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EXTRACELLULAR POLYSACCHARIDES FROM SUSPENSION CULTURES OF *NICOTIANA PLUMBAGINIFOLIA*

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Key Word Index—*Nicotiana plumbaginifolia*; Solanaceae; tobacco; cell-suspension cultures; carboxyl reduction; linkage analysis; extracellular polysaccharide.

Abstract—The soluble polymers secreted by cell-suspension cultures of *Nicotiana plumbaginifolia* contained 78% carbohydrate, 6% protein and 4% inorganic material. The extracellular polysaccharides were separated into three fractions by anion-exchange chromatography using a gradient of imidazole–HCl at pH 7 and the individual polysaccharides in each fraction were then isolated by selective precipitation and enzymic treatment. Monosaccharide and linkage compositions were determined for each polysaccharide after reduction of uronic acid residues and the degree of esterification of the various uronic acid residues in each polysaccharide was determined concurrently with the linkage types. Six components were identified: an arabinoxyloglucan (comprising 34% of the total polysaccharide) and a galactoglucomannan (15%) in the unbound neutral fraction, a type II arabinogalactan (an arabinogalactan-protein, 11%) and an acidic xylan (3%) in the first bound fraction, and an arabinoglucuronomannan (11%) and a galacturonan (26%) in the second bound fraction.

INTRODUCTION

A mixture of high M_r compounds, including (glyco) proteins, and neutral and acidic polysaccharides, accumulate in the medium surrounding suspension-cultured plant cells. Some of these extracellular polysaccharides (ECPs) and (glyco)proteins are structurally similar to components of plant primary cell walls [1], but can be isolated from the culture medium without the harsh chemical treatments often employed to extract similar molecules from walls [1, 2]. Cell-suspension cultures are thus ideal sources of material for structural characterization of wall polysaccharides.

The composition of ECPs from cell-suspension cultures of *Nicotiana tabacum* has been reported previously [3, 4]. The ECPs are composed of 70–80% (w/w) neutral sugars, 20–28% (w/w) uronic acids and 2–11% (w/w) protein [3, 4]. Akiyama and Kato [3] showed that the ECPs from *N. tabacum* cells contained predominantly Glc, Ara, Gal, Xyl and Man, and that the uronic acid present was mostly GlcA. Iraki *et al.* [4] showed that the ECPs from *N. tabacum* cells grown to stationary phase contained the same neutral monosaccharides as those reported by Akiyama and Kato [3], but contained more Ara and almost no Man; Iraki *et al.* [4] did not analyse the type of uronic acid present in their preparations. Five major polysaccharides were present in *N. tabacum* ECPs [3], and Kato and coworkers subsequently purified a xyloglucan [5], a galactoglucomannan [6], an acidic xylan [7], an arabinogalactan-protein (AGP) [8, 9] and an arabinoglucuronomannan [10] from this material. Iraki *et al.* [4] suggested that the *N. tabacum* ECPs contained predominantly AGPs, xyloglucans and arabinoxylans, as well as uncharacterised, high M_r uronic acid-rich material.

In this paper, we report the fractionation and characterization of ECPs from cell-suspension cultures of *N. plumbaginifolia*. The ECPs were fractionated by anion-exchange chromatography with a gradient of imidazole–HCl to improve the recovery of pectic polysaccharides from the column. Methylation analysis following reduction of esterified and non-esterified uronic acids enabled the linkage types of these residues to be identified concurrently with the linkage types of the neutral sugars present.

RESULTS AND DISCUSSION

The polymers secreted by *N. plumbaginifolia* cells were composed of *ca* 78% (w/w) carbohydrate and 6% (w/w) protein and 4% (w/w) inorganic material; preliminary experiments indicated that most of the remaining dry matter could be accounted for by the presence of methyl esters and *O*-acetyl groups. The protein content was between that found in *N. tabacum* extracellular material by Iraki *et al.* (1.5%) [4] and by Akiyama and Kato (10.7%) [3]. The nature of the protein in the *N. plumbaginifolia* extracellular material was not investigated.

