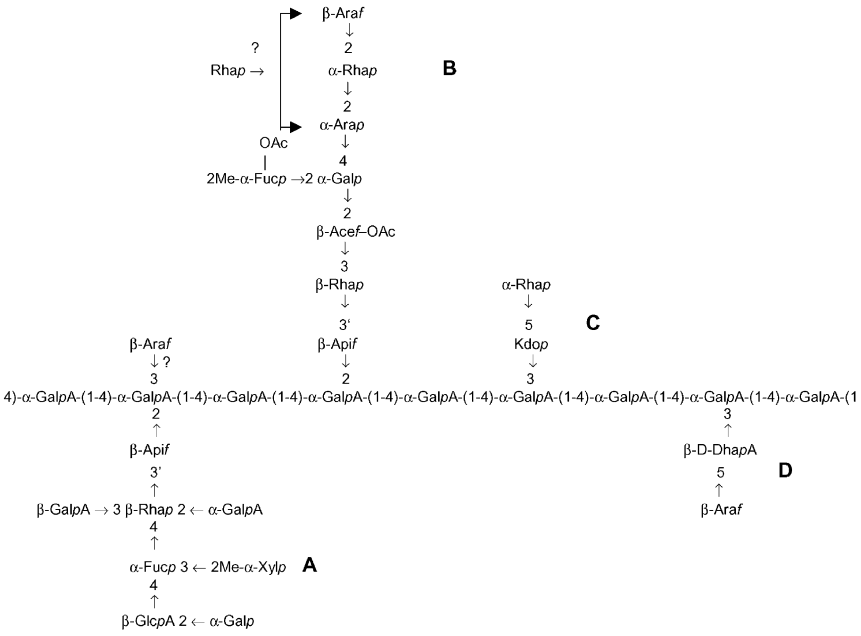

Fig. 1. Structural model of RG-II (from Vidal et al., 2000).

length of the homogalacturonan backbone or from the loss of terminal non-reducing residues attached to the side-chains (Brett & Waldron, 1990; Shin et al., 1997).

The biological function of RG-II is related to its ability to form complexes with boron in the cell wall. Boron being an essential micronutrient for plants, the importance of RG-II as boron carrier is evident. RG-II predominantely exists as a dimer, covalently linked by a 1:2 borate diol ester (Matoh et al., 1996; O'Neill et al., 1996; Kobayashi, Matoh & Azuma, 1996). This cross-linking is thought to result in the formation of a covalently cross-linked pectic matrix (Albersheim et al., 1996; Ishii et al., 1999) which could be involved in the regulation of pore size and other physical properties of the primary wall (Fleischer, O'Neill & Ehwald, 1999; Kobayashi, Nakagawa, Asaka & Matoh, 1999).

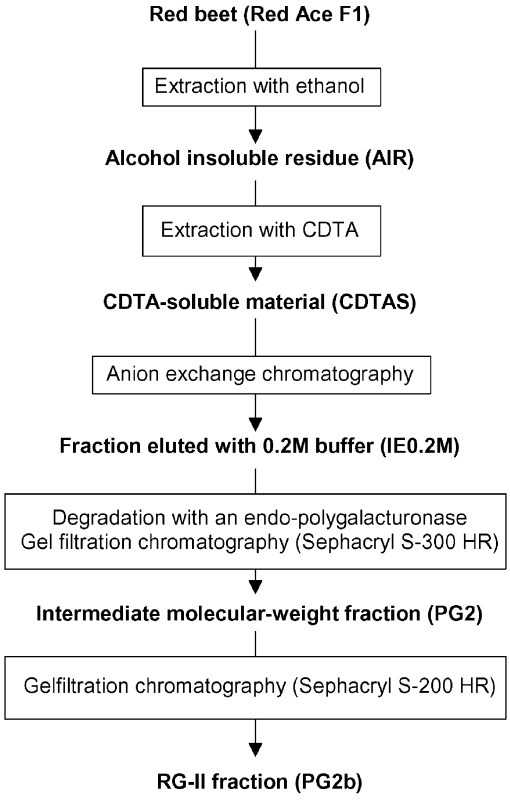

Fig. 2. Scheme for the isolation of the RG-II fraction from red beet cell wall material.

