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Investigation of the occurrence of pectic-xylan–xyloglucan complexes in the cell walls of cauliflower stem tissues

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Abstract

Selected fractions of cauliflower stem, obtained after ethanol graded precipitation of 0.5 M KOH cell wall extractions, were further purified by anion-exchange and gel-permeation chromatographic methods. Pure xyloglucans and various xylan-rich polysaccharide complexes were obtained, particularly from the most mature section of the stem. A pectic-xylan–xyloglucan complex, tentatively characterised by methylation analysis, was digested separately with purified and highly specific *endo*-xylanase and also *endo*-polygalacturonase. Digestions degraded the xylan and pectic polysaccharides moieties, and caused a decrease in the molecular weight of the xyloglucan moieties, thus demonstrating the strong, probably covalent interactions between the different polymer species. The formation of polysaccharide complexes during secondary thickening and the structure of the pectic-xylan–xyloglucan complexes in cauliflower stem tissues are discussed.

Keywords: Cell walls; Complexes; Cauliflower

1. Introduction

In a previous paper (Femenia et al., 1999), we reported that maturation of cauliflower stem tissues was accompanied by an increase in the levels of (1 → 4)-linked xylose in several sequentially-extracted fractions of the cell wall. More detailed studies suggested that some of the sub-fractions from the 0.5 M KOH extracts may contain polysaccharide complexes comprising xylans and other polymer species. These proposed complexes may have arisen from the cell walls of tissues capable of undergoing secondary thickening. However, there is some debate on the occurrence of polysaccharide complexes in vivo. It could be argued that the small amounts of the complexes isolated from cell walls may, in fact, be artefacts of the isolation procedure.

In the past we have reported on the isolation of small amounts of complexes from a range of edible plant organs including, runner bean parenchyma (O'Neill and Selvendran, 1985; Ryden and Selvendran, 1990a), runner bean parchment layer (Selvendran and King, 1989), potatoes (Ryden and Selvendran, 1990b) and onions (Redgwell and Selvendran, 1986). Previous reports by other researchers have also indicated the presence of complexes in cell walls, for example in the leaves of *Vicia faba* (Puzstai et al., 1971), from cultured tissues of sycamore (Bauer et al., 1973), rice endosperm cell walls (Shibuya and Iwasaki, 1978; Shibuya and Misaki, 1978) and poplar cells (Karacsonyi and Kovacik, 1989).

Recently, we have demonstrated the presence of xylan–xyloglucan complexes in the cell walls of olive pulp (Coimbra et al., 1995), and our work on asparagus stems during maturation and storage has provided fairly firm evidence for the occurrence of xylan–pectic polysaccharide complexes and their association with tissue maturation (Waldron and Selvendran, 1992).

In order to shed more light on the possible occurrence of polysaccharide complexes, particularly in the walls of tissues which have a potential to undergo secondary thickening, we have investigated in some detail selected fractions from the 0.5 M KOH extracts of immature and mature stems of the cauliflower plant.

2. Materials and methods

2.1. Preparation and sequential extraction of CWM

Alcohol-insoluble cell-wall-rich residues were prepared from immature and mature stem tissues of cauliflower, and sequentially extracted using techniques developed by

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Redgwell and Selvendran (1986) as reported (Femenia et al., 1999). The polysaccharides present in the 0.5 M KOH extracts were subjected to graded precipitation with increasing concentrations of ethanol (Femenia et al., 1999).

2.2. Anion-exchange chromatography

Selected fractions obtained by precipitation with graded ethanol were subjected to anion-exchange chromatography. Each fraction was suspended in water and stirred at 20°C, and the insoluble residue was removed by centrifugation. To the supernatant fractions potassium-phosphate buffer (pH 6.5) was added to give a final concentration of 50 mM phosphate and containing about 1 mg/ml of polysaccharide material. The solution was passed through a column of DEAE-Trisacryl-M (15 × 1 cm, phosphate form), at a flow rate of 15 ml/h. The column was first eluted with the same buffer and then with a 0–1.0 M NaCl gradient containing 50 mM buffer. Fractions (1.5 ml) were collected and aliquots (20 μl) were assayed for carbohydrate by the phenol–sulphuric acid method (Dubois et al., 1956) and total phenolic content was measured at 280 nm. The appropriate fractions were combined, dialysed, concentrated, and an aliquot was freeze-dried for sugar and methylation analysis and the remaining material was stored at − 20°C.