The monosaccharide composition of the ECPs from *N. plumbaginifolia* was determined either directly by methanolysis, or directly by acid hydrolysis after carboxyl reduction with sodium borodeuteride, or indirectly from the sum of the linkage types of each monosaccharide residue determined by methylation analysis after carboxyl reduction with sodium borodeuteride (Table 1). The relative proportions of the monosaccharides determined as alditol acetates and as partially methylated alditol acetates were similar. However, the methanolysis procedure detected about half as much Glc as that detected by the other two methods, with only a trace of GlcA. In previous studies, the monosaccharide composition of ECPs has been determined either by methanolysis [2], which, thus, may have resulted in the underestimation of certain residues, or as alditol acetates without carboxyl reduction [3, 4], which does not detect uronic acids. The ECPs from *N. plumbaginifolia* were thus determined to contain a mixture of Ara, Xyl, Man, Gal, Glc, GalA and GlcA, with a trace amount of Rha, comprising 77% (w/w) neutral sugars and 23% (w/w) uronic acids (Table 2). The proportions of the neutral monosaccharides were similar to those reported by Akiyama and Kato [3] for *N. tabacum* ECPs, whereas Iraki *et al.* [4] found only minor amounts of Man in *N. tabacum* ECPs. The ECPs from *N. plumbaginifolia* contained significant amounts of both GalA and GlcA (Table 2), whereas Akiyama and Kato [3] detected mostly GlcA in *N. tabacum*. Iraki *et al.* [4] did not characterise the type of uronic acid present in their *N. tabacum* ECPs.

Linkage analysis of *N. plumbaginifolia* native ECPs without prior carboxyl reduction gave similar results (Table 3) to those obtained for *N. tabacum* [3] suggesting that similar polysaccharides were present in the culture media of these two species. However, polysaccharides which contained uronic acid were not detected by this method and the complete linkage composition of neutral and acidic material was thus determined after carboxyl reduction. This showed that ECPs from *N. plumbaginifolia* were complex, with high proportions of terminal Ara*f* (19 mol%), 4-Glc*p* (14%) and 4-Gal*p*A (12%) (Table 3). Selective reduction of carboxylic esters to their respective 6,6′-dideuterio neutral sugars, followed by activation of free uronic acids with carbodiimide and reduction of free uronic acids with sodium borohydride, enabled the degree of esterification of the uronic acids to be determined concurrently with the linkage types [11]. The 4-Gal*p*A residues were *ca* 5% esterified, whereas the 3,4-Gal*p*A residues were present only as free uronic acids. The 4-Glc*p*A and 3,4-Glc*p*A residues were *ca* 40% esterified, whereas the terminal Glc*p*A residues were present only as free uronic acids. The types of polysaccharides

Table 1. Monosaccharide composition of *Nicotiana plumbaginifolia* ECPs

Method used for analysis	Monosaccharide composition (wt%)*							
	Rha	Ara	Xyl	Man	Gal	Glc	GalA	GlcA
Methanolysis	1	26	11	13	15	13	21	tr
Alditol acetates‡	—	18	10	11	17	22	18	4
Partially methylated alditol acetates†‡	tr	20	11	12	11	22	15	8

*Average of duplicate determinations.
tr, Trace (< 1%).
—, Not detected.
†Calculated from methylation data in Table 3.
‡Uronic acids and their esters reduced to 6,6′-dideuterio neutral sugars.

Table 2. Composition of *Nicotiana plumbaginifolia* ECPs and fractions obtained by anion-exchange chromatography

Fraction	Total sugar (mg)	Uronic acid* (wt%)	Monosaccharide composition (wt%)†							
			Rha	Ara	Xyl	Man	Gal	Glc	GalA	GlcA
ECP	780	23	tr	20	11	12	11	22	15	8
A	305	—	—	7	10	11	10	62	—	—
B	85	1	1	38	15	tr	42	3	—	1
C	230	41	—	21	4	25	7	2	28	13

*From monosaccharide composition.
†From sum of partially methylated alditol acetates.
tr, Trace (< 1%).
—, Not detected.

Table 3. Linkage composition of *Nicotiana plumbaginifolia* ECPs and fractions obtained by anion-exchange chromatography

Deduced glycosidic linkage*		Linkage composition (mol%)†				
		ECP		Fraction		
		Native	Carboxyl reduced‡	A‡	B‡	C‡
Rha*p*	terminal	—	tr	—	1	—
Ara*f*	terminal	20	19	8	16	25
	terminal*p*	2	2	tr	10	—
	2-	tr	tr	—	tr	—
	5-	5	2	tr	14	tr
	3,5-	—	—	—	1	—
Xyl*p*	terminal	5	6	6	2	2
	2-	5	6	6	—	—
	4-	2	tr	—	12	3
	2,4-	tr	—	—	2	—
Man*p*	2-	tr	2	—	—	5
	4-	3	1	3	—	4
	2,3-	3	5	—	—	15
	4,6-	5	3	8	tr	—
Gal*p*	terminal	8	4	7	1	7
	2-	2	2	3	—	—
	3-	tr	1	—	9	—
	6-	tr	tr	—	1	—
	3,6-	3	3	tr	27	—
Glc*p*	terminal	tr	tr	1	—	tr
	4-	24	14	39	2	2
	4,6-	11	7	19	1	—
GalA*p*	4-	n.d.	12	—	—	23
	3,4-	n.d.	1	—	—	2
GlcA*p*	terminal	n.d.	tr	—	1	—
	4-	n.d.	2	—	—	2
	3,4-	n.d.	5	—	—	10

*Terminal Ara*f* is deduced from 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc.