The objective of our work was the characterisation of the chelator-soluble pectic substances from ripe red beet (*Beta vulgaris* L. var. *conditiva*). In part I of this paper, the isolation and characterisation of RG-I was described (Strasser & Amadò, 2001). The present paper describes the characterisation of an RG-II present in the chelator-soluble pectic substances of ripe red beet. RG-II has been obtained from the alcohol-insoluble residue (AIR) by extraction with CDTA, fractionation by a anion-exchange chromatography and gel filtration, enzymatic degradation with a purified *endo*-α-(1 → 4)-polygalacturonase and methylation analysis as previously described (Strasser & Amadò, 2001).

2. Experimental

Isolation of RG-II fractions. Preparation of the AIR, extraction of the chelator-soluble pectin fraction with 1,2-diaminocyclohexane-*N*,*N*,*N'*,*N'*-tetraacetic acid (CDTA-soluble pectin), ion-exchange chromatography (AEC) and its degradation with *endo*-α-(1 → 4)-polygalacturonase (*endo*-PG) was described in part I of this paper (Strasser

Table 1
Glycosyl-residue composition (mol%), yield (%) and reference data for the methylation analysis of red beet pectic fractions (see Fig. 2)

Glycosyl residue	Pectic fraction			
	CDTAS	IE0.2M	PG2	PG2b
Arabinose	19.7	16.8	29.7	14.2
Fucose	0.3	0.3	3.6	6.1
Galactose	10.1	7.1	12.2	11.6
Glucose	1.4	0.9	1.1	0.6
Mannose	0.0	0.0	0.5	0.0
Rhamnose	2.4	2.1	10.1	12.8
Xylose	0.4	0.4	2.4	3.4
Apiose	0.6	0.6	5.6	8.5
GalA	63.2	70.3	32.1	37.4
GlcA	1.8	1.5	2.7	5.5
$r_{B/T}$	1.07	1.07	1.01	0.75
$r_{L/T}$	6.0	7.3	1.5	1.1
$r_{L/B}$	5.6	6.8	1.5	1.7
r_L	5.8	7.1	1.5	1.4
Yield[a]	6.6	60.0	11.0	62.0

[a] Expressed as % dry matter of the preceeding fraction.

& Amadò, 2001). The *endo*-PG was free of lyase activity (Elgorriaga, 1994). The intermediate molecular-weight fractions obtained by gel filtration chromatography (GFC, PG2-fractions, Fig. 2 in Strasser and Amadò, 2001) were further fractionated by gel filtration chromatography using Sephacryl S-200 HR (Pharmacia Biotech, Uppsala, S) on a $95 \times 2.6\ cm^2$ column using 0.05 M sodium acetate buffer (pH 5.0, containing 0.01% NaN_3) as eluent. The separations were monitored using a HP 1037A RI-Detector (Hewlett Packard) at 30°C. Corresponding fractions were pooled, dialysed, and freeze-dried (Fraction: PG2b). A schematic view of the isolation procedure is shown in Fig. 2.

Enzymatic degradation with α-L-*arabinofuranosidase*. Fraction PG2b was enzymatically degraded as described in Strasser and Amadò (2001).

Linkage analysis. Linkage analysis was performed after carbodiimide-activated reduction with $NaBD_4$ by methylation analysis as described in Strasser and Amadò (2001).

Qualitative determination of 3-*deoxy*-D-*manno*-2-*octulosonic acid* (*Kdo*), *and* 3-*deoxy*-D-*lyxo*-2-*heptulosonic acid* (*Dha*). The sum of Kdo and Dha was determined by the thiobarbituric acid method described by (York, Darvill, McNeill, & Albersheim, 1985). The method is based on the oxidative cleavage of the carbohydrate with periodate. The liberated 2,4-dioxo-butyric acid reacts in an acidic medium with two molecules of thiobarbituric acid to form a red-coloured complex which is measured at a wavelength of 548 nm.

