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Rhamnogalacturonan I in *Solanum tuberosum* tubers contains complex arabinogalactan structures

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Abstract

A rhamnogalacturonan I polysaccharide was isolated from potato (*Solanum tuberosum* cv. Posmo) tuber cell walls and characterised by enzymatic digestion with an endo-β-1 → 4-galactanase and an endo-α-1 → 5-arabinanase, individually or in combination. The reaction products were separated using size-exclusion chromatography and further analysed for monosaccharide composition and presence of epitopes using the LM5 anti-β-1 → 4-galactan and LM6 anti-α-1 → 5-arabinan monoclonal antibodies. The analyses point to distinct structural features of potato tuber rhamnogalacturonan I, such as the abundance of β-1 → 4-galactan side chains that are poorly substituted with short arabinose-containing side chains, the presence of α-1 → 5-arabinan side chains substituted with β-1 → 4-galactan oligomers (degree of polymerisation >4), and the presence of α-1 → 5-arabinans that resist enzymatic degradation. A synergy between the enzymes was observed towards the degradation of arabinans but not towards the degradation of galactans. The effect of the enzymes on isolated RG I is discussed in relation to documented effects of enzymes heterologously expressed in potato tubers. In addition, a novel and rapid method for the determination of the monosaccharide and uronic acid composition of cell wall polysaccharides using high-performance anion exchange chromatography with pulsed amperometric detection is described.

Keywords: *Solanum tuberosum*; Solanaceae; Potato; Pectin; Rhamnogalacturonan I; Endo-galactanase; Endo-arabinanase; *Aspergillus niger*; ELISA; HPAEC-PAD

1. Introduction

Rhamnogalacturonan I (RG I) is a pectic polysaccharide found abundantly in primary cell walls of dicotyledonous plants (Schols et al., 1994), including the cell walls of potato tuber cells (Jarvis et al., 1981; van Marle et al., 1997). RG I is a structurally complex molecule, consisting of a backbone of -4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1- repeating units. The rhamnose residues often carry side chain additions of neutral sugars attached at the 4 position. These side chains typically contain β-1 → 4-linked-galactan, α-1 → 5-linked arabinan or branched type I arabinogalactans (Carpita and Gibeaut, 1993; Ridley et al., 2001). Experimental evidence suggests that RG I is covalently linked to other pectic polysaccharides including homogalacturonan, rhamnogalacturonan II (RG II), and xylogalacturonan since they can all be extracted from cell walls using endo-polygalacturonase (endo-PG; Schols et al., 1995). However, the exact molecular arrangement of the different pectic molecules relative to one another is not certain (Vincken et al., 2003).

The occurrence of RG I, and its side chains, are temporally regulated during potato tuber development (Bush et al., 2001) and spatially regulated in both stolon (Bush et al., 2001) and tuber cell walls (Bush and McCann, 1999). For instance, in potato tuber parenchyma,

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the LM6-arabinan epitope (Jones et al., 1997) was detected throughout the cell wall, while the LM5-galactan epitope (Willats et al., 1998) was detected in regions of the primary cell wall close to the plasma membrane. In contrast, homogalacturonan epitopes were detected throughout the cell wall, but most abundantly in the middle lamellae and at cell corners. The precise functions of RG I during potato tuber development are still not known. A number of enzymes have been isolated that degrade RG I (for a review see Prade et al., 1999). The backbone may be fragmented with RG-endohydrolases and RG-endolyases. Galactan is degraded by endo-galactanases and galactosidases, whereas arabinan can be degraded by endo-arabinanases and arabinofuranosidases. These enzymes are widely used for the structural characterisation of pectin preparations (Schols et al., 1995; Prade et al., 1999). Some of these enzymes have also been used in transgenic experiments with the goal of modifying pectin structure in native potato cell walls. Over-expression of a secreted *Aspergillus aculeatus* endo-galactanase decreased the RG I-galactose content in the tubers by 70% (Sørensen et al., 2000). Despite of this, the transgenic plants did not show alterations in plant or tuber development. In a similar experiment, expression of a secreted *A. aculeatus* endo-arabinanase resulted in a severely altered growth and inability of the plants to initiate tuber formation (Skjøt et al., 2002). Targeting of the same enzyme to the Golgi apparatus resulted in viable plants with no visual phenotype and normal tuber formation. The tubers showed a reduction of 70% in the level of RG I-arabinose (Skjøt et al., 2002). Expression of a secreted *A. aculeatus* RG lyase resulted in tubers with clear morphological alterations, including radial swelling of the periderm cells and development of intercellular spaces in the cortex. Sugar compositional analysis of the isolated cell walls showed a large reduction in galactosyl and arabinosyl residues in transgenic tubers (Oomen et al., 2002). These results point to diverse functions of RG I in potato tuber development, and to the ability of plants to functionally compensate for the lack of one cell wall component.