†Average of duplicate determinations.

‡Uronic acids and their esters reduced to 6,6′-dideuterio neutral sugars before methylation.

tr, Trace (< 1%).

—, Not detected.

n.d., Not determined.

present in ECPs from *N. plumbaginifolia* were deduced by comparison of the linkage analyses to the linkage analyses of individual polysaccharides purified from ECPs [5–10] and cell walls [12] of *N. tabacum*; see Shea *et al.* [13]. The *N. plumbaginifolia* ECPs contained linkages consistent with a mixture of polysaccharides, including arabinoxyloglucan [5], galactoglucomannan [6], AGP [8, 9], glucuronomannan [10] and galacturonan [11].

Polysaccharides in the *N. plumbaginifolia* ECPs were fractionated to confirm these deductions and to define precisely the fine structure of the individual polysaccharides. Fractionation of the ECPs by anion-exchange chromatography on DEAE–Sepharose CL-6B in phosphate buffer eluted with a gradient of NaCl gave very low recoveries of uronic acid (18% of the total uronic acid applied to the column), similar to those described previously for polysaccharides from onion walls [14]. However, elution with a gradient of imidazole–HCl buffer (pH 7) [1, 2] resulted in much higher yields of uronic-acid-containing polysaccharides and gave an unbound, neutral fraction (A) and two bound fractions (B and C; Fig. 1). The monosaccharide composition of the fractions obtained from anion-exchange chromatography was determined by summing the various linkage types deduced from partially methylated alditol acetates after carboxyl reduction (Table 2). The overall yield of total carbohydrate from the anion-exchange column was 79% (w/w), whereas the yield of uronic acid was 53% (w/w; calculated from the relative proportions of Gal*p*A and Glc*p*A in the three fractions), indicating that *ca* one third of the uronic acids was selectively lost when compared to the yield of total carbohydrate. Fraction A accounted for 49% (w/w)

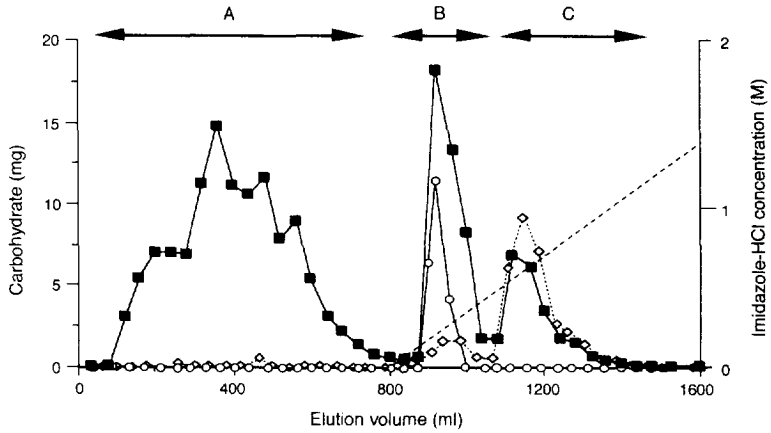

Fig. 1. Anion-exchange chromatography of *Nicotiana plumbaginifolia* ECPs. ECPs were fractionated on a column of DEAE-Sepharose CL-6B with a gradient of imidazole–HCl (pH 7) and fractions (10 ml) assayed for hexose by the anthrone method (■—■), for uronic acid residues using 3-phenylphenol (◇—◇) and for AGP by radial diffusion (○—○). Fractions were pooled as indicated to give fractions A–C.

of the recovered carbohydrate and contained only neutral carbohydrate. Fraction B accounted for 14% (w/w) of the recovered carbohydrate and contained 1% (w/w) uronic acid; the β-glucosyl Yariv diffusion assay detected AGP only in this fraction and did not detect AGP in fractions A or C. Fraction C accounted for 37% (w/w) of the recovered carbohydrate and contained 41% (w/w) uronic acid; almost all of the recovered uronic acid was thus eluted in this fraction.