MALDI-TOF-MS. Matrix-assisted laser desorption time-of-flight mass spectrometry was performed at the Laboratory of Organic Chemistry ETH (Dr W. Amrein) on a Bruker Reflex spectrometer (Bruker Instruments Inc., Billerica, MA, USA) in the positive ion mode. The fraction PG2bAF1 was dissolved in water/acetonitrile (5:1). This solution was diluted 2:1 in a mixture consisting of two parts of 2,4,6-trihydroxy-acetophenone (0.5 M in ethanol) and one part of diammonium citrate (0.1 M in water).

3. Results and discussion

The intermediate molecular weight fraction PG2 was obtained as described by Strasser and Amadò (2001) and shown schematically in Fig. 2. The yield, the glycosyl-residue composition and the methylation analysis reference data of the different pectic fractions are shown in Table 1. The low r_L-value of fraction PG2 indicated this fraction to be less linear as the fractions CDTAS and IE0.2M ($r_L = 5.8$ and 7.1, respectively). This was attributed to an almost exclusive degradation of the homogalacturonan region by the *endo*-PG. Within the neutral sugars, the relative amount of arabinose and galactose was reduced, whereas the values for

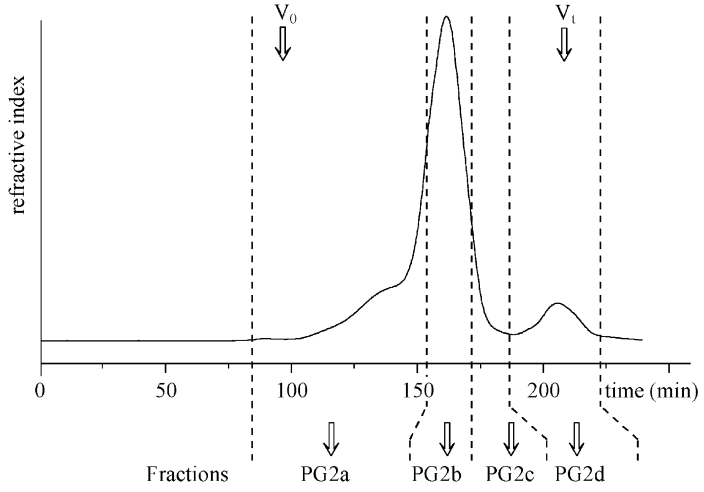

Fig. 3. Gelfiltration chromatography (Sephacryl S-200 HR) of the intermediate molecular-weight fraction (PG2) obtained from CDTA-soluble red beet pectin after degradation with a purified PG.

Table 2
Glycosyl-linkage composition (mol%) of red beet pectic fractions (details on the different fractions see text and Fig. 2)