2.3. Gel-permeation chromatography

Selected fractions were subjected to gel permeation chromatography. The elution of these fractions was performed on a column (100 × 1 cm) of Sephacryl S-400-HR at a flow rate of 15 ml/h. The fractions were dissolved in 2 ml, 50 mM potassium-phosphate buffer, pH 6.5, containing 0.2 M NaCl. Fractions (1.5 ml) were collected and aliquots (20 μl) were assayed for carbohydrate by the phenol–sulphuric acid method, and total phenolics were also monitored. To calibrate the column, standard dextrans having molecular weights of 2000, 487, 266 and 72 kDa were used.

2.4. Xylanase purification

Bioxylanase, a preparation from cultures of *Tricoderma viride* (Biocon, Australia) was first purified according to the procedure described by Gibson and McCleary (1987). The enzyme aforementioned was subjected to further chromatography on a column of Sephacryl S-200 (Coimbra et al., 1995). The final enzyme was shown to be pure by electrophoresis and did not exhibit any activity against tamarind xyloglucan and ball-milled cellulose.

2.5. Xylanase digestion

Selected fractions (LS60-A1; 6 mg, and LS.SN-A1; 8 mg, obtained by gel filtration of fractions LS60-A and LS.SN-A, respectively), were digested with xylanase (50 units) in 1 ml 100 mM sodium acetate buffer, pH 5.0. The mixture containing the LS60-A1 fraction and xylanase fraction was incubated overnight in a water-bath at 36–38°C and the digested mixture was heated at 90°C for 10 min to inactivate the enzyme. The mixture containing the LS.SN-A1 fraction and xylanase was allowed to react at 20°C for 16 h. In this case the final heating step to inactivate the enzyme was omitted to prevent a likely degradation of the constituent pectic polysaccharides by β-elimination.

The solutions were cooled, centrifuged (12 000 rpm, 10 min) and the supernatants were chromatographed on Sephacryl S-400-HR column as described previously.

For comparison, a blank containing xylanase and buffer was treated as for the LS.SN-A1 sample and eluted from the column.

2.6. Polygalacturonase purification

Crude A. Niger pectinase (Sigma, UK) was desalted by diafiltration with a 10 kDa membrane (Amicom, UK). Separation by gel filtration on a column of Sephacryl S-200 superfine (Pharmacia, Sweden) generated two peaks of pectin degrading activity, the peak corresponding to *endo*-polygalacturonase (PG) was further separated on a column of hydroxyapatite (BioRad, UK). The peak of polygalacturonase activity was desalted by dialysis and applied to a column of DEAE-Trisacryl-M. Pure polygalacturonase was eluted with a salt gradient. SDS-PAGE showed the PG contained only a single band.

2.7. Polygalacturonase digestion

Selected fractions (LS60-A1; 6 mg and LS.SN-A1; 5 mg) were digested with *endo*-polygalacturonase (10 units) in 1 ml 100 mM sodium acetate buffer, pH 4.0, as described for the xylanase.

For comparison a blank containing *endo*-polygalacturonase and buffer was treated as for the LS.SN-A1 fraction and eluted from the gel filtration column.

2.8. Methylation analysis

The polysaccharides were methylated by a modified sequential method as described by Femenia et al., 1999.

2.9. General methods

Neutral sugars and total uronic acid content of the polysaccharides were determined using standard techniques (Femenia et al., 1999).