In industrial applications, potato tuber pectin is an abundant residual material after starch extraction. However, it has low economical value due to poor gelling properties, which have been associated with an elevated content of neutral sugars (Mayer and Hillebrandt, 1997). Recently, possible new applications of RG I-rich pectins as pharmaceutical agents have been put forward. A galactoside containing molecule that was derived from pectin has been shown to interact with a galectin 3-type lectin at the surface of proliferating mammalian cancer cells. The interaction prevented cell-cell interactions and therefore tumor growth (Nangia-Makker et al., 2002; Inohara and Raz, 1994). Galactoside containing pectins may also have positive effects on human immunity (Duan et al., 2003). It is therefore of interest to study the structural properties of potato RG I in more detail, and with the use of enzymes, to specifically modify its structure (in planta or in vitro) in order to obtain RG I molecules with interesting functionalities. RG I rich fractions that are highly substituted with β-1 → 4-galactans and α-1 → 5-arabinans have been extracted from potato cell wall preparations using sequential solvent extraction (Jarvis et al., 1981; van Marle et al., 1997). However, despite interest in potato cell walls and RG I modification using transgene technology, the structural features of potato RG I have not been reported in detail.

This paper describes the isolation and purification of RG I from potato tuber cell walls. The purified RG I preparation was analysed for monosaccharide composition and used as a substrate for enzymatic degradation with *Aspergillus niger* endo-β-1 → 4-galactanase and endo-α-1 → 5-arabinanase, individually or in combination. The effect of degradation on the composition of RG I and presence of epitopes using anti-pectin antibodies were tested. The implications of our findings for the elucidation of the structural properties of RG I in potato tubers cell walls is discussed. In addition, a novel rapid method for the determination of the monosaccharide and uronic acid composition using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is described.

2. Results

2.1. An improved and rapid method for cell wall composition analysis by HPAEC-PAD

An HPAEC method was developed that separated most common cell wall monosaccharides and uronic acids in one chromatographic run using the newly available PA-20 column. The advantage of the method presented here over previously published methods, as described for example by Gardner et al. (2003), is that PAD detection of the sugars is achieved without the need for post-column base addition as the separation is achieved with a base concentration sufficient to ensure PAD detection (see Experimental for details). Using the standard PA-1 column, baseline separation of Rha/Ara and Xyl/Man is not possible as conditions for the separation of one of the pairs contradict the separation of the other pair of sugar. In contrast, baseline separation of all four sugars is possible using the presented method and the PA-20 column. PAD response factors were linear up to 0.2 μg monosaccharide injected. This method was used for the analysis of the monosaccharide composition of TFA-hydrolysed cell wall material and TFA-hydrolysed size-exclusion chromatography (SEC) fractions (Fig. 1). The method was also used for the

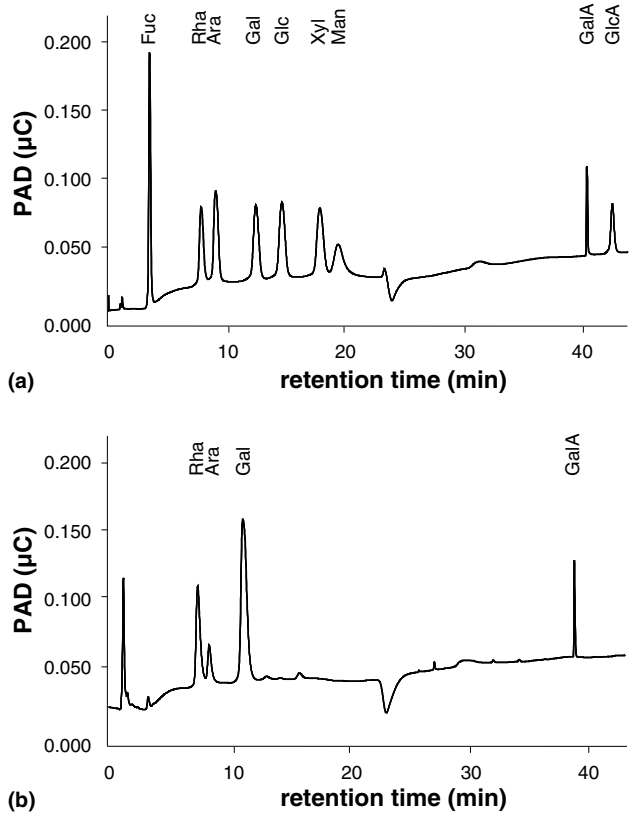

Fig. 1. HPAEC separation of common cell wall monosaccharides on a PA-20 column. Linear response PAD factors were obtained for up to 0.2 μg monosaccharide without post-column addition of base. (a) Standard run showing resolution of neutral monosaccharides and uronic acids (0.2 μg of each.). (b) TFA hydrolysate of purified RG I showing the abundance of Rha, Ara, Gal and GalUA.

quantification of monosaccharides produced upon enzymatic digestion of polysaccharide (data not shown). The method can be used to resolve neutral sugars in H_2SO_4-hydrolysed material; however uronic acids are lost during the required desalting step (data not shown).

