ELSEVIER

0144-8617(95)00094-1

Carbohydrate Polymers 28 (1995) 159–166

0144-8617/95/$09.50+.00

Structural features of CDTA-soluble pectins from flax hypocotyls

Christophe Rihouey[a]*, Claudine Morvan[a], Irina Borissova[a], Alain Jauneau[a], Maurice Demarty[a] & Michael Jarvis[b]

[a]*S.C.U.E.O.R., URA 203 CNRS, Faculté des Sciences de Rouen, 76821 Mont-Saint-Aignan, Cedex, France*
[b]*Agricultural, Food and Environmental Chemistry, Chemistry Department, The University, Glasgow G128QQ, UK*

(Received 24 August 1994; revised version received 20 November 1994; accepted 1 December 1994)

Polymers extracted with CDTA Na_2 (*trans*-diaminocyclohexane-*N*,*N*,*N*,*N'*-tetra-acetic acid partially neutralized with NaOH) after a boiling water treatment from cell walls of 3-day-old seedlings of flax, germinated on water and in darkness, contained three main components: (1) rhamnogalacturonan I (RG-I)-like polymers with ratios of rhamnose to galacturonic acid which increased with their molecular mass; (2) neutral polysaccharides including galactans as well as arabinans; and (3) galacturonic-acid enriched polymers.

High degrees of acetylation were estimated for most of the rhamnogalacturonans but a minor RG-I-like fraction with a ratio of rhamnose to galacturonic acid of unity was acetylated (29%) as well as methylesterified (40%).

However, the calcium activity coefficients of the fractionated polymers measured in solution were quite high which implies that these polyelectrolytes did not behave like linear ones. These polymers which reacted in solution as well as *in situ* not only with ruthenium red but also with ferric hydroxylamine, and which contained large amounts of acetylated RG-I-like blocks might be considered as early markers of the differentiation of the cellulosic fibre cells in flax.

INTRODUCTION

The differentiation of secondary cell walls is one of the major events in plant development, but the mechanisms involved are still not completely understood. The flax seedling has been shown (Morvan *et al.*, 1991*a*; Goubet *et al.*, 1993) to be a good model for studying the differentiation of cellulosic fibres. Using analytical imaging by secondary ion mass spectrometry, Ripoll *et al.* (1993) noticed a dramatic increase in the sodium to calcium ratio in these fibres during the early steps of the cell wall secondary depositions. On the other hand, before any secondary deposits had been laid down in the fibres of 3-day-old flax seedlings, an unexpected constituent in the core of their tricellular junctions was detected using ruthenium red and ferric hydroxylamine staining reactions (Jauneau *et al.*, 1994). In other words, the hydroxylamine staining after the boiling water treatment of hypocotyl sections was attributed to the presence of acetylesterified hydroxyl functions of galacturonic acids, these molecules being considered as early markers for potential fibre differentiation.

In the present work, using flax seedlings in the same state of development as above (3-day-old plantlets), we have extracted these 'markers' with the CDTA calcium chelator after a boiling water treatment, tested their reactivity in solution with ruthenium red and ferric hydroxylamine and analyzed them for (1) their sugar composition; (2) their esterification; and (3) their interactions with calcium.

*Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Plant Material

Flax seeds (*Linum usitatissimum* var. Ariane) were treated for 1 min in 90% ethanol, soaked for 20 min in 2% sodium hypochlorite, rinsed in distilled water then placed for germination on moist paper and covered with an aluminium foil. After 3 days of growth, hypocotyls (10 ± 5 mm long) were excised and placed for 15 min in boiling 90% ethanol to inactivate enzymes.

Preparation of pectins

Cell walls were isolated as previously reported (Morvan *et al.*, 1991*b*): after grinding in a Tenbroek Potter homogenizer, the plant fragments were washed with

ethanol, chloroform–methanol 1:1 (V/V) and then with acetone. The cell walls were dried overnight at 45°C, then for 1 h at 80°C and weighed. After a washing of the cell walls with cold water which solubilized a substantial amount of oligosaccharides, the first pectic fraction was extracted over 2 h with boiling water (three times). The fraction under study was solubilized, using a solution of *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA–Na_2 0.5%, pH 4.5, overnight, at 4°C). Cell wall polymers, solubilized with CDTA-Na_2 after a boiling water treatment (P_{CDTA2}) were obtained after ultrafiltration through a Pellicon membrane (10 kDa, Millipore) or after dialysis, and then freeze-dried.

Ion exchange chromatography

P_{CDTA2} was submitted to ion exchange chromatography on DEAE Sepharose CL 6B (Pharmacia) column (17 × 1.8 cm) equilibrated in H_2O at pH 5.5. After sample loading, the column was washed with 100 ml of the same eluent, giving the fraction NF. Bound polysaccharides were then eluted (400 ml) with a NaCl gradient (0–1 M). Appropriate acid fractions were pooled (AF1–AF4) according to their 214 and 280 nm absorbance, ultrafiltered and concentrated according to the needs of the further analyses.