3. Results and discussion

3.1. Anion exchange chromatography

Selected graded-ethanol-precipitated sub-fractions from the 0.5 M KOH extracts of the CWM of immature and mature stems were further resolved by chromatography on DEAE-Trisacryl M. The subfractions examined were the

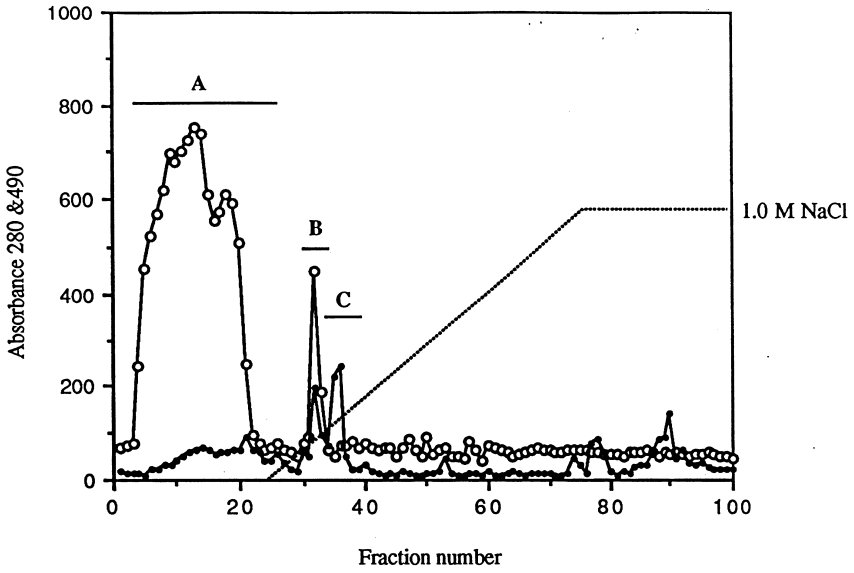


Fig. 1. Ion-exchange chromatography of fraction US60 on DEAE-Trisacryl M. Symbols: ◦—◦, OD 280 nm (carbohydrate); · — ·, OD 490 nm (phenolics).

40–60% (US60) and 60–80% (US80) ethanol cuts from the upper stem, and 40–60% (LS60), 60–80% (LS80) and 80% supernatant (LS.SN) fractions from the lower stem. Sugar and methylation analysis suggested that these sub-fractions may contain complexes containing acidic-xylans, xyloglucans and pectic polysaccharides (Femenia et al., 1999).

DEAE-Trisacryl M was chosen for further fractionation of the polymers, as it has been found to give good recovery of polysaccharides from onion (Redgwell and Selvendran, 1986) and asparagus stems (Waldron and Selvendran, 1992). All the sub-fractions with the exception of the 40–60% ethanol fraction from the lower stem, dissolved efficiently in the application buffer, and less than 1% of the material remained insoluble.

The elution profiles of the soluble materials from the upper and lower stem are shown in Figs. 1–5. Data containing the recoveries of the fractions from the columns and their carbohydrate composition are shown in Table 1. The recovery of the material applied to the columns ranged from 80 to 95%. The high recovery is because of the fact that neutral (e.g. xyloglucans) or slightly acidic polysaccharides comprised the bulk of the applied materials.

3.2. Upper stem; sub-fractions from the 40–60% ethanol fraction (US60)

The fraction US60 gave rise to three fractions (US60-A-B-C, Fig. 1). The major neutral fraction (US60-A), which accounted for 75% of the applied material, was composed almost entirely of carbohydrate (Table 1). Sugar analysis