2.2. Preparation of RG I from potato cell wall material

A procedure was designed to extract RG I from the cell wall material that included a mild base treatment to remove methyl and acetyl esters without pectin degradation by β-elimination, followed by degradation of de-esterified homogalacturonan with endo-polygalacturonase (endo-PG) from *A. niger*. An extensive dialysis step was carried out after the endo-PG digestion on both the extract and the residue in order to remove low molecular mass products. The crude RG I represented ca. 40% of the cell wall (dry wt). The extract and residue were analysed for sugar composition (Table 1). The crude RG I extract was enriched in Gal, Ara, GalUA and Rha, indicating that the procedure extracted branched RG I. Glucose indicative of residual starch was present in both extract and residue. The crude RG I preparation contained only traces of Xyl, suggesting that xylogalacturonan was not extracted by the procedure. The residue (about 60% of the cell wall) still contained high quantities of GalUA, Rha, Gal, Ara, indicating that the procedure did not extract all RG I polysaccharide. The residual RG I may be cross-linked in the cell wall by covalent interactions with cell wall components other than homogalacturonan or by physical entanglement. Interactions of RG I polysaccharide with xyloglucan and cellulose have been suggested to occur (van Marle et al., 1997). The ratio of GalUA to Rha in the crude RG I extract was of 3:1. SEC fractionation of the extract showed that the extract contained three populations of carbohydrate (Fig. 2), one high-M_r polysaccharide population (pool I, 50–>500 kDa) containing both neutral sugars and uronic acids, a medium M_r and less abundant polysaccharide population (pool II, 12–50 kDa) also containing both neutral sugars and uronic acids, and a low M_r oligosaccharide population (pool III, 0.2–12 kDa) containing mainly uronic acids. Pool II has been suggested to be RG II oligosaccharides (Sørensen et al., 2000) and was not analysed further. Pool III represented negatively charged oligogalacturonides, which did not effectively dialyse out of the pectic extract, as has been observed by Mort et al. (1990). Pool I corresponded to about 90% (w/w) of the crude RG I extract and was analysed for sugar composition (Table 1). This polysaccharide population contained GalUA, Rha, Gal and Ara in a ratio 1:0.8:12:3. Glucose could not be detected confirming that residual starch had been efficiently removed. The ratio of GalUA:Rha larger than 1 pointed to the presence of GalUA oligomeric units in the RG I backbone which are nonetheless not susceptible to endo-PG cleavage, and are therefore smaller than a tetramer. The ratio of Rha: Gal + Ara suggests that the purified RG I is heavily branched, and the ratio of Gal:Ara of 4:1 suggests that galactans are the most abundant substitutions. This has been previously suggested (van Marle et al., 1997).

Table 1
Monosaccharide composition (mol%) of cell wall material (CWM), crude RG I extract, residue after RG I extraction, and purified RG I

Monosaccharides	CWM	RG I extract	Residue	Purified RG I
Fuc	2.6	n/d	4.1	n/d
Rha	3.4	4.7	2.7	5.0
Ara	10.7	18.2	15.3	17.5
Gal	32.2	62.1	45.3	71.6
Xyl	3.0	n/d	7.9	n/d
Man	0.9	n/d	0.8	n/d
GalUA	47.2	15.0	23.7	5.9
GlcA	n/d	n/d	n/d	n/d

n/d, not detected.

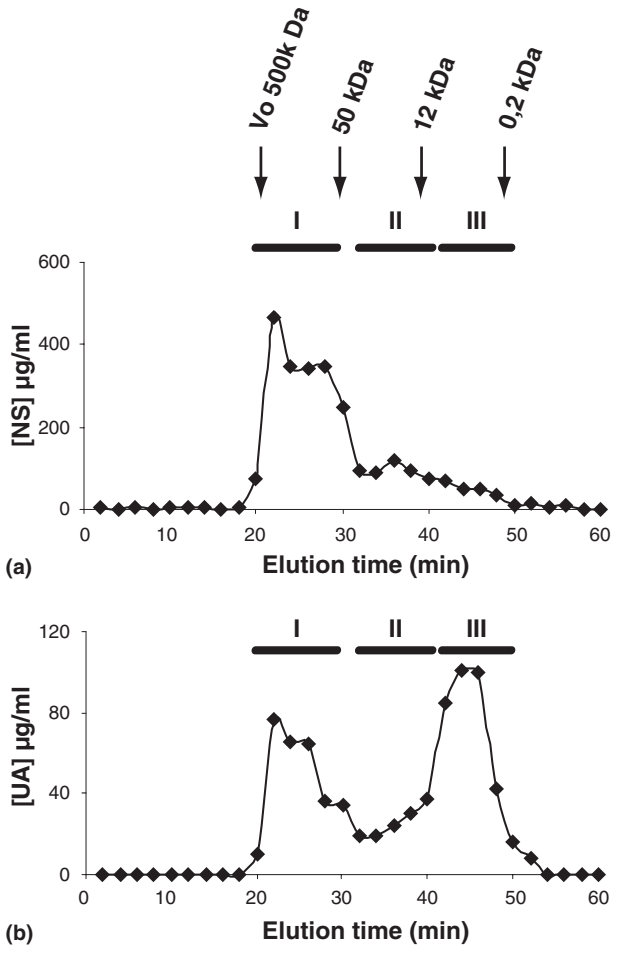

Fig. 2. SEC of crude RG I preparation on a Superose 12 column. Fractions were analysed for neutral sugar (NS) content (a) and uronic acid (UA) content (b). Three populations of carbohydrate were identified as I (RG I), II (possibly RG II) and III (oligogalacturonides). Pool I was used as purified RG I in subsequent analyses. Calibration was performed using dextran standards and glucose. V_0: void volume.