Size exclusion chromatography

Size exclusion chromatography was run in 1 M NaCl on a column of Sephacryl S200 (Pharmacia, 60 × 2.5 cm, 100 ml/h). Polysaccharides were detected by absorption at 214 nm. The exclusion and inclusion volumes were estimated with dextran blue and galacturonic acid, respectively.

Sugar composition

In order to analyse the total sugar composition, including uronic acids, the samples were methanolysed (24 h at 80°C) and methyl-silylated (4°C, overnight) in 1% trimethylchlorosilane in N, O Bis (trimethylsilyl-fluoroacetamide) (Quememer & Thibault, 1990) and analyzed by gas liquid chromatography on a DB 225 capillary column (J.W. Instrument) as previously described (Goubet *et al.*, 1993). An equivalent molar mass (M_{eqGA}) was estimated from the ratio m/N_{GA}, N_{GA} being the number of galacturonic acid corresponding to the mass (m) of total sugars.

Esterification

The degrees of methylation and acetylation were determined, according to Voragen *et al.* (1986), by high performance liquid chromatography (HPLC) using a CCOA, SFCC column (eluent: 0.05 M H_2SO_4; flow rate: 1 ml/min).

Potentiometric measurements

After ultrafiltration against distilled water, pectic fractions were transformed into their H-form by H-exchange chromatography (Amberlite IR 120 H). Potentiometric measurements were then carried out as previously described (Morvan *et al.*, 1985, Goldberg *et al.*, 1986). The calcium activity coefficient γCa was measured with a specific electrode (Métrohm 6-0504-100) (Morvan *et al.*, 1985) and the ζ linear charge parameter was calculated according to Manning (1978).

Reactivity of the polymers with ruthenium red

Ruthenium red was a Prolabo product (Cat. No. 27 418 084) used without further purification. The dye concentration was approximately 10^{-2} M to allow the precipitation of complexes with the polyanions P_{CDTA2}, the concentration of their galacturonic acids being in the range 10^{-3}–10^{-4} M. The absorption spectrum of ruthenium red was in the range 300–650 nm and complex formation with the polyanions was followed through the decrease of the major band centred at 533 nm. The assay consisted of 0.1 ml ruthenium red and 1 ml of pectin, stirred and then centrifuged for 15 min at 3000 RPM, the absorbance at 533 nm of the supernatant then being read without further treatment.

Reactivity of the polymers with alkaline hydroxylamine reagent

The procedure was first described by Bergman (1952) as a colorimetric determination of amides and was slightly modified. The reagents were (1) hydroxylamine hydrochloride NH_2OH, HCl 2 N; (2) sodium hydroxide 3.5 N; (3) HCl 3.5 N; and (4) ferric chloride 0.74 M in 0.1 N HCl. The alkaline hydroxylamine reagent (1 ml) was prepared by mixing equal volumes of solutions 1 and 2 and a graded volume of the pectic fraction was added, with further addition of water to give a total volume of 3 ml. The reaction mixture was maintained at 25°C for 2 h and 1 ml each of solutions 3 and 4 were added. The absorption spectrum of the reagent was in the range 450–650 nm and complex formation with the methylated citrus pectin was monitored via the increase in absorbance at 600 nm (A_{600}). Polygalacturonic acid gave no reaction while citrus pectin reactivity was characterized by a linear relation $A_{600} = 0.01 + 0.7x$ in the range 10–200 mg/l.

RESULTS

Pectic material

The calcium chelator, CDTA, solubilized 9.1 ± 1.1% ($n = 7$) of the mass of the dry cell walls when they had

previously been treated with boiling water. After dialysis or ultrafiltration, the polymeric fraction P_{CDTA2} represented between 20 and 60% of the extracted material, depending on the experiment. Such a variation, obviously originating from the variability in the molecular mass distribution, might be due to some differences in (1) the grinding treatment; (2) the β-elimination during the boiling water treatment if the degree and/or the distribution of the methylesterification of galacturonic acids varied; and/or (3) the extent of reticulation of these polymers with the primary cell walls.

When P_{CDTA2} was submitted to fractionation on DEAE exchange chromatography (Fig. 1), unbound material was first recovered (fraction NF), which accounted for $30 \pm 5\%$ of the mass of P_{CDTA2}. Bound acid fractions (AF1–4) were eluted with increasing ionic strength of the NaCl eluent. Fraction AF1 was a minor fraction ($5 \pm 2\%$ of the total amount of P_{CDTA2}) eluted between 0 and 0.15 M NaCl. The next fraction, AF2, eluted between 0.15 and 0.3 M NaCl and represented $22 \pm 2\%$ of P_{CDTA2}. Fraction AF3, the major acid fraction, accounting for more than $33 \pm 5\%$ of P_{CDTA2}, had an absorbance at 214 as well as 280 nm maximum when eluted at an ionic strength of 0.32 M, i.e. very close to AF2. Consequently some contamination of each fraction by the others was to be expected. Lastly, fraction AF4 ($10 \pm 5\%$ of P_{CDTA2}) with a ratio between absorbances at 280 and 214 nm larger than unity and a specific absorbance at 280 nm in its UV spectrum may contain some phenolic components, and/or proteinaceous material.