Fraction A was composed entirely of neutral monosaccharides (Table 2), in proportions similar to those of the neutral fraction obtained from anion-exchange chromatography of *N. tabacum* ECPs [3]. The linkage composition of this fraction (Table 3) consisted predominantly of 4-Glc*p* (39 mol%) and 4,6-Glc*p* (19%), with smaller amounts of terminal Ara*f*, terminal Xyl*p* and 2-Xyl*p*, 4-Man*p* and 4,6-Man*p* and terminal Gal*p* and 2-Gal*p*, consistent with the presence of an arabinoxyloglucan [5] and a galactoglucomannan [6].

The two polysaccharides present in fraction A were separated by the addition of ammonium sulphate to saturation [5, 6]. The fractions insoluble (A-1) and soluble (A-2) in saturated ammonium sulphate accounted for *ca* 60 and 40% (w/w) of the recovered carbohydrate, respectively. Linkage analysis of fraction A-1 showed that it contained mostly 4-Glc*p* and 4,6-Glc*p*, terminal Ara*f* and terminal Xyl*p* and 2-Xyl*p*, and it was thus calculated to contain 96% arabinoxyloglucan (Table 4). The linkage composition of this material was similar to that of arabinoxyloglucan from *N. tabacum* [5] and, thus, the arabinoxyloglucan from *N. plumbaginifolia* was deduced to consist of a backbone of 4-Glc*p* residues, *ca* 40% of which are branched at O-6 to Xyl*p* or to Ara*f*-(1 → 2)-Xyl*p*.

Linkage analysis of fraction A-2 showed that it contained predominantly 4-Man*p* and 4,6-Man*p*, terminal Gal*p*, and 4-Glc*p*, consistent with the major polysaccharide being a galactoglucomannan (80% of fraction A-2; Table 4) [6]. The linkage composition of this galactoglucomannan was similar to that from *N. tabacum* [6], and, therefore, we deduce that the galactoglucomannan from *N. plumbaginifolia* consists of a backbone of *ca* equal amounts of 4-Man*p* and 4-Glc*p* residues; *ca* 70% of the Man*p* residues are branched at O-6 with Gal*p* units and, less frequently, with Gal*p*-(1 → 2)-Gal*p* side-chains.

Fraction B, eluted from the anion-exchange column at low concentrations of imidazole–HCl, contained mostly Ara, Xyl and Gal, and low levels of Glc, Rha and GlcA (Table 2). Linkage analysis by methylation showed that there was a high proportion of terminal Ara*f* and 5-Ara*f*, terminal Ara*p*, and 3-Gal*p* and 3,6-Gal*p* (Table 3), consistent with fraction B containing *ca* 80% type II arabinogalactans (AGs) [8, 9, 15], probably present as AGPs. The linkage composition suggested that the other major polysaccharide present in fraction B was a xylan composed of terminal Xyl*p*, 4-Xyl*p* and 2,4-Xyl*p* [7].

Fraction B was further fractionated by precipitation with β-glucosyl Yariv reagent [16]. The insoluble fraction (B-1) contained *ca* 65% (w/w) and the soluble fraction (B-2) contained *ca* 35% (w/w) of the recovered carbohydrate, respectively. Linkage analysis of fraction B-1 showed that it contained linkages consistent with the presence of an AGP (98% of fraction B-1). The linkage composition of the Yariv-insoluble AGP from *N. plumbaginifolia* was similar to that of AGPs from the ECPs of *N. tabacum* suspension cultures [8, 9] and contained predominantly terminal Ara*f* and 5-Ara*f*, terminal Ara*p*, and 3-Gal*p* and 3,6-Gal*p*. The high proportion of 3,6-linked Gal*p* suggested that, in common with other AGPs [15, 19–21], the AGP from *N. plumbaginifolia* cell-suspension cultures is a highly branched molecule.