2.3. Effect of fungal endo-galactanase and endo-arabinanase treatment on RG I composition

In order to gain additional information on the structural properties of the SEC-purified RG I (pool I), this polysaccharide was treated with endo-β-1 → 4-D-galactanase and endo-α-1 → 5-L-arabinanase, individually or in combination. Both enzymes are mono-component enzymes derived from the filamentous fungus *A. niger*. The RG I degradation products were fractionated by SEC (Fig. 3). Two polysaccharide pools were identified: a high-M_r pool (Ia) and a low-M_r pool (Ib). The Ara, Gal and GalUA content relative to Rha content of pool Ia is shown in Table 2. Endo-galactanase treatment decreased the Gal content in pool Ia by 84%, compared to the buffer control treatment. The extent of degradation is 14% higher than was observed when a similar fungal enzyme from *A. aculeatus* was heterologously expressed in potato

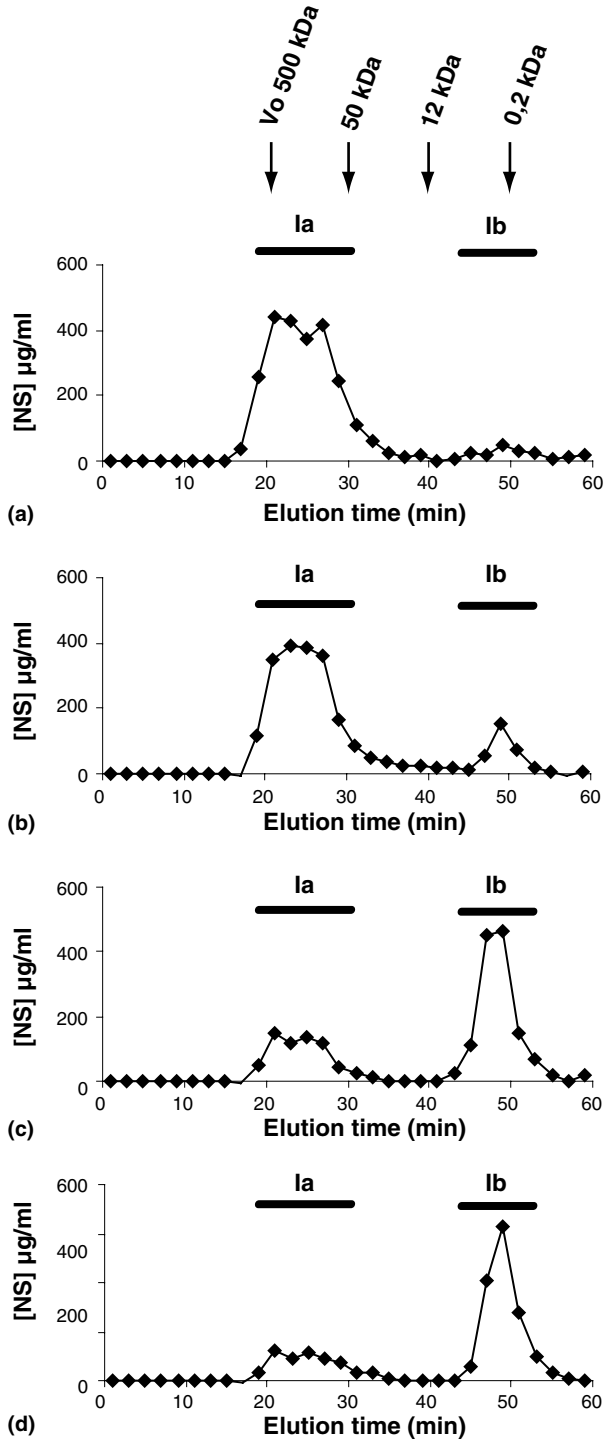

Fig. 3. SEC of products after enzymatic degradation of purified RG I. Purified RG I was incubated with buffer alone (a), endo-arabinanase (b), endo-galactananase (c) or both enzymes in combination (d), and then separated on a Superose 12 column. The fractions were analysed for their neutral sugar (NS) content. Two populations of carbohydrate were identified as Ia and Ib. Calibration was performed using dextran standards and glucose. V_0: void volume.

tubers (Sørensen et al., 2000). In addition, endo-galactanase decreased the level of Ara residues in pool Ia by 20%, indicating the presence of Ara substitutions on

Table 2
Monosaccharide composition of high-M_r fractions (pool Ia) obtained after SEC of enzyme-degraded RG I

Treatment	Rha	Ara	Gal	GalUA
No enzyme	100	433	1511	137
Arabinanase	100	72	1284	133
Galactanase	100	349	247	139
Arabinase + galactanase	100	40	217	158

Rha content was set to 100 as a reference compound, since its content did not change following any of the treatments.

galactan side chains. A significant decrease in Ara of RG I was not observed when endo-galactanase was expressed in potato tubers (Sørensen et al., 2000). Endo-arabinanase treatment resulted in a decrease in the content of Ara in pool Ia by 83%, when compared to the buffer control treatment. Surprisingly, the enzyme treatment also reduced the level of Gal in pool Ia by 15%. In contrast, when endo-arabinanase from *A. aculeatus* was expressed in the Golgi apparatus of potato tuber cells, the Ara content of RG I decreased by 70% in transformed plants compared to the wild type, without affecting the levels of Gal (Skjøt et al., 2002). When both enzymes were used in combination, the Ara content decreased by a further 8%, while the Gal content did not decrease significantly. Thus, the two enzymes appear to work synergistically towards the degradation of arabinans, but not towards the degradation of galactans. In vivo data using transgenic plants supporting these observations is not available.