All fractions apart from NF reacted with both hydroxylamine (although to a lesser extent than citrus pectin) and ruthenium red, indicating that the labelling observed in microscopy (Jauneau *et al.*, 1994) was mainly due to the acidic fractions of P_{CDTA2}.

Sugar composition of P_{CDTA2}

Fractions recovered after P_{CDTA2} fractionation on anion exchange chromatography were methanolysed and their sugar composition estimated from GLC analysis. As shown in Table 1, fraction NF mainly comprised neutral chains, including arabinose, galactose and glucose. The high percentage of glucose may have originated from contamination with either a hemicellulose-like component or some residual starch. Otherwise, the significant level of galactose as well as arabinose, compared to rhamnose suggests the presence of arabinans and galactans as has been previously described in the cell walls of mung bean hypocotyl (Hervé du Penhoat *et al.*, 1987).

Similarly, the fraction first eluted, AF1, contained high percentages of glucose, galactose and arabinose. However, rhamnose and galacturonic acid were detected in almost equal percentages which indicates the presence of a rhamnogalacturonan I (RG-I)-like polysaccharide as described by McNeil *et al.* (1980). The ratio of galactose and arabinose to rhamnose was 7:1 and 3:1, respectively.

In constrast, in the three other fractions, AF2–AF4, the percentage of neutral sugars was lower and decreased significantly during the elution process. In these fractions, rhamnose, galactose and arabinose were the main neutral sugars, but there were differences between them especially in the ratio of galactose to rhamnose.

Conversely, the percentage of galacturonic acid was larger in the latest eluted fractions. Hence, the

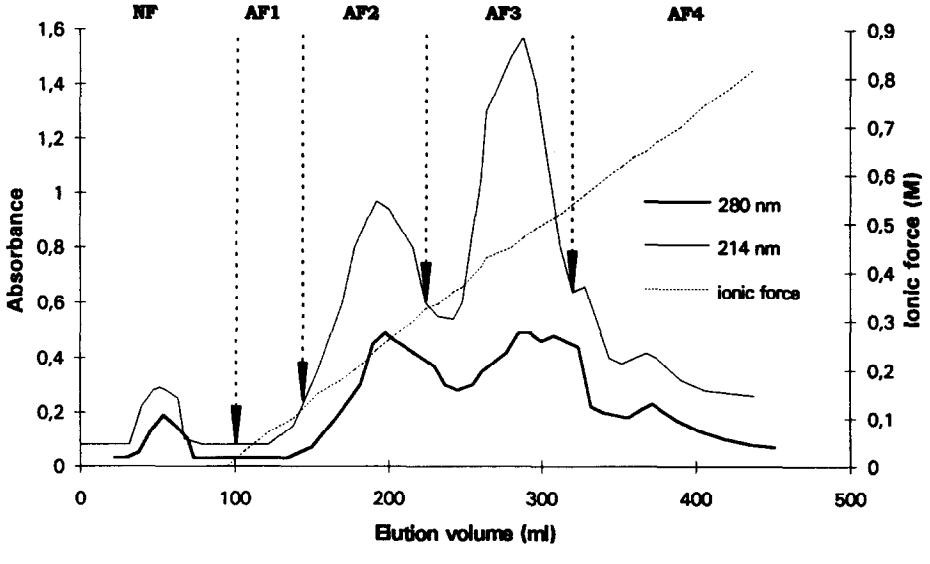

Fig. 1. Fractionation of P_{CDTA2} on DEAE exchange chromatography. The elution profiles were followed using UV light at (1) 214; and (2) 280 nm. NF constituted the unbound fraction while AF1–AF4 were the fractions recovered when increasing the ionic strength of the NaCl eluent.

Table 1. Sugar composition of P_{CDTA2} polymers fractionated on a DEAE exchange chromatography

Fraction[a]	NF	AF1	AF2	AF3	AF4
Mass (%)	30±5	5±2	22±2	33±5	10±5
Sugar[b]					
Rhamnose	1	5	10	7	6
Arabinose	24	17	15	10	10
Xylose	8	6	6	5	3
Mannose	5	8	1	4	2
Galactose	21	37	49	17	45
Glucose	41	22	5	4	8
Galacturonic acid	—	5	14	53	26
M_{eqGA}[c]	—	3115	585	325	635

[a]NF, AF1–AF4 were the fractions pooled from ion exchange chromatography as described in *Material and Methods*.
[b]Sugar composition was expressed in mol % of the sugars, neutral and acidic.
[c]M_{eqGA}, the equivalent molar mass was calculated from the ratio m/N_{GA}, N_{GA} being the number of galacturonic acid corresponding to the mass m of total sugars.