Linkage analysis of fraction B-2 (Table 4), not precipitated with β-glucosyl Yariv reagent, indicated that this fraction contained an acidic xylan (52% of this fraction), with a backbone of 4-Xyl*p* branched at O-2 of *ca* 12% of the residues. Xylans are frequently branched

Table 4. Linkage composition of *Nicotiana plumbaginifolia* ECP fractions

Deduced glycosidic linkage*		Linkage composition (mol%)† Fraction A-1	A-2	B-1‡	B-2‡	C-1‡§	C-2‡
Rha*p*	terminal	—	—	1	—	—	—
Ara*f*	terminal	15	2	24	13	18 (39)	6
	terminal*p*	—	2	10	6	—	—
	2-	—	—	—	—	—	—
	5-	—	tr	18	10	2 (tr)	—
	3,5-	—	—	—	—	—	—
Xyl*p*	terminal	10	3	—	5	4 (3)	6
	2-	11	2	2	—	—	—
	4-	—	tr	—	22	6 (2)	3
	2,4-	—	—	—	3	—	—
Man*p*	terminal	—	1	—	—	—	—
	2-	—	—	—	—	4 (4)	—
	4-	—	8	—	3	12 (5)	7
	2,3-	—	—	—	—	10 (15)	2
	4,6-	—	20	—	2	1 (—)	—
Gal*p*	terminal	1	19	1	2	5 (9)	3
	2-	—	6	—	—	—	—
	3-	—	—	9	3	—	—
	6-	—	—	1	1	—	—
	3,6-	—	—	31	14	—	—
Glc*p*	terminal	tr	1	1	—	4 (tr)	2
	4-	37	31	—	9	6 (2)	4
	4,6-	25	4	—	3	2 (—)	—
GalA*p*	terminal	n.d.	n.d.	—	—	14 (3)	58
	3,4	n.d.	n.d.	—	—	2 (2)	8
GlcA*p*	terminal	n.d.	n.d.	1	3	—	—
	4-	n.d.	n.d.	1	—	3 (2)	—
	3,4-	n.d.	n.d.	—	—	7 (13)	1

*Terminal Ara*f* is deduced from 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinitol, etc.
†Average of duplicate determinations.
‡Uronic acids and their esters reduced to 6,6′-dideuterio neutral sugars before methylation.
§Numbers in parentheses are for sample incubated with endo-polygalacturonase.
—, Not detected.
n.d., Not determined.

with side-chains of Glc*p*A and 4-*O*-Me-Glc*p*A [22, 23], and Stevenson *et al.* [2] have shown that arabinoxylans from ECPs of sycamore cell-suspension cultures contain both of these residues. However, Akiyama *et al.* [7] concluded that xylans from *N. tabacum* ECPs contain only Glc*p*A residues. The xylan-containing fraction from *N. plumbaginifolia* fraction contained 3 mol% terminal Glc*p*A; however, we did not investigate whether any 4-*O*-Me-Glc*p*A residues were included in this value. Linkage analysis of fraction B-2 suggested that a significant amount of AG (48%) was also present. The linkage composition of this Yariv-soluble AG appeared to be similar to that of the Yariv-insoluble AGP of fraction B-1. However, this soluble AG could not be isolated by a further precipitation with β-glucosyl Yariv reagent and, thus, may represent an AG-peptide [17], AG or AGP [18] that does not interact with β-glucosyl Yariv reagent.

Fraction C was eluted from the anion-exchange column between 0.5 and 1.0 M imidazole–HCl (Fig. 1). The linkage composition of this fraction (Table 3) was consistent with the presence of an arabinoglucuronomannan, containing 2-Man*p* and 2,3-Man*p*, 4-Glc*p*A and 3,4-Glc*p*A, terminal Ara*f* and terminal Gal*p* [10, 24, 25]. In addition, fraction C contained 4-Gal*p*A and 3,4-Gal*p*A which indicated that a galacturonan was also present. The very low recovery of uronic acid observed on elution of ECPs from the DEAE-Sepharose CL-6B column with a gradient of NaCl in phosphate buffer suggested that fraction C could be further fractionated on this column. Fraction C was thus loaded on to the anion-exchange column in 20 mM phosphate buffer (pH 7) and eluted

with 1 M NaCl in this buffer, giving fraction C-1, followed by 2 M imidazole–HCl (pH 7), giving fraction C-2. Fractions C-1 and C-2 contained *ca* 70% (w/w) and 30% (w/w) of the recovered material, respectively.

Linkage analysis of fraction C-1 (Table 4) revealed a range of linkage types consistent with this fraction containing an arabinoglucuronomannan similar to that isolated from *N. tabacum* by Akiyama *et al.* [10], as well as residual galacturonan. Fraction C-1 was incubated with endo-polygalacturonase to degrade the remaining galacturonan present in this fraction. Linkage analysis by methylation of the enzyme-resistant material revealed predominantly terminal Ara*f*, 2-Man*p*, and 2,3-Man*p*, 4-Glc*p*A and 3,4-Glc*p*A, and terminal Gal*p* (Table 4), suggesting that this material was composed of more than 80% arabinoglucuronomannan. Selective reduction of carboxylic esters showed that *ca* 40% of both the 4-Glc*p*A and 3,4-Glc*p*A residues was esterified. The arabinoglucuronomannan from *N. plumbaginifolia* probably consists of a backbone of alternating 4-linked Glc*p*A and 2-Man*p*, branched at O-3 of a high proportion of both residues with terminal Ara*f* and, less frequently, terminal Gal*p* residues.