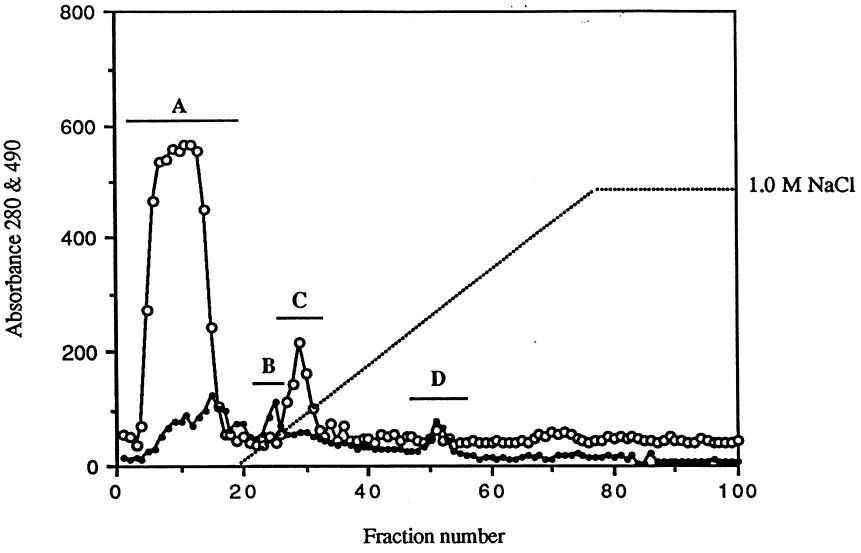


Fig. 2. Ion-exchange chromatography of fraction US80 on DEAE-Trisacryl M. Symbols: ◦—◦, OD 280 nm (carbohydrate); · – ·, OD 490 nm (phenolics).

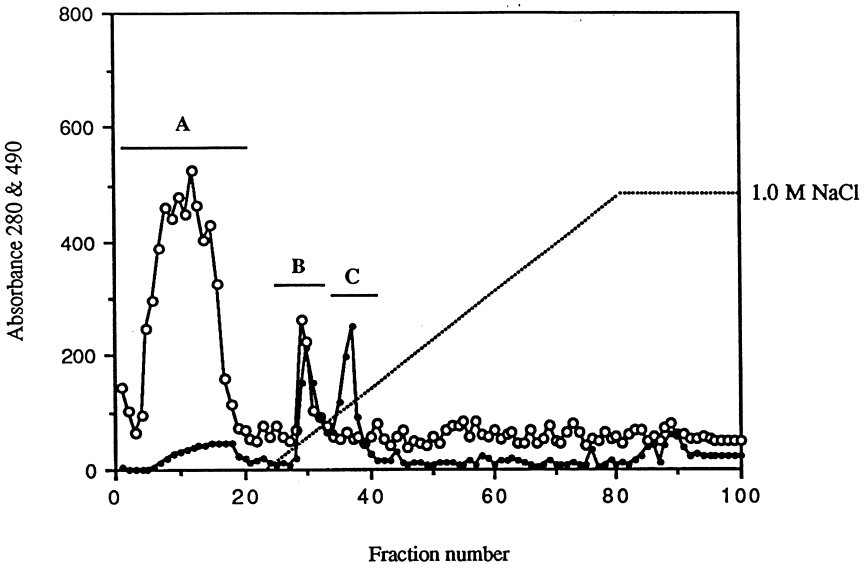


Fig. 3. Ion-exchange chromatography of fraction LS60 on DEAE-Trisacryl M. Symbols: ◦—◦, OD 280 nm (carbohydrate); · – ·, OD 490 nm (phenolics).

clearly suggested that xyloglucan was the major component of this fraction, and this inference was confirmed by methylation analysis (Table 3, column 1). The quantitative recovery of per-methylated alditol acetates is demonstrated in Tables 3–6 by the proportions of neutral sugars (in parentheses) quantified as alditol acetates. The preponderance of (1 → 4,6)-linked glucose residues over (1 → 4)-linked glucose residues, showed that the xyloglucan was very highly branched. Also, there was a close correspondence between the (1 → 4,6)-linked glucose residues and terminal residues of xylose, galactose and fucose. The close correspondence between (1 → 2)-linked galactose and terminal fucose residues is also to be noted. This finding is of particular interest as we have seldom observed such good correlation between branch and terminal residues in xyloglucans (Ryden and Selvendran, 1990a; Coimbra et al., 1994; Gooneratne et al., 1994), and it may be a reflection on the improved methylation procedure used in this study. It is also interesting to note the presence of small amounts of (1 → 4)- and (1 → 4,6)-linked mannose units, although their structural significance is not clear. They may be an integral part of the xyloglucan, but the presence of a contaminating glucomannan cannot be excluded.