The specificity of the enzyme preparations used in this study was investigated by the analysis of the lower M_r fractions (pool Ib). It has been reported that endo-arabinanases from *A. niger* will release oligomers with a degree of polymerisation (DP) of 2–3 residues from linear arabinans, suggesting the minimal substrate is a linear tetramer (Pitson et al., 1997). Similarly, it has been reported that endo-galactanase from *A. niger* degrades unbranched galactans to Gal, Gal_2 and Gal_3, suggesting that the minimal substrate is Gal_4 (Lahaye et al., 1991). It was also observed that Ara substitutions hinder endo-galactanase activity (Christau et al., 1995). Pool Ib was analysed for the presence of monomers and oligomers by HPAEC-PAD and further hydrolysed and analysed for monosaccharide composition (data not shown). The endo-arabinanase treatment released small amounts of Ara monomers; however the most abundant products were larger oligomers. The profiles showed that the endo-arabinanase treatment did not release Gal monomers, confirming that the enzyme preparation did not contain detectable galactanase or galactosidase activity. After hydrolysis, the pool was shown to contain Ara and Gal in a 1:1 ratio, indicating that the arabinan oligomers carry Gal substitutions, thereby confirming the decrease of Gal in pool Ia after endo-arabinanase treatment (Table 2). The endo-galactanase treatment released Gal, Gal_2 and Gal_3 plus a larger product (maybe a tetramer) that could not be structurally identified. After hydrolysis, the pool was shown to contain mainly Gal, with some Ara in a ratio of 20:1, confirming that some of the oligomers released by endo-galactanase contained Ara substitutions. In all three treatments the released Ara and Gal from pool Ia could be recovered in pool Ib. These observations point to the presence of mixed sugar oligomers that are released specifically by either endo-galactanase or endo-arabinanase, and also confirm that the enzyme preparations do not contain detectable side activities.

2.4. Effect of fungal endo-galactanase and endo-arabinanase treatment on RG I immunological properties

The changes in composition after digestion were supported by the changes in the immunological properties of the digested RG I (Fig. 4). Digested RG I SEC fractions were analysed by ELISA using the antibodies LM5, LM6 and JIM5. LM5 recognises a tetramer of β-1 → 4-galactan (Jones et al., 1997), LM6 recognises a pentamer of α-1 → 5-arabinan (Willats et al., 1998) and JIM5 recognises a range of small GalUA oligomers with a low degree of methyl esterification (Willats et al., 2000; Clausen et al., 2003). It should be noted that oligomers with a DP < 10 will generally not be immobilised on the ELISA plate, and therefore small M_r products (corresponding to fractions eluting at 44–50 min) cannot be detected in this way. The JIM5 epitope was not detected in any of the fractions, either before or after enzymatic degradation (data not shown), confirming that stretches of GalUA are not present in the RG I preparation. The LM5 and LM6 epitopes were found abundantly in the high-M_r fractions (corresponding to pool Ib) in buffer-control treated RG I (Fig. 4(a) and (e)). RG I treatment with endo-arabinanase decreased both the level of the LM5 epitope (by 28%; Fig. 4(b)) and the level of the LM6 epitope (by 54%; Fig. 4(f)) in the high-M_r fractions. Moreover, the endo-arabinanase treatment resulted in the detection of the LM5 epitope in lower M_r fractions (<12 kDa; Fig. 4(b)), indicating that the enzyme has released some β-1 → 4-galactan-containing oligomers from the high-M_r material. This suggests that β-1 → 4-galactan oligomers with a DP of at least 4 (LM5 epitope) are attached to arabinan branch points that are at least four residues long (the minimum substrate size for endo-arabinanase cleavage). Galactanase treatment of the lower M_r fraction of products of arabinanase treatment and analysis by HPAEC demonstrated the release of galactooligomers (DP 1–3) thereby confirming the presence of galactan side chains on the arabinans (data not shown). The endo-arabinanase treatment did not eliminate the LM6 epitope in the high-M_r fraction (Fig. 4(f)). This may be due to decoration of arabinans