Linkage analysis of fraction C-2 showed that this material was composed primarily of Gal*p*A, comprising 4-Gal*p*A (58 mol%) and 3,4-Gal*p*A (8%) (Table 4). In addition, this material contained terminal Ara*f*, terminal Xyl*p* and 4-Xyl*p*, 2-Man*p* and 4-Man*p*, terminal Gal*p*, terminal Glc*p* and 4-Glc*p*, and 3,4-Glc*p*A (Table 4). The linkage composition suggested that this material contained predominantly a galacturonan composed of a backbone of 4-Gal*p*A, *ca* 12% of which was branched at O-3. However, it was not possible to determine whether the other linkages present were neutral side-chains attached to the galacturonan, or represented polysaccharides bound to the column non-specifically. Selective reduction of carboxylic esters in fraction C-2 showed that the degree of esterification of the 4-Gal*p*A residues was *ca* 5%; the 3,4-Gal*p*A residues were not methyl esterified. Kato and Noguchi [26] isolated a uronic acid-rich fraction from *N. tabacum* ECPs which, on partial acid hydrolysis, gave aldobiouronic acid and oligogalacturonides. A pectic polysaccharide from the cell walls of *N. tabacum* mesophyll contained almost 90% GalA, together with small amounts of Gal, Ara and Rha [12]. High M_r polysaccharides rich in galacturonic acid have been isolated from the ECPs of sycamore cell-suspension cultures [2] and high M_r uronic acid-rich material has also been shown to be released into the extracellular medium of *N. tabacum* cell-suspension cultures [4].

The compositional and linkage analyses showed that the ECPs from suspension-cultured cells of *N. plumbaginifolia* contained at least six types of polysaccharide. The amounts of the different polysaccharides present were estimated from the relative proportions of the fractions obtained and by summing the mol% of the individual monosaccharide residues using linkages diagnostic of the purified polysaccharides [13]. The ECPs contained predominantly arabinoxyloglucan and galacturonan, with smaller amounts of galactoglucomannan, arabinoglucuronomannan and AGP, with a trace of acidic xylan (Table 5).

The relative proportions of arabinoxyloglucan, galactoglucomannan, AGP and arabinoglucuronomannan in the ECPs from *N. plumbaginifolia* were similar to those in *N. tabacum* ECPs investigated by Akiyama and Kato, but *N. plumbaginifolia* ECPs contained significantly less xylan than found in *N. tabacum* ECPs [3, 5, 6]. The major difference between the ECPs from *N. plumbaginifolia* and the ECPs from *N. tabacum* was the presence of galacturonan in *N. plumbaginifolia* ECPs. In contrast to this present study on *N. plumbaginifolia*, and the work of Akiyama and co-workers on *N. tabacum*, Iraki *et al.* [4] showed that the ECPs from *N. tabacum* contained mostly AGPs and an uncharacterized high-M_r pectic material, with smaller amounts of xyloglucan and arabinoxylan. The ECPs from these cultures also contained linkages consistent with the presence of only a small amount of galactoglucomannan and linkages characteristic of arabinoglucuronomannan were not detected. The reasons for the differences in polysaccharide composition between this present study and the work of Iraki *et al.* [4] are unclear, although it is possible that they may reflect differences in the age of the cultures when ECPs were harvested; in our study, the cells were grown for *ca* 6 days, whereas Iraki *et al.* [4] grew cells for more than 15 days, although in each case the cultures had reached stationary phase.

The composition of ECPs from cell-suspension cultures has been studied in a number of species, including sycamore [2], soybean [27, 28], blackberry [20, 29] and white campion [30]. The overall features of ECPs from these species were similar to the ECPs from *N. plumbaginifolia*, containing a mixture of neutral and acidic polysaccharides, including xyloglucan, arabinoxylan, AGP and pectic polysaccharides, together with galactoglucomannan in blackberry and white campion. In addition, AGPs have been isolated from the medium of cell-suspension cultures of ryegrass [19] and rose [31]. However, arabinoglucuronomannan has been reported previously only in ECPs from *N. tabacum* [3, 10].