The fraction US60-B, which eluted as a homogeneous peak with 0.125 M NaCl, was rich in xylose, and contained significant amounts of uronic acids (galacturonic and glucuronic acids), glucose and arabinose, suggesting the presence of acidic xylans, xyloglucans and pectic polysaccharides. Methylation analysis of this fraction (Table 3, column 2) confirmed the earlier observation. Although

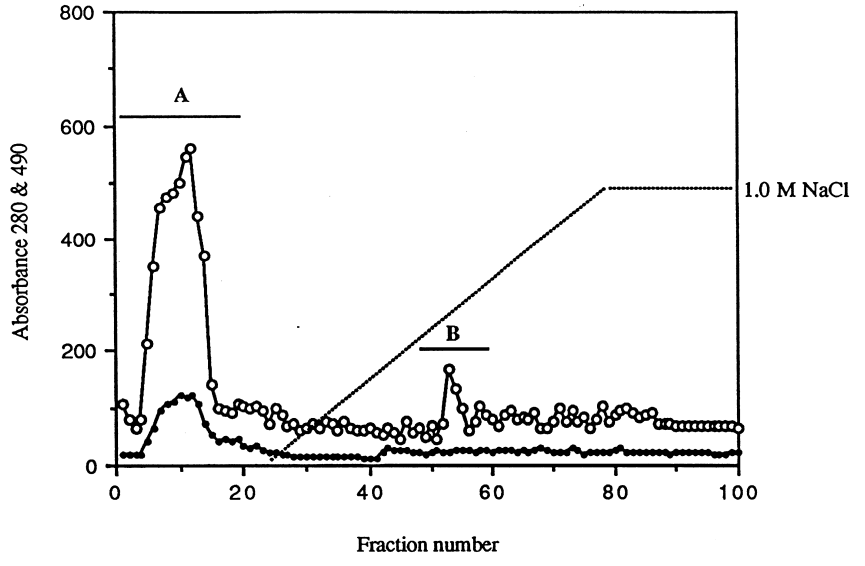


Fig. 4. Ion-exchange chromatography of fraction LS80 on DEAE-Trisacryl M. Symbols: ◦—◦, OD 280 nm (carbohydrate); · — ·, OD 490 nm (phenolics).

Table 1
Sugar composition of graded ethanol fractions from cauliflower stem obtained on DEAE-Trysacryl M anion-exchange chromatography

Fraction	Recovery (%)	Cell wall sugars (mol%)								
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Ur.A	Total sugar[a] (μg/mg)
Upper stem										
60% EtOH										
US60-A	74.9	0.9	5.3	0.7	28.0	1.6	10.3	49.6	3.7	998.1
US60-B	10.4	0.5	0.0	10.5	61.8	0.8	2.1	9.6	14.7	763.6
US60-C	2.6	1.7	0.0	3.1	18.5	5.0	1.8	62.9	7.1	546.8
80% EtOH										
US80-A	59.1	0.7	5.0	1.7	31.3	2.8	10.3	40.7	7.5	692.3
US80-B	3.5	2.4	0.0	11.1	14.5	1.9	10.9	35.6	23.6	179.3
US80-C	16.7	0.7	0.0	10.5	69.3	1.0	3.1	1.0	1.0	695.5
US80-D	9.2	3.4	0.0	16.5	64.8	2.3	5.6	7.5	7.5	58.5
Lower stem										
60% EtOH										
LS60-Ins.	(17.1)	0.3	0.0	2.4	60.7	3.6	1.8	24.4	7.0	399.7
LS60-A	60.3	0.5	4.8	0.7	30.1	1.7	9.5	38.2	5.5	958.2
LS60-B	15.3	0.4	0.0	4.4	64.5	0.5	3.2	8.8	18.2	737.5
LS60-C	3.0	2.0	0.0	3.8	35.7	5.3	1.6	32.7	19.0	281.9
80% EtOH										
LS80-A	92.4	2.2	3.0	1.7	48.1	0.9	7.3	26.1	10.7	786.4
LS80-B	6.3	9.9	2.7	1.1	14.5	0.1	14.0	36.6	21.3	987.6
80% Supt.										
LS.SN-A	54.1	1.3	1.9	2.0	61.3	0.8	3.6	18.5	10.6	533.6
LS.SN-B	21.2	4.3	0.0	5.1	45.4	1.4	10.2	10.6	23.0	383.4
LS.SN-C	2.2	4.3	0.0	4.2	38.8	2.1	3.5	17.9	29.2	188.9

[a] Values are expressed as μg of ''anhydrosugar''/mg dry polymers.