Glycosyl residue	CDTAS	IE0.2M	PG2	PG2b
T-Ara*f*	7.7	6.3	13.1	8.2
T-Ara*p*	0.3	0.3	0.3	2.6
1,2-Ara*f*	0.4	0.4	0.4	0.0
1,3-Ara*f*	0.7	0.6	1.4	0.3
1,3-Ara*p*	0.0	0.0	0.4	0.4
1,5-Ara*f*	5.8	4.7	6.1	1.1
1,2,5-Ara*f*	0.0	0.0	1.6	1.3
1,3,5-Ara*f*	4.0	3.8	5.5	0.0
1,2,3,5-Ara*f*	0.7	0.6	1.1	0.2
T-Fuc*p*	3.0	0.3	2.1	3.9
1,3,4-Fuc*p*	0.1	0.1	1.4	2.2
T-Gal*p*	0.9	0.6	2.8	4.8
1,3-Gal*p*	2.3	1.4	0.7	0.4
1,4-Gal*p*	1.2	1.0	2.8	0.0
1,6-Gal*p*	0.8	0.6	1.4	0.7
1,2,4-Gal*p*	0.3	0.3	1.9	2.8
1,3,4-Gal*p*	0.3	0.2	1.9	2.9
1,3,6-Gal*p*	4.4	2.9	0.8	0.0
T-Glc*p*	0.3	0.1	0.0	0.3
1,4-Glc*p*	1.1	0.8	1.1	0.4
1,4-Man*p*	0.0	0.0	0.5	0.0
T-Rha*p*	0.5	0.4	1.3	2.6
1,2-Rha*p*	0.6	0.6	2.1	2.9
1,3-Rha*p*	0.3	0.4	1.7	2.2
1,2,3-Rha*p*	0.1	0.1	0.6	0.6
1,2,4-Rha*p*	1.0	0.7	1.8	0.8
1,2,3,4-Rha*p*	0.0	0.0	2.5	3.7
T-Xyl*p*	0.3	0.3	2.0	3.4
1,4-Xyl*p*	0.1	0.1	0.4	0.0
1,3′-Api*f*	0.6	0.6	5.6	8.5
T-GalA*p*	0.6	0.7	7.0	11.3
1,4-GalA*p*	60.7	67.4	18.4	19.6
1,2,4-GalA*p*	0.4	0.5	3.9	3.5
1,3,4-GalA*p*	0.9	0.8	2.1	2.2
1,4,6-GalA*p*	0.7	0.8	0.7	0.0
1,2,3,4-GalA*p*	0.0	0.0	0.0	0.9
T-GlcA*p*	1.6	1.5	0.6	0.0
1,2-GlcA*p*	0.0	0.0	1.9	5.5
Total	100.0	100.0	100.0	100.0

fucose, rhamnose, xylose and particularly apiose were increased. Fraction PG2 was further fractionated by gel filtration to yield the fractions PG2a (27%), PG2b (62%), PG2c (7%), and PG2d (4%), respectively (Fig. 3). Fraction PG2b consisted almost entirely of sugar residues, which are known to occur in RG-II (Table 1), furthermore methylation analysis revealed PG2b to be a RG-II-like polysaccharide (Table 2). The $r_{B/T}$-value of 0.75 was low, but it has to be taken into account that the theoretical value of 1.0 can only be attained by high-molecular-weight, branched polysaccharides. However, the value was also too low, when compared to the calculated $r_{B/T}$-value of 0.88 for the RG-II structural model presented in Fig. 1.

Fucose and xylose. T-2-*O*-Me-Fuc*p*, T-2-*O*-Me-Xyl*p*, and 1,3,4-Fuc*p* are expected to be part of RG-II (Fig. 1). Due to the permethylation with CH_3I naturally methylated and naturally non-methylated sugars cannot be distinguished. Therefore, an additional methylation analysis was performed using CD_3I instead of CH_3I as methylation reagent. The resulting mass spectra allowed the identification of T-2-*O*-Me-Fuc*p* and T-2-*O*-Me-Xyl*p*. Other *O*-methylated sugars were not detected. 1,3,4-Fuc*p* was present in small amounts as well. This could be due to the instability of deoxy sugars during acid hydrolysis. Traces of 1,4-Xyl*p* could be identified as impurity.

Apiose. 1,3′-Api*f* is a typical residue of RG-II and is expected to occur twice per RG-II-molecule (Fig. 1). 1,3′-Api*f* was found in the PG2b-fraction in a relative amount of 8.5 mol%. This result indicates the presence of two Api*f* residues in the red beet RG-II molecule.

Galactose. T-Gal*p* and 1,2,4-Gal*p* are typical residues of RG-II and could be detected in amounts corresponding to the structural model given in Fig. 1. Moreover 1,3,4-Gal*p* was found in surprisingly high amounts. This residue has not been postulated to be present in the structural model for RG-II. However 1,3,4-Gal*p* has been identified in comparable amounts in RG-II-fractions of red wine (Doco & Brillouet, 1993; Pellerin et al., 1996), sugar beets (Ishii & Matsunaga, 1996), and carrots (Doco et al., 1997), respectively. It may be postulated that this sugar residue represent an additional component of some RG-II varieties.