Akiyama and coworkers have isolated polysaccharides similar to those in ECPs from leaves of *N. tabacum* [32–36] and from the walls of suspension-cultured *N. tabacum* cells [12, 37–40]. Thus, it is probable that the six

Table 5. Relative proportions of polysaccharides present in *Nicotiana plumbaginifolia* ECPs

Polysaccharide	Relative proportion (%)*
Arabinoxyloglucan	34
Galactoglucomannan	15
Arabinogalactan-protein (AGP)	11
Acidic xylan	3
Arabinoglucuronomannan	11
Galacturonan	26

*Calculated as sum of mol% of individual monosaccharide residues.

polysaccharides present in ECPs from *N. plumbaginifolia* are components of the walls of the suspension-cultured cells and also of the wall of intact plants. The detailed structural analysis of the individual components of the *N. plumbaginifolia* ECPs is currently in progress.

EXPERIMENTAL

Material. ECPs from *N. Plumbaginifolia* were supplied by Dr David McManus (Sirius Biotechnology, Altona, Melbourne, Australia). Suspension cultures were grown for 6 days in an airlift fermenter (1000 l) on liquid CSV medium [41] at 27°; dissolved O_2 was controlled at 20% (± 5%) of air saturation. The cells were removed by rotary-drum vacuum filtration and the ECPs recovered from the medium by ultrafiltration and diafiltration (M_r cut-off 3000), then spray-dried. ECPs were dissolved in aq. Na_2-EDTA (1 mg ml^{-1}) containing NaN_3 (0.1 mg ml^{-1}) as preservative, by stirring overnight at 20°. The soln was filtered under vacuum (Whatman 541 filter paper), dialysed extensively against deionized H_2O (M_r cut off 6000–8000), concd under red. pres. (40°), then freeze-dried.

Fractionation of ECPs. A soln of ECPs (750 mg) in 20 mM imidazole–HCl buffer (500 ml, pH 7) was applied to a column (15 × 4.4 cm) of DEAE–Sepharose CL-6B equilibrated in the same buffer and eluted at 40 ml hr^{-1} until no carbohydrate was detected in the eluate by the anthrone assay. Material which bound to the column was then eluted by a linear gradient (0.02–2 M) of imidazole–HCl (pH 7) over 1 l and frs (10 ml) assayed for hexose, uronic acids and AGP as described below. Appropriate frs were pooled to give frs A–C (see Fig. 1), concd, dialysed extensively against deionized H_2O and freeze-dried.

Fr. A from anion-exchange chromatography (305 mg redissolved in 150 ml deionized H_2O) was treated by addition of $(NH_4)_2SO_4$ to satn [5, 6], left for 1 hr at 20° and centrifuged (10 000 *g*, 20 min). The pellet was dissolved in deionized H_2O (75 ml) and the pellet (A-1) and supernatant (A-2) dialysed extensively against deionized H_2O, then freeze-dried.

Fr. B from anion-exchange chromatography (85 mg) was dissolved in 25 ml 1% NaCl (giving 2 mg ml^{-1} AGP as estimated by the β-glucosyl Yariv diffusion assay), an equal vol. of β-glucosyl Yariv reagent (2 mg ml^{-1} in 1% NaCl) added and the mixt. left overnight at 4° [16]. The resultant insol. complex was collected by centrifugation (10 000 *g*, 20 min, 4°) and the pellet washed × 3 with 1% NaCl and × 3 with MeOH. The pellet was dissolved in deionized H_2O (25 ml) and both the pellet (B-1) and the original supernatant (B-2) were reduced and decolourized by stirring with Na dithionite (30% w/v) for 2 hr, dialysed extensively against deionized H_2O and freeze-dried.

Fr. C from anion-exchange chromatography (230 mg) was redissolved in 115 ml of 20 mM Na–Pi buffer (pH 7), rechromatographed on the same column of DEAE–Sepharose CL-6B equilibrated in phosphate buffer and eluted with 1 M NaCl in this buffer, followed by 2 M imidazole–HCl (pH 7). The two frs (C-1, 140 mg, eluted with NaCl and C-2, 53 mg, eluted with imidazole–HCl) were concd, dialysed extensively against deionized H_2O and freeze-dried. Fr C-1 (20 mg) was dissolved in 20 mM NH_4OAc buffer (pH 4) and incubated with *endo*-polygalacturonase (0.05 mg, 44.5 units; Megazyme Australia) for 24 hr at 25°, then heated at 100° for 10 min. The undegraded material was pptd with 80% v/v EtOH, collected by centrifugation, redissolved in deionized H_2O and freeze-dried.