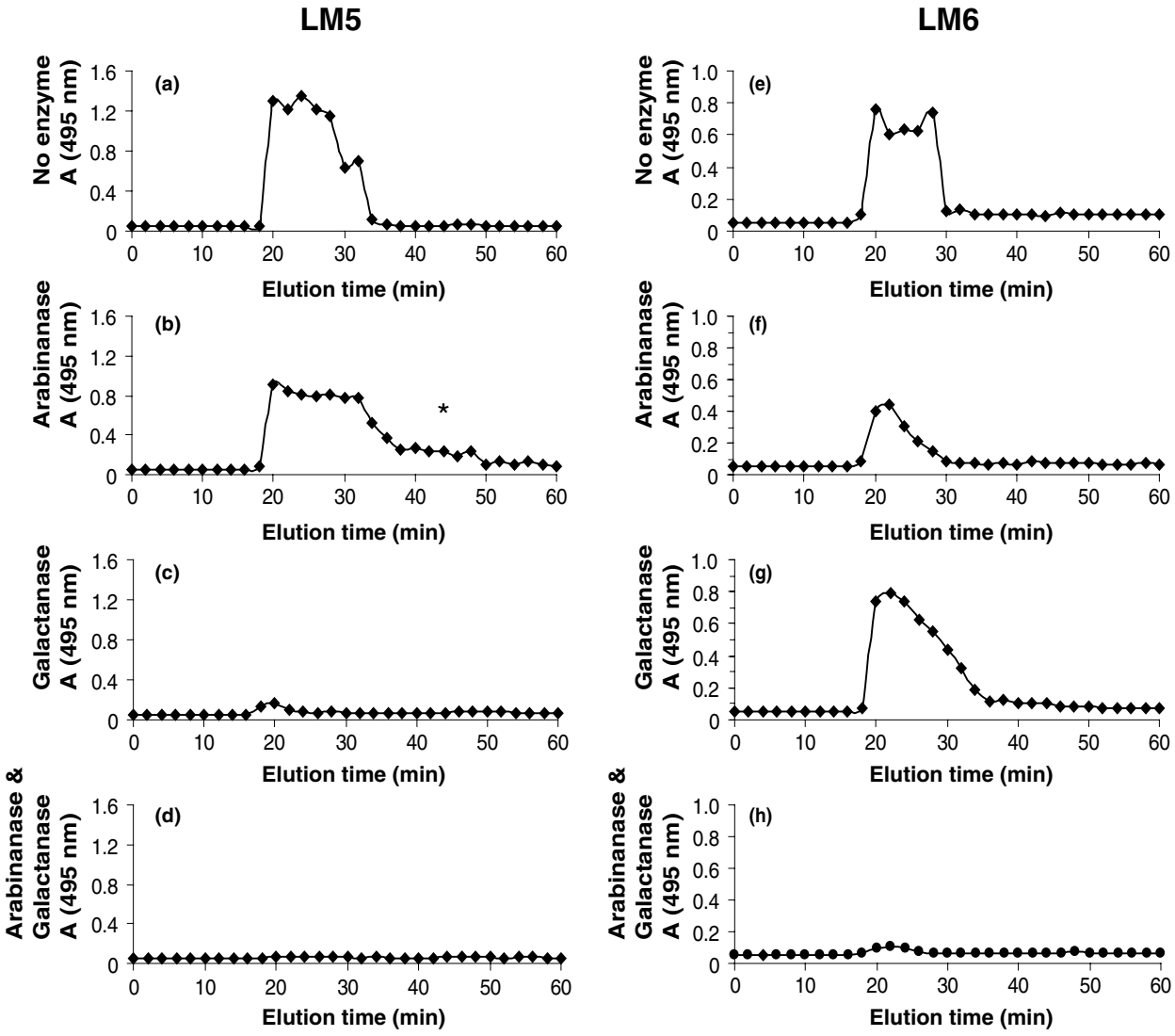

Fig. 4. SEC of products after enzymatic degradation of purified RG I. Purified RG I was incubated with buffer alone (a and e), endo-arabinanase (b and f), endo-galactananase (c and g), both enzymes in combination (d and h), and then separated on a Superose 12 column. The fractions were analysed by ELISA using the LM5 (a–d) and LM6 (e–h) antibodies. (*) indicates the presence of LM5 reactive material released upon endo-arabinase treatment. Small oligosaccharides (fractions 40–50 min) do not immobilise effectively onto the plate surface and are therefore underestimated.

with substitutions that affect their degradation, but not the binding of the LM6 antibody. These decorations could be arabinan side chains, as observed in type I and II arabinogalactans, or arabinans decorated with galactans. The endo-galactanase treatment dramatically reduced the content of the LM5 epitope by 91% in the high-M_r fractions (Fig. 4(c)), indicating that the enzyme digested β-1 → 4-galactans to oligomers with a DP smaller than about 10. The residual Gal measured in the high-M_r fractions after galactanase digestion (Table 2) is likely to originate from β-1 → 3- or β-1 → 6-linked Gal residues occurring in branched type II arabinogalactans, which are not recognised by LM5. Moreover, the endo-galactanase treatment did not affect the level of the LM6 epitope (Fig. 4(g)), but changed the distribution of the epitope to lower molecular mass fractions (12–50 kDa). The polymers present in those fractions could be RG I polymers that have been reduced in size due to the removal of the β-1 → 4-galactan side chains. This also indicates that arabinans are directly attached to the RG I backbone, or through galactan side chains shorter than three residues (and therefore not degraded by endo-galactanase), whereas longer galactans do not contain arabinan side chains with a DP >4. When the endo-galactanase was expressed in potato tuber cells and targeted to the apoplast, the LM5 epitope was significantly reduced in tissue sections. However, the effect of the expressed enzyme of the LM6 epitope was not investigated. Finally, a combination of the enzymes resulted in the elimination of the LM5 epitope (Fig. 4(d))

and a reduction of 85% in the level of the LM6 epitope (Fig. 4(h)) in all fractions, confirming a synergy of the two enzymes towards the degradation of arabinans. The residual LM6-reactive polymers may be type II arabinogalactans that are not degraded by either enzyme. The level of protein in the fractions (pointing to the presence of arabinogalactan proteins) was not investigated.