Table 2. Acetylation and methylesterification of P_{CDTA2} polymers fractionated on a DEAE exchange chromatography

	QCH_3COOH (μmol/mg)	QCH_3OH (μmol/mg)	QGalU (μmol/mg)	d.a.	d.m.
P_{CDTA2} DEAE	0.56	0.40	2	28	20
NF	—	—	—	—	—
AF1	0.13	0.18	0.45	29	40
AF2	0.63	0.18	0.87	72	21
AF3	0.62	0.29	1.66	37	17
AF4	0.69	0.26	1.12	83	31

d.a. and d.m. which are expressed in mol per 100 mol of galacturonic acid are the degree of acetylation and degree of methylesterification, respectively.

equivalent mass of galacturonic acid (M_{eqGA}) decreased with elution volume. Most importantly, the ratio of galacturonic acid to rhamnose was highest in the major fraction AF3. In other words, this fraction most probably contained a large amount of homogalacturonan blocks comprising between 30 and 50% of the mass of AF3, depending on the experiment) whereas AF2 was mainly made up of RG-I-like sequences, with a galacturonic acid to rhamnose ratio generally close to 1.5:1. The experimental variation in the percentage of homogalacturonan blocks in the main fraction AF3 might be due—as may the variation of the P_{CDTA2} yield, see above—to some discrepancies in molecular masses. It may also have originated from some variations in the yield of rhamnogalacturonan. Nevertheless in all experiments ($n=13$) the elution patterns were very similar as regards the ratio of galacturonic acid to rhamnose.

Finally, the fraction AF4 was unusual because, although it was the last to be eluted, its equivalent mass was not the lowest. As noted above, this fraction had a strong absorbance at 280 nm which might originate from the presence of some protein or phenolic groups and explain the absorption on the DEAE gel.

In all fractions, xylose (3–8%) and mannose (1–8%) were minor sugars.

Degrees of esterification

As shown by HPLC, the acidic fractions were more acetylated than methylated. The total degree of esterification (acetyl + methyl) of P_{CDTA2}, before any fractionation, varied from 25 to 65% depending on the experiment. In the reported example (Table 2), the degree of acetylation (mol of acetic acid per 100 mol of galacturonic acid) varied from 29% in AF1 to 83% in AF4. The minor but nearly pure RG-I fraction that was first eluted, as well as the most acidic fraction AF3 were the least acetylated. Similar degrees of acetylation (26–32%) have been previously reported for beet pectins extracted with acid buffer or calcium chelators (Renard & Thibault, 1993). However, the degree of acetylation of fractions AF2 and AF4 were significantly larger. RG-I, rather than polygalacturonan was thought by O'Neill *et al.* (1990) to be acetylated, although the position of the ester groups was not exactly established. Komalavilas & Mort (1989) reported that up to 30% of the galacturonic acid residues in a RG-I-like polymer isolated from suspension-cultured cotton cells had acetyl groups linked through 0–3. On the other hand, Lerouge *et al.* (1993) established by a combination of ^{1}H-NMR and periodate oxidation that the backbone repeating unit of rhamnogalacturonan I from suspension-cultured sycamore cells contained a single *O*-acetyl substituent on C2 or C3 of each galactosyluronic acid residue.

According to O'Neill *et al.* (1990), RG-I is characterized by a backbone of the alternating disaccharide →4)-α-D-GalpA-(1→2)-α-L-Rhap-(1→. Hence, we estimated the amount of galacturonic acid in RG-I blocks to be equal to the percentage of rhamnose; the amount of homogalacturonan was then calculated as the difference between the percentages of galacturonic acids and rhamnose (the presence of RG-II being neglected). Now, if we consider the hypothesis that all the acetyls substituted galacturonic acids in RG-I sequences, then the degree of acetylation of these blocks in fractions AF2-AF4 would vary between 100 and 200%. Such high values of acetylation have been previously reported only in the modified hairy region of potato after fractionation by size exclusion chromatography (Schols & Voragen, 1994).

In contrast, the degree of methylation in all fractions was rather low. Moreover, a purified pectin methylesterase (Gaffé *et al.*, 1992) was unable to de-esterify them. According to Mort *et al.* (1993), methylesterification is confined to galacturonan regions. Surprisingly, the degree of methylesterification (d.m.)

was highest in fraction AF1 where most of galacturonic acid residues were linked in dimers with two rhamnosyl residues. This result suggests that methylesterification as well as acetylation occurs in some RG-I-like polymers. Such a conclusion has been already drawn for pectins extracted from mung bean hypocotyl cell walls (Goldberg *et al.*, 1994). Otherwise, even when the degree of methylation was calculated relative only to the galacturonic acids in homogalacturonan blocks, it remained rather low in the fractions rich in galacturonic acids, AF3 and AF4, while it was higher (60%) in AF2, a fraction enriched in RG-I-like polymers. Overall, one fact seems relevant, i.e. the less methylesterified the homogalacturonan blocks, the more they were retained on anion exchange chromatography.

Size exclusion chromatography

The molecular mass distributions of P_{CDTA2} as well as of fractions NF and AF collected from DEAE are reported in Table 3. All the polymers had a fairly similar elution pattern (see Fig. 2 as an example) showing a wide polydispersity from 6000 to 80,000, according to the calibration for the Sephacryl S200 column of Hourdet & Muller (1987).