Analytical methods. Total carbohydrate was determined by the PhOH–H_2SO_4 method [42] using galactose as standard. Uronic acid was determined by the 3-phenylphenol method [43] using galacturonic acid as standard. Hexoses were determined by the anthrone method [44] using galactose as standard. Arabinogalactan-protein (AGP) was determined by radial diffusion against β-glucosyl Yariv reagent [45] using gum arabic (Sigma) as standard. Total nitrogen (N) was determined by National Analytical Laboratories (Melbourne, Australia) using the Kjeldahl method and expressed as protein (N × 6.25). Inorganic material was determined as ash content and was performed by National Analytical Laboratories. Monosaccharide compositions were determined by GC-MS following methanolysis and conversion of the Me glycosides to their corresponding TMSi derivatives [46], and by carboxyl reduction (see below) and subsequent conversion of the neutral sugars to their corresponding alditol acetates [47]. The proportion of acidic to neutral hexose in the alditol acetate prepns was determined by measurements of *m/z* 219 (dideuterated, carboxyl-reduced uronic acid) and *m/z* 217 (neutral hexose); the ratio *m/z* 219 to the sum of *m/z* 217 and *m/z* 219 was calculated, and the percentage of uronic acid was obtained from a standard response curve. Monosaccharide compositions were also determined by summation of values from linkage analysis of carboxyl-reduced polysaccharides (see below).

Carboxyl reduction. Reduction of uronic acids and esterified uronic acids was based on the methods of refs [11] and [48]. Polysaccharides (5 mg) were dissolved in 500 mM imidazole–HCl (5 ml, pH 8), cooled in an ice-bath, and esterified uronic acids reduced by three additions of $NaBD_4$ (each 1 ml, 100 mg ml^{-1} in 500 mM imidazole–HCl, added at 5 min intervals) and left for 1 hr on ice. Excess $NaBD_4$ was then destroyed with HOAc and the samples dialysed for 24 hr against deionized H_2O and freeze-dried. Samples were then dissolved in 50 mM MES–KOH (2 ml, pH 4.75) and free uronic acid residues were derivatized with 1-cyclo-3-(2-morpholinoethyl) carbodiimide *metho-p*-toluenesulphonate (0.4 ml, 500 mg ml^{-1}) for 2–3 hr at 30°; Tris–HCl buffer (1 ml, pH 8) and 4 drops of *n*-octanol were then added and samples reduced (18 hr, 4°) with either $NaBD_4$ (for determination of the linkage analysis of total uronic acids) or $NaBH_4$ (to yield the proportion of esterified uronic acids compared to the total uronic acids) (1 ml, 70 mg ml^{-1} in 0.05 M NaOH). Excess reductant was then destroyed with HOAc and the samples dialysed extensively against deionized H_2O and freeze-dried.

Linkage analysis. Methylation was performed using the NaOH method of ref. [49] as described in ref. [50]. Methylated polysaccharides were hydrolysed with 2.5 M TFA (60 min, 121°), except for purified galactoglucomannan (hydrolysed for 100 min at 100°) and frs containing arabinoglucuronomannan and galacturonan (hydrolysed for 4 hr at 100°). After hydrolysis, samples were reduced with 1 M $NaBD_4$ in 2 M NH_4OH (1 hr, 60°) and acetylated using perchloric acid as catalyst [51]. The partially methylated alditol acetates were sepd on a fused-silica capillary column (25 m × 0.22 mm i.d.) with bonded phase BPX70 (SGE, Australia) on a Finnigan MAT 1020B GC-MS [52]. Identifications were based on peak R_ts and by comparison of EI-MS with published spectra. The relative proportion of terminal Glc*p*A was determined from the ratio of *m/z* 205 (neutral terminal Glc) to *m/z* 207 (dideuterated, carboxyl-reduced terminal Glc*p*A) and the percentage of uronic acid calculated. Similarly, the percentages of 4-Gal*p*A and 4-Glc*p*A were calculated from the ratios of *m/z* 233 (neutral 4-linked hexose) to *m/z* 235 (dideuterated, carboxyl-reduced 4-linked uronic acid), and the percentages of 3,4-Gal*p*A and 3,4-Glc*p*A were calculated from the ratios of *m/z* 305 (neutral 3,4-linked hexose) to *m/z* 307 (dideuterated, carboxyl-reduced 3,4-linked uronic acid).

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