Table 2
Sugar composition of US60, LS60, LS80 and LS.SN fractions after S-400-HR Sephacryl chromatography

Fraction	Recovery (%)	Cell wall sugars (mol%)								
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Ur.A	Total sugar[a] (μg/mg)
Upper stem										
US60.A1	68.5	0.2	5.3	0.6	28.4	2.7	10.0	47.0	6.1	695.6
Lower stem										
LS60-A1	77.1	0.1	4.2	0.5	36.3	1.8	8.7	39.6	8.8	659.3
LS60-A1X	88.0	0.1	5.9	0.8	34.0	2.1	8.5	38.3	7.9	641.4
LS60-A1P	87.3	0.0	6.1	0.4	38.1	1.3	6.4	41.7	4.3	618.3
LS.SN-A1	65.8	1.4	1.9	1.8	60.6	0.1	5.5	16.9	11.9	842.6
LS.SN-A2	13.1	2.9	1.1	2.4	57.1	2.6	2.9	16.0	15.1	278.0
LS.SN-A1X1	11.5	0.9	1.9	2.1	25.7	1.6	7.1	36.7	24.2	272.5
LS.SN-A1X2	24.8	6.0	1.5	2.8	45.2	2.4	4.5	20.0	17.6	603.6
LS.SN-A1X3	12.0	3.0	0.3	2.9	16.2	3.9	5.8	48.5	19.5	149.7
LS.SN-A1P	76.0	0.2	2.6	1.1	68.2	0.1	4.7	18.5	4.6	657.4

[a] Values are expressed as μg of ''anhydrosugar''/mg dry polymers.

Table 3
Glycosyl linkage composition of polysaccharides from upper Stem 60% (US60) and 80% (US80) EtOH ppt. fractions after anion exchange chromatography (values expressed as relative mol%)

Linkage types	Relative mol%									
	US60-A		US60-B		US80-A		US80-C		US80-D	
Rhamnose										
1,2	0.11		0.33		0.35		0.34		1.17	
1,3	0.06	[0.90]	0.20	[0.63]	0.14	[0.79]	0.30	[0.87]	0.37	[3.38]
1,2,4	0.07		0.42		0.05		0.53		1.02	
Fucose										
Terminal	4.60	[5.54]	1.60	[0.00]	5.37	[5.42]	0.14	[0.00]	1.67	[0.00]
Arabinose										
Terminal-f	0.94		3.21		0.52		1.57		3.07	
1,2	0.19		0.32		0.51		0.38		0.16	
1,3	0.13		0.18		0.14		0.18		0.20	
1,5	0.92	[0.67]	5.04	[12.32]	0.57	[1.78]	1.39	[12.27]	3.37	[16.46]
1,2,5	0.05		0.13		0.05		0.26		0.34	
1,3,5	0.61		0.40		1.75		0.67		1.18	
1,2,3,5	0.02		0.29		0.00		0.00		0.00	
Xylose										
Terminal	18.98		6.31		15.43		9.59		8.19	
1,2	6.80		3.99		9.92		4.60		6.21	
1,4	2.23		53.07		2.96		61.13		47.80	
1,2,4	1.12	[29.09]	6.57	[72.40]	0.37	[33.86]	7.05	[80.98]	5.25	[64.78]
1,3,4	0.00		0.00		0.00		0.00		0.00	
1,2,3,4	0.07		0.40		0.10		0.44		0.44	
Mannose										
1,4	1.41		0.40		2.21		0.10		0.85	
1,2,4	0.00	[1.71]	0.00	[0.88]	0.00	[3.01]	0.00	[1.13]	0.00	[2.34]
1,4,6	0.87		0.28		1.76		1.02		1.39	
Galactose										
Terminal	5.67		0.83		0.05		0.59		2.54	
1,2	5.58		1.08		5.85		0.21		1.03	
1,4	0.00		0.00		0.00		0.00		0.35	
1,6	0.11		0.24		0.25		1.36		0.44	
1,3,6	0.56	[10.64]	0.39	[2.49]	0.65	[11.14]	1.19	[3.60]	0.64	[5.57]
1,4,6	0.00		0.42		0.37		0.42		0.16	
Galactitol	0.06		0.29		0.15		0.25		0.53	
Glucose										
Terminal	0.26		0.30		0.52		0.42		0.99	
1,4	14.69		5.12		14.25		2.89		4.23	
1,4,6	33.60		5.37		30.08		0.69		5.16	
1,3	0.00	[51.46]	0.00	[11.27]	0.00	[44.01]	0.00	[1.16]	0.00	[7.47]
1,3,4	0.00		0.00		0.00		0.00		0.00	
1,2,4	0.00		0.00		0.00		0.00		0.00	
Glucitol	0.29		2.85		0.94		2.30		1.16	