The high molecular mass polymers of P_{CDTA2}, i.e. those eluted in the void volume, originated mainly from fractions AF2 to AF4. These large molecules were of two kinds. The first type was assembled from long RG-I-like sequences (galacturonic acid/rhamnose: 1.2:1 to 1.4:1 from AF3-Vo and AF4-Vo, respectively) and short lateral chains of galactans as was previously inferred from nuclear magnetic resonance (NMR) analysis for polymers extracted with EDTA from mature fibre cells (Davis *et al.*, 1990). These polymers might account for 5–10% of the total mass of P_{CDTA2}. The second type of high molecular mass molecules was enriched in galacturonic acids with a high ratio to rhamnose (15:1 from AF2-Vo), and associated in some way with galactans. Those polymers might account for 2–5% of

Table 3. Sugar composition of P_{CDTA2} and its components after DEAE exchange and S200 size exclusion chromatographies

Polymer	P_{CDTA2}					NF			AF2					AF3					AF4		
S200 fraction	Vo	Ve1	Ve2	Vt1	Vt2	Vo	Ve	Vt	Vo	Ve1	Ve2	Vt1	Vt2	Vo	Ve1	Ve2	Ve3	Vt	Vo	Ve	Vt
Yield (%)	16	27	30	24	3	—	M[a]	—	8	55	16	13	8	12	3	30	10	45	M[a]	—	—
% from P_{CDTA2}[b]	16	27	30	24	3		(30)		2	12	4	3	1	4	1	10	3	15	(10)		
Sugar																					
Rhamnose	5	4	3	5	—	—	1	4	3	7	3	3	8	7	10	10	4	4	13	—	13
Arabinose	11	14	11	12	4	—	21	39	6	14	10	6	7	27	11	13	4	5	16	—	30
Xylose	11	9	7	7	6	—	7	12	2	5	9	4	2	3	3	4	2	3	8	—	13
Mannose	2	2	1	3	2	5	6	11	1	1	2	8	2	1	1	1	1	6	2	—	5
Galactose	40	42	35	25	54	52	10	17	33	45	29	13	26	37	35	43	11	15	36	—	15
Glucose	8	11	7	14	29	43	56	13	5	3	10	16	41	10	16	4	8	36	4	—	1
Galacturonic acid	22	18	36	34	2	—	—	2	47	25	34	55	12	14	23	25	69	31	18	—	2

[a]M indicates the major fraction recovered after size exclusion chromatography.
[b]After the first step chromatography of P_{CDTA2} onto DEAE, each fraction, NF–AF4 (apart from AF1 which only represented a minor part (5%) of P_{CDTA2}) was further separated on S200 and the yield was expressed per 100 g of P_{CDTA2}. The parentheses denote the total yields of NF and AF4.

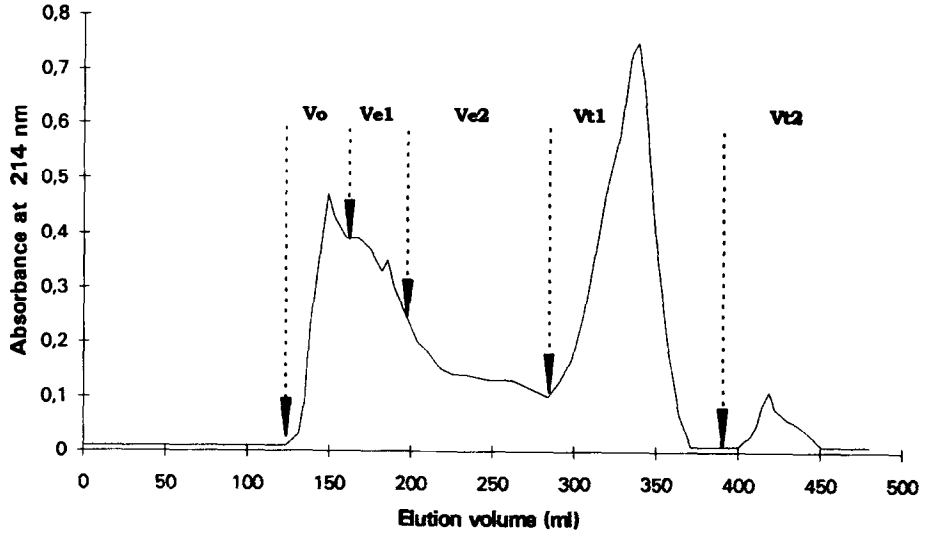

Fig. 2. Size exclusion chromatography of P_{CDTA2} on Sephacryl S200. The elution profile was followed using UV light at 214 nm.

the total mass of P_{CDTA2}. These two results explain why a simple fractionation of P_{CDTA2} on S200 led to high molecular mass polymers with a mean galacturonic to rhamnose ratio of approximately 4:1, which might have been interpreted as alternating sequences of RG-I and homogalacturonan blocks rather than as two independent polymers. It should be noted that the yields of high molecular mass polymers were close both after the two chromatography steps or only the size exclusion chromatography.

The neutral fraction, NF, as well as the major acidic fractions, AF2 and AF3, mainly contained intermediate and low molecular mass pectins. In fraction NF of intermediate molecular mass as well as in the whole NF fraction, there were very few rhamnose and galacturonic acid units, glucose being the main sugar. Thus, it seems that the glucose-rich 'contaminant' was mainly characterized by an intermediate molecular mass. Galactose was the main sugar in the high molecular mass fraction while arabinose was most abundantly represented in the low molecular mass fraction. Hence, as in mung bean seedlings (Hervé du Penhoat *et al.*, 1987), high molecular mass galactans (probably in a (1→4) galp structure) could be separated from low molecular mass arabinan (probably in a (1→5) araf structure) if they had been previously co-fractionated as fraction NF on anion exchange chromatography. Altogether with some part of AF1, these neutral polymers accounted for 30–35% of the total mass of P_{CDTA2}.