Arabinose. According to the published structural models, two different types of arabinose residues are present in RG-II, namely a β-glycosidically linked T-Ara*f* and a 1,2- or 1,2,3-linked Ara*p*. In order to evaluate the presence of terminally α-linked Ara*f* residues, fraction PG2b was additionally incubated with an α-L-arabinofuranosidase. By this treatment, only a few terminal Ara*f* residues could be removed. Therefore, it can be concluded that terminal arabinose residues present in red beet RG-II are β-glycosidically linked. 1,2- or 1,2,3-linked arabinopyranose could not be detected in any sample. Since Wechsler (1997) could identify 1,2,3-Ara*p* in apple pectic substances using exactly the same analytical technique as here, losses due to methodological inconveniences can most probably be excluded. T-Ara*p* was present in surprisingly high amounts, indicating that some variations within RG-II may take place within the aceric acid containing side-chain. Similar results were described by Ishii and Matsunaga (1996) for sugar beet RG-II.

Rhamnose. Several differently linked rhamnose residues are expected in RG-II molecules (Fig. 1). Our results were consistent with the expectations and revealed the presence of T-, 1,2-, 1,3-, and 1,2,3,4-Rha*p*, respectively. Impurities of 1,2,3-, and 1,2,4-Rha*p* were detected as well.

Glucuronic acid. 1,2-GlcA*p*, an other characteristic constituent of RG-II, was identified in the PG2b-fraction in amounts corresponding to the structural model shown in Fig. 1.

Galacturonic acid. The high content of galacturonic acid (37.4 mol%) in the PG2b-fraction is due to the backbone of RG-II which contains exclusively galacturonic acid.

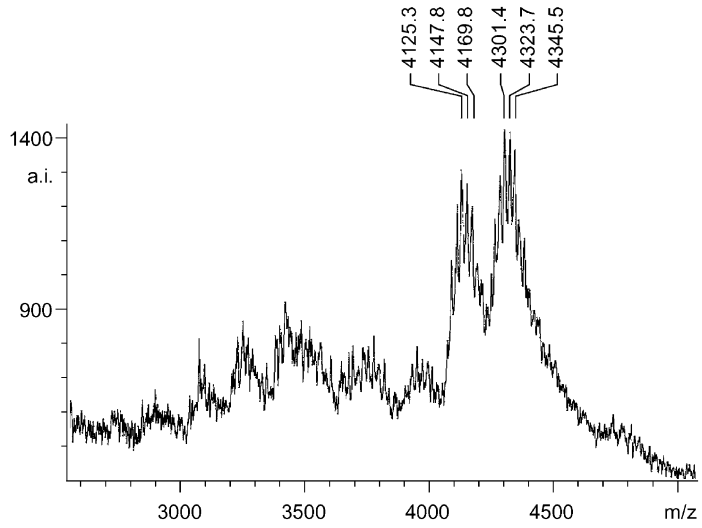

Fig. 4. Detail of a MALDI-TOF-mass spectrum of the RG-II fraction of red beet (PG2b after α-L-arabinofuranosidase incubation).

Besides the linear 1,4-linked GalA*p,* the branched residues 1,2,4- and 1,3,4-GalA*p* have been shown to be present in considerable amounts in the backbone. Additionally some 1,2,3,4-GalA*p* were detected, indicating the presence of double branched residues. This residue has also been identified RG-II from red wine (Doco & Brillouet, 1993; Pellerin et al., 1996), apples (Wechsler, 1997), and carrots (Doco et al., 1997). The presence of considerable amounts of T-GalA*p* in the PG2b-fraction may be explained by the fact that the RG-II molecule was obtained by endo-PG degradation. Moreover, according to the structural model of RG-II, two terminally linked galacturonic acid residues are present in the uronic acid rich side-chain.