3. Discussion

The analysis of a purified RG I preparation from *Solanum tuberosum* tubers indicated that this polymer, which represents about 36% of the dry wt of the cell wall, contains a RG backbone interceded with GalUA oligomers that are smaller than a tetramer. The RG backbone is highly decorated with β-1 → 4-galactans that may carry few substitutions of short Ara-containing oligomers. The RG backbone is also decorated with α-1 → 5-arabinans that may be directly attached to the RG I backbone or through short galactan anchor chains (DP <3). The arabinan side chains may carry decorations that hinder the degradation of the arabinan backbone with endo-arabinanase. Some of these decorations may be galactan side chains with a DP >4. This feature is supported by the positive effect of endo-galactanase digestion on the degradation of arabinans by endo-arabinanase. Arabinans may also be substituted with Ara side chains, which may be present in type I, as well as type II arabinogalactans. The most surprising finding was the release of β-1 → 4-galactans by arabinanase treatment. Single arabinofuranosyl residues in the middle of β-1 → 4-galactans have been described in soybean (Huisman et al., 2001) but longer stretches of arabinans found internally in galactan chains have not been described. That such longer internal arabinans are present seems to be excluded by the absence of low-M_r and LM6-positive products released by galactanse. Therefore, we conclude that the galactans released by arabinanase are present as side-chains or extensions of the arabinans. Arabinan substitutions with single Gal residues have been suggested in sugar beet pectin (Sakamoto and Sakai, 1995) but arabinans with longer galactan extensions or side chains have never been described before. The structural features of the RG I characterised in this paper are shown schematically in Fig. 5. The degradation in vitro of RG I side chains by the two enzymes was of somewhat higher magnitude than was observed when similar enzymes were expressed in potato tubers. This could reflect a reduced accessibility of the enzymes to their substrate in the cell wall in the case of galactanase (Sørensen et al., 2000) or in the Golgi apparatus in the case of the arabinanase (Skjøt et al., 2002). The complex structure of this RG I population raises interesting question concerning the biosynthetic machinery required to construct and assemble these polymers. Until now, none of the enzymes involved in RG I biosynthesis have been identified.

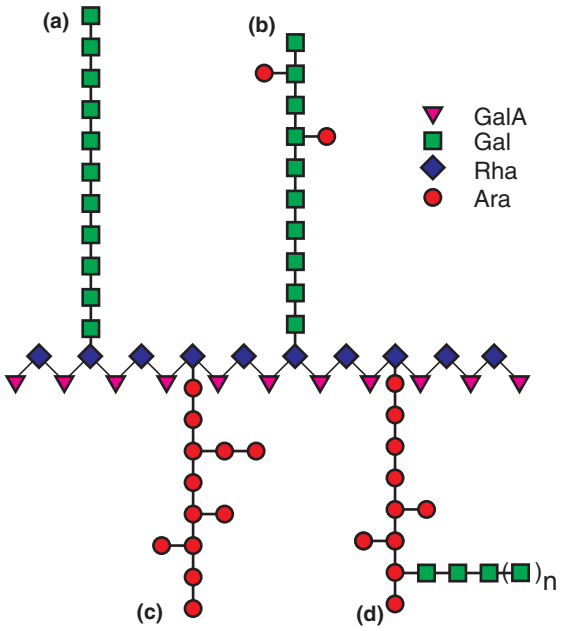

Fig. 5. Diagrammatic representation of the structure of *Solanum tuberosum* RG I. (a) Linear galactan, (b) arabinogalactan I, (c) branched arabinan and (d) hypothesised branched arabinan with galactan decorations ($n \geqslant 1$).

4. Experimental

4.1. Plant material

Potato (*S. tuberosum* cv. Posmo) plants were grown in soil in a greenhouse under a 16 h light (21 °C, between 2 and 8 klx) and 8 h dark regime (18 °C).Tubers from several plants were collected and immediately peeled, cut into small cubes (1 cm^3), frozen in liquid N_2 and stored at −80 °C until needed.

4.2. Preparation of cell wall material from potato tubers

Cell wall material was prepared from potato tubers using a method optimised for commercial high-starch potatoes (Sørensen et al., 2000; Jardine et al., 2002) with minor modifications. Frozen material was ground to a fine powder using an electric coffee grinder (Brown, Germany). The powder (200 g) was suspended in 800 ml of 1% SDC buffer [1% sodium deoxycholate, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 5 mM Na_2SO_3, pH 7.0]. The suspension was