In addition to these neutral molecules, polymers of intermediate mass with rather little galacturonic acid and a low ratio of galacturonic acid to rhamnose constituted the major proportion of fraction AF2 (3.7:1) and were present in smaller quantities in AF3 (2.5:1). These polymers, which accounted for a quarter of the total mass of P_{CDTA2} were hence enriched with RG-I-like blocks, most probably associated with relatively long chains of galactose (gal/rha: 4:1–6:1) and some blocks of galacturonic acids as described for mung bean pectins (Goldberg *et al.*, 1994).

Lastly, the smallest molecules, which were retained close to Vt in the elution volume from AF3 but also to a lesser extent from AF2, contained large amounts of galacturonic acid in high ratios with rhamnose (from 9:1–18:1). The two major neutral sugars associated with these polyacids were glucose and galactose, and the ratios galactose to rhamnose and to arabinose were in the range 3–4. Altogether, these polymers accounted for approximately 25% of the total mass of P_{CDTA2} although the purely galacturonic acid sequences may only have represented a mean of 15%.

Interaction with calcium ions

Pectins and especially polygalacturonic acids show a very high affinity for calcium ions, which can partly be explained through condensation of the cations onto the negative charges of the polymers. According to Manning (1978) the affinity of a linear polyelectrolyte for ions depends on ξ, its linear charge density which is inversely related to b, the average distance between two charges ($\xi = 7.14/b$). In the case of polygalacturonic acid, $b = 4.35$ Å and $\xi = 1.64$, while in the case of RG-I, $b = 6.5$ Å and $\xi = 1.1$ (Patte-Boucrel, 1992).

The mean range of values for the crude P_{CDTA2} was between 0.25 and 0.45. However, the mean calcium activity coefficient was estimated as 0.65 ± 0.20 from 20–25 samples (the activity being measured at least 3–5 times for each sample) that had been fractionated by either ion exchange or size exclusion chromatography (Table 4). The difference between crude P_{CDTA2} and DEAE fractionated polymers most probably originated from the loss of some polygalacturonic acid from the mixture. Indeed it is known that polygalacturonic acids are difficult to recover from DEAE exchangers. Consequently, most of the recovered fractions were enriched in RG-I rather than polygalacturonic acids. Naturally, the lowest values of γCa were measured for fractions enriched in galacturonic acid. However, they were never, even after deesterification, as low as the theoretical value calculated from the following relationship (Goldberg *et al.*, 1994):

$$\gamma Ca^{M} = n1.\gamma 1Ca + n2.\gamma 2Ca$$

where $n1$ and $n2$ represent, respectively, the proportion of galacturonic acids in polygalacturonic acid and in RG-I blocks, within the total main chains containing galacturonic acid, while γ1Ca and γ2Ca are the theoretical values of their activity coefficient, calculated from the relationship $\ln \gamma Ca = -0.5 - \ln 2\xi$, (Manning, 1978), with $\xi = 1.64$ in the case of polygalacturonic acid and $\xi = 1.1$ for RG-I (Patte-Boucrel, 1992).

As suggested by Mort *et al.* (1993), some contaminating acids without any affinity for calcium ions might have remained despite the dialysis steps. However, the equivalent molar mass estimated potentiometrically (data not shown) being close to that calculated from sugar analysis, this last hypothesis is unlikely.

Moreover, the fractions which were relatively rich in galacturonic acids were mainly low molecular molecules (eluted close to the total volume of Sephacryl S200) in which cation condensation might not have occurred (Kohn, 1975, 1987).

Coincidentally, most of the experimental values were close to another theoretical value γCa^{sn} estimated when it was hypothesized that galacturonic acid units were randomly distributed in the whole polymer (including the neutral sugars) as, for example, glucuronic acids in arabic gum (Vandevelde & Fenyo, 1987) rather than linearly linked in a principal chain of RG-I or polygalacturonic acid. We do not assume such a structure to be probable; the relatively high value of γCa

Table 4. Interaction of P_{CDTA2} polymers with calcium ions

	γCa	γ'Ca	*n*1 (%)	*n*2 (%)	% GA	γCa^{M}	γCa^{sm}
DEAE exchange chromatography							
NF	1.0	1.0	—	—	—	—	—
AF1	0.85±0.15	0.80±0.10	0	100	5	0.28	0.92
AF2	0.75±0.15	0.60±0.15	29	71	14	0.25	0.78
AF3	0.45±0.15	0.45±0.15	87	13	53	0.19	0.40
AF4	0.80±0.15	0.80±0.15	77	23	26	0.20	0.66
Size exclusion chromatography							
S200-1	0.70±0.10	0.70±0.10	75	25	23	0.21	0.69
S200-2	0.90±0.10	0.55±0.15	80	20	18	0.20	0.75
S200-3	0.90±0.10	0.50±0.15	90	10	37	0.19	0.54
S200-4	0.80±0.10	0.75±0.15	85	15	34	0.20	0.54