Aceric acid, Kdo, Dha. These rare constituents are characteristic for RG-II. The method for methylation analysis used in this work is known to destroy Kdo and Dha (Wechsler, 1997), it was therefore not possible to identify them together with the other constituents. However, it was possible to determine qualitatively Kdo and Dha after oxidative cleavage with periodate by a photometric method using thiobarbituric acid (York et al., 1985). The presence of Kdo and Dha in the PG2b-fraction has been clearly demonstrated.

Glucose and mannose. Glucose and mannose are not thought to be components of RG-II. The detected traces might originate from impurities.

Molecular weight of RG-II. The molecular weight of RG-II was determined by MALDI-TOF-MS on the PG2b-fraction after treatment with an α-L-arabinofuranosidase (Fig. 4). Two signals, corresponding to a molecular weight

Table 3
Glycosyl-residue composition (mol%) of RG-II fractions isolated from different sources (n.d.: not determined)

Glycosyl residue	RG-II sources				
	Sycamore[a]	Sugar beet[b]	Red wine[c]	Apple juice[c]	Red beet[d]
Ara	10.8	10.9	11.2	16.8	14.2
Rha	12.4	11.3	16.8	17.4	12.8
Gal	9.4	12.4	6.6	6.4	11.6
Fuc	2.8	1.6	3.0	5.5	2.2
2-*O*-Me-Fuc	3.5	3.3	3.7	4.8	3.9
2-*O*-Me-Xyl	4.8	4.9	2.8	2.9	3.4
Api	12.2	12.4	6.2	5.4	8.5
GalA	31.2	37.7	38.2	33.0	37.4
GlcA	3.2	7.0	3.3	2.8	5.5
Aceric acid	3.5	–[e]	1.2	1.5	n.d.
Kdo	3.5	5.3	2.6	1.1	–[e]
Dha	3.5	5.3	4.4	2.5	–[e]

[a] Stevenson, Darvill and Albersheim (1988).
[b] Ishii and Matsunaga (1996).
[c] Doco et al. (1997).
[d] PG2b-fraction (see Fig. 2 and Table 1).
[e] Present but not quantified.

of 4301.4 and 4125.3 Da, respectively, and differing in *m/z* 176, were obtained. This result reflects the presence of two RG-II-populations with a difference of one residue in the backbone (176 corresponds to the mass of an anhydro uronic acid residue). The fine structure of the exceptionally broad signals showed mass differences of 22. This can be explained by the presence of salt adducts ($[M + H]^+$, $[M + Na]^+$, $[M - H + 2Na]^+$,…) often found in anionic carbohydrates (O'Neill et al., 1996). Both signals indicated distinctly lower molecular weights than those measured for RG-II of sycamore ($M_w = 4941$ Da) and red wine ($M_w = 4713$ Da), respectively (O'Neill et al., 1996). This might be due to differences within the side-chain structures of RG-II.

4. Conclusions

Though the structure of RG-II is highly conserved (Mazeau & Pérez, 1998), a variability of this complex polysaccharide from species to species was demonstrated by several authors. RG-II from red beet showed a similar pattern of sugar residues when compared to RG-II isolated form other sources (Table 3). Some differences, particularly the missing branched and linear arabinopyranose residues, the presence of a terminal arabinopyranose, and the low molecular-weight indicate differences within the aceric acid containing side-chain (side-chain B in Fig. 1). It could be that the postulated terminal β-Ara*f*→2-α-Rha*p* residues in the B-chain are absent in the red beet RG-II. Additional variations are likely to occur because of the presence of residues such as 1,3,4-Gal*p* and 1,2,3,4-GalA*p*, respectively. Further studies are necessary to elucidate in more details the nature of the side-chains of RG-II isolated from red beet.

Acknowledgements

We thank Novo-Nordisk AS (Bagsvaerd, Denmark) for the generous gift of enzymes and Dr W. Amrein for carrying out the MALDI-TOF-MS measurements.

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