homogenised using a Polytron (Kinematica, Switzerland) at 14,000 rpm in five bursts of 2 min each. The homogenate was filtered through a metal sieve (58 μm mesh) and the residue on the sieve was washed sequentially with 4 litre of 0.5% SDC buffer [0.5% (w/v) sodium deoxycholate, 20 mM Hepes, 5 mM Na_2SO_3, pH 7.0], 8 litre of H_2O and 2 litre of salt buffer (10 mM NaOAc, 3 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 5.0). The residue was re-suspended in 50 ml of salt buffer and homogenised again for 2 min at 14,000 rpm. The homogenate was filtered through a metal sieve (38 μm mesh) and washed with 1 litre of salt buffer. Up to this point, all manipulations were carried out at 4 °C using pre-cooled solutions. The residue was suspended in 400 ml of PhOH–HOAc–H_2O (2:1:1) and stirred for 2 h at room temp. The suspension was filtered through a sintered glass filter (grade 3), and washed extensively with salt buffer. The residue was suspended in 500 ml of buffer (10 mM K-Pi, pH 6.5, 1 mM $CaCl_2$, 0.05% NaN_3) that had been pre-heated to 95 °C. Starch was allowed to gelatinise for 30 s before 16 mg of thermostable α-amylase (Novozymes, Denmark) was added and the suspension was incubated at 85 °C for 15 min. After the incubation, 1 litre of cold salt buffer was added. Amyloglucosidase and pullulanase (1 U/ml of each, both from Megazyme, Ireland) were added and the suspension was incubated for 16 h at 40 °C, with continuous shaking at 150 rpm. The suspension was filtered thought a metal sieve (38 μm mesh) and the residue containing cell wall material was washed with 10 litre of H_2O and subsequently freeze-dried.

4.3. Isolation of RG I from cell wall material

Cell wall material (500 mg) was suspended in 200 ml of cold H_2O and then 200 ml of cold 0.2 M Na_2CO_3 containing 10 mM $NaBH_4$ was slowly added to the suspension. The suspension was incubated for 24 h at 4 °C with continuous agitation at 200 rpm. After incubation, the pH was adjusted to 4.0 with HOAc and NaN_3 was added to a final concentration of 0.05%. Endo-PG (Megazyme) was added at 0.05 U/ ml and the suspension incubated for 24 h at 35 °C with continuous agitation at 200 rpm. After the incubation, the suspension was boiled for 5 min and filtered through a nylon mesh (30 μm). The filtrate and the residue were extensively dialysed (twice against 50 mM NaOAc buffer, pH 5.5 containing 0.05% NaN_3, twice against H_2O containing 0.05% NaN_3, and once against H_2O). All dialysis steps were carried out at 4 °C using tubing with a M_r cut off of 6–8 kDa (Medicell London, UK). After dialysis, the extract and the residue were concentrated under vacuum and freeze-dried. This extract was the crude RG I preparation. High-M_r RG I was purified by SEC.

4.4. General experimental procedures

4.4.1. Size-exclusion chromatography (SEC)

SEC was carried out on a Superose 12 column (1 × 30 cm^2, Amersham Pharmacia, Sweden) equilibrated in 0.05 M NH_4COOH. 100 μl of sample solution (10 mg/ml carbohydrate) were applied to the column and eluted with the same buffer at a flow rate of 24 ml/h. 0.8 ml fractions were collected. The eluent was monitored using a refractive index detector (model 131, Gilson, USA).

4.4.2. Hydrolysis of polysaccharide

Samples were hydrolysed in 2 M TFA for 1 h at 120 °C. TFA was removed by evaporation under vacuum.

4.4.3. HPAEC-PAD

Monosaccharide composition was determined by HPAEC-PAD of hydrolysed material. Chromatography was on a PA20 column (Dionex, CA, USA) at a flow rate of 0.5 ml/min. Before injection of each sample (up to 0.2 μg monosaccharide), the column was washed with 200 mM NaOH for 10 min, then equilibrated with 10 mM NaOH for 10 min. The elution program consisted of a linear gradient from 10 mM NaOH to 5 mM NaOH from 0 to 1.5 min, followed by isocratic elution with 5 mM NaOH from 1.5 to 20 min, followed by a linear gradient up to 800 mM NaOH from 20 to 43 min. Monosaccharides were detected using a pulsed amperometric detector (gold electrode) set on waveform A according to manufacturers instructions. Monosaccharide standards were from Sigma Chemical Co. and included L-Fuc, L–Rha, L-Ara, D-Gal, D-Glc, D-Xyl, D-Man, D-GalUA and D-GlcA. A standard mixture run was performed before analysis of a batch of samples for verification of the response factors. Oligosaccharides were analysed on a PA100 column according to Blennow et al. (1998).

4.4.4. Colorimetric assays

The uronic acid content in SEC fractions was measured using the *m*-hydroxybiphenyl colorimetric assay (Blumenkrantz and Asboe-Hansen, 1973) and neutral sugars were measured using the phOH–H_2SO_4 colorimetric assay (DuBois et al., 1956).

4.4.5. Enzymatic degradation

Polysaccharide solutions were prepared to 1 mg/ml in 0.1 M $NaHPO_4$/citric acid buffer, pH 5.5. *A. niger* endo-α-1 → 5-L-arabinanase and endo-β-1 → 4-D-galactanase were purchased from Megazyme. Digestions were carried out with 0.05 U/ml of each enzyme, individually or in combination, at 30 °C with continuous agitation for 18 h. The control incubation was in buffer alone. The amount of enzyme used was determined empirically to be sufficient for the end-point degradation of pectic

standards (potato galactan or sugar-beet arabinan, both from Megazyme) within the same incubation time.

4.4.6. Enzyme-linked immunosorbent assays (ELISAs)

ELISAs were performed in 96-well microtitre plates (Maxisorb, NUNC, Denmark) coated with 100 μl of antigen per well. The assays were performed as described in Willats et al. (1998). The monoclonal antibodies used were LM5, LM6 and JIM5. All antibodies were a kind gift of Dr. J.P. Knox (University of Leeds, UK).

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