γCa and γ'Ca were the experimental values of calcium activity coefficients, estimated for polymers before and after de-esterification. Calcium activity with a specific electrode (see *Materials and Methods*) was measured at least three times for each sample. Three independent series of P_{CDTA2} polymers fractionated on DEAE and one series fractionated on S200 were examined. The ratio of homogalacturonan to rhamnogalacturonan type I (RG-I) was *n*1:*n*2, calculated from Tables 1 and 3 when considering the % RG-I being equal to the rhamnose percentage and % PGA being galacturonic acid percentage (GA) minus rhamnose %.

A theoretical value of the calcium activity coefficient was calculated according to the relation $\gamma Ca^{M} = n1.\gamma 1Ca + n2.\gamma 2Ca$ (Goldberg *et al.*, 1994) where γ1Ca and γ2Ca were the theoretical values 0.185 and 0.275 calculated from the relationship $\ln \gamma Ca = -0.5 - \ln 2\xi$ (Manning, 1978) with $\xi = 1.64$ in the case of PGA and $\xi = 1.1$ in the case of RG-I (Patte-Boucrel, 1992). γCa^{sm} was calculated from the Manning relationship but considering the mean distance between two charges being equal to 4.35×100/GA.

in the presence of P_{CDTA2} fractions that contained large amounts of neutral sugars simply indicates that Manning's theory cannot be applied because they did not strictly behave as linear polysaccharides.

DISCUSSION

Two successive chromatography steps (anion exchange and then size exclusion), have shown the complexity of these 'unexpected' components extracted from flax seedling with the CDTA calcium chelator after a boiling water treatment, in terms of polydispersity as well as heteromolecularity. In addition, the two steps were necessary to fractionate most of the polymers; nevertheless, DEAE chromatography was more effective than size exclusion chromatography, since not only the neutral polymers but also a RG-I-like polymer, acetylated as well as methylated, could be isolated after one DEAE chromatography step. Conversely, size exclusion chromatography could not fractionate different kinds of polymers because some were of similar molecular mass. To summarize, three main classes of polymers were obtained on fractionation: (1) RG-I-like polymers, highly acetylated and having a rhamnose to galacturonic acid ratio which increased with their molecular mass, accounted for almost half of the mass of P_{CDTA2}; (2) neutral polymers (rather more than one-third of the mass of P_{CDTA2}) contained high molecular mass galactans, intermediate molecular mass glucans as well as low molecular mass arabinans; (3) the most acidic fractions enriched in low methylated galacturonic acid were of low molecular mass and represented only 15% of the total mass of P_{CDTA2}. Some oligomers, of lower molecular mass than the cut-off of dialysis were nevertheless not counted. In addition, a few per cent were high molecular mass polymers enriched in galacturonic acids.

Hence, the majority of polymers of P_{CDTA2} were rich in RG-I-like sequences and shown to be highly acetylated. This type of esterification, together with some methyl esterification, was responsible of the reactivity of P_{CDTA2} with hydroxylamine. Most importantly, these pectins and especially those of intermediate molecular mass were shown to have a structure close to the acetylated RG-I extracted with EDTA from mature flax fibre cells (Davis *et al.*, 1990). Finding this polymer before any deposition of cellulose had occurred indicates that it is an early marker of cells destined to give rise to the fibres in mature flax plants.

Although a large amount of calcium is associated, *in vivo*, with those components extractible with CDTA (Jauneau, pers. commun.), the role of calcium in retaining the RG-I-like polymers in the core of the cell junctions remains unknown since these polyanions, *in vitro*, showed relatively low affinity for calcium ions. Moreover, some covalent linkages might have been broken during the boiling water treatment.

On the other hand, the homogalacturonan blocks, as well as the oligomers of galacturonic acids, were most

probably responsible for the labelling with ruthenium red and for some interactions with calcium ions. Hence, they might have a role in cross-linking between, on the one hand, the RG-I in the core of the cell junctions and on the other, with polyanions (mainly pectic as previously shown by Morvan *et al.*, 1991*b*) in the primary cell walls. Indeed, it was previously reported (Jauneau *et al.*, 1994) that labelling by an endopolygalacturonase gold probe was located mainly at the periphery of the tricellular junctions. Further investigations are now needed to test this hypothesis.

REFERENCES

Bergman, F. (1952). Colorimetric determination of amides as hydroxamic acids. *Analyt. Chem.*, **24,** 1367–1369.

Davis, E.A., Derouet, C., Hervé du Penhoat, C. & Morvan C. (1990). Isolation and N.M.R. study of pectins from flax (*Linum usitatissimum* L.). *Carbohydr. Res.*, **197,** 205–215.

Gaffé, J., Morvan, C., Jauneau, A. & Demarty, M. (1992). Partial purification of flax cell wall pectin methylesterase *Phytochemistry*, **31,** 761–765.

Goldberg, R., Morvan, C. & Roland, J.C. (1986). Composition, properties and localisation of pectins in young and mature cells of the mung bean hypocotyl. *Plant Cell Physiol.*, **27,** 417–429.

Goldberg, R., Prat, R. & Morvan, C. (1994). Structural features of water-soluble pectins from mung bean hypocotyls. *Carbohydr. Poly.*, **23,**

Goubet, F., Martini, F., Thoiron, B. & Morvan, C. (1993). Incorporation of D-(U-^{14}C) glucose and $^{14}CO_2$ into the cell wall polymers of flax hypocotyl in the course of the fibre differentiation. *Plant Cell Physiol.*, **34,** 841–848.

Hervé du Penhoat, C., Michon, V. & Goldberg, R. (1987). Development of arabinans and galactans during the maturation of hypocotyl cell of mung bean (*Vigna radiata* Wilczeck). *Carbohydr. Res.*, **165,** 31–42.

Hourdet, D. & Muller, G. (1987) Solution properties of pectin polysaccharides. 1-Aqueous size exclusion chromatography of flax pectins. *Carbohydr. Polym.*, **7,** 301–312.

Jauneau, A., Cabin-Flaman, A., Morvan, C., Pariot, C., Ripoll, C. & Thellier, M. (1994). Polysaccharide distribution in the cellular junction of immature fibre cells of flax seedlings. *Histochem. J.*, **26,** 226–232.

Kohn, R. (1975). Ion binding on polyuronates—alginate and pectin. *Pure Appl. Chem.*, **42,** 371–397.

Kohn, R. (1987). Binding of divalent cations to oligomeric fragments of pectin. *Carbohydr. Res.*, **160,** 343–353.

Komalavilas, P. & Mort, A.J. (1989). The acetylation at *O*-3 of galacturonic acid in the rhamnose-rich portion of pectins. *Carbohydr. Res.*, **189,** 261–272.

Lerouge, P., O'Neill, M.A., Darvill, A.G. & Albersheim, P. (1993). Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. *Carbohydr. Res.*, **243,** 359–371.

Manning, G.S. (1978). The molecular theory of polyelectrolyte solutions with application to the electrostatic properties of polynucleotides. *Quart. Rev. Biophys.*, **11,** 179–246.

McNeil, M., Darvill, A.G. & Albersheim, P. (1980). Structure of plant cell walls. X. Rhamnogalacturonan I, a structurally complex pectic polysaccharide in the walls of suspension cultured sycamore cells. *Plant Physiol.*, **66,** 1128–1134.

Mort, A.J., Qiu, F. & Manness, N.O. (1993). Determination of the pattern of methyl esterification in pectin. Distribution of contiguous nonesterified residues. *Carbohydr. Res.*, **247,** 21–35.

Morvan, C., Abdul Hafez, A.M., Jauneau, A., & Demarty, M. (1991*a*) Les composés pectiques, marqueurs de la croissance du lin. *Bull. Soc. Bot. Fr.*, **138,** 339–350.

Morvan, C., Abdul Hafez, A.M., Jauneau, A., Thoiron, B. & Demarty, M. (1991*b*). Incorporation of D[U^{14}C] glucose in the cell walls of *Linum* plantlets during the first steps of growth. *Plant Cell Physiol.*, **32,** 609-621.

Morvan, C., Demarty, M. & Thellier, M. (1985) Propriétés physicochimiques des parois de Lemna minor L.: échanges entre les ions calcium et lithium. *Physiol. Vég.*, **63,** 1117–1122.

O'Neill, M.A., Albersheim, P. & Darvill, A. (1990). The pectic polysaccharides of primary cell walls. In *Methods in Plant Biochemistry*, pp. 415–441.

Patte-Boucrel, V. (1992). Propriétés physicochimiques de pectines de lin, gélification par réticulation et application à la gélification de milieux de culture. Thèse de l'Université de Rouen.

Quememer, B. & Thibault, J.F. (1990). Assessment of methanolysis for the determination of sugars in pectins. *Carbohydr. Res.*, **206,** 277–287.

Renard, M.G.C. & Thibault, J.F. (1993). Structure and properties of apple and sugar beet pectins extracted by chelating agents. *Carbohydr. Res.*, **244,** 99-114.

Ripoll, C., Pariot, C., Jauneau, A., Verdus, M.C., Catesson, A.M., Morvan, C., Demarty, M. & Thellier, M. (1993). Involvement of sodium in a process of cell differentiation in plants. *C.R. Acad. Sc. Paris, Sciences de la vie*, **316,** 1433–1437.

Schols, H.A. & Voragen, G.J. (1994). Occurrence of pectic hairy regions in various plant cell wall materials and their degradability by rhamnogalacturonase. *Carbohydr. Res.*, **256,** 83–95.

Vandevelde, M.-C. & Fenyo, J.-C. (1987). Estimation of the charge density of arabic acid by potentiometry and dye binding. *Polym. Bull.*, **18,** 47–51.

Voragen, A.G.J., Schols, H.A. & Pilnik, W. (1986). Determination of the degree of methylation and acetylation of pectins by h.p.l.c. *Food Hydrocoll.*, **1,** 65–70.