this fraction, unlike US60-A, was not examined any further because of insufficient material, the results of the previous analysis clearly suggested the occurrence of a xylan–xyloglucan–pectic-polysaccharide complex, similar to those described in the asparagus stem tissues (Waldron and Selvendran, 1992). In addition, a small but significant amount of phenolics co-eluted with the carbohydrate moiety, suggesting that phenolics may be involved in cross-linking the polysaccharide moieties (O'Neill and Selvendran, 1985; Biggs and Fry, 1987; Waldron and Selvendran, 1992).

The fraction US60-C was rich in xyloglucans and phenolics. The small quantity of this fraction precluded further analysis.

3.3. Upper stem; 60–80% ethanol fraction, (US80)

US80 gave one main and three smaller fractions on anion exchange chromatography (Fig. 2). The main neutral fraction (A) contained about 60% of the applied material. Sugar analysis, particularly the ratio of xylose to glucose, suggested that xyloglucan is probably the major component (Table 1) and this inference was confirmed by methylation analysis (Table 3, column 3). From the linkage composition, it can be inferred that the associated xyloglucan is highly comparable with that present in the US60A. The second fraction US80B, was rich in phenolics and contained only about 18% of carbohydrate and was not investigated any further. The third fraction US80C, accounted for 17% of

Table 4
Glycosyl linkage composition of polysaccharides from lower stem 60 (LS60), 80 (LS80) and 80% EtOH supernatant (LS.SN) fractions after anion exchange chromatography (values expressed as relative mol%)

Linkage types	Relative mol%											
	LS60-Ins		LS60-A		LS60-B		LS80-A		LS.SN-A		LS.SN-B	
Rhamnose												
1,2	0.49		0.24		0.35		0.61		0.84		1.56	
1,3	0.60	[0.46]	0.12	[0.54]	0.53	[0.27]	0.24	[2.42]	0.19	[1.42]	0.21	[5.62]
1,2,4	0.85		0.35		0.25		0.95		0.92		3.87	
Fucose												
Terminal	0.07	[0.00]	4.17	[5.11]	0.13	[0.00]	3.21	[3.45]	2.32	[2.08]	0.24	[0.00]
Arabinose												
Terminal-f	2.69		0.45		1.72		1.56		1.38		2.32	
1,2	0.02		0.00		0.24		0.23		0.39		0.48	
1,3	0.12		0.00		0.18		0.14		0.20		0.21	
1,5	0.87	[5.38]	0.46	[0.73]	0.50	[2.54]	0.61	[1.85]	0.98	[2.23]	2.47	[6.57]
1,2,5	0.04		0.12		0.18		0.04		0.60		0.55	
1,3,5	0.21		0.14		0.96		0.38		0.00		0.05	
1,2,3,5	0.00		0.00		0.00		0.34		0.00		0.00	
Xylose												
Terminal	4.17		18.12		2.09		8.65		6.64		1.23	
1,2	4.49		9.42		3.79		5.63		4.65		4.19	
1,4	59.68		5.56		50.39		36.12		48.44		52.30	
1,2,4	5.32	[78.86]	1.87	[41.34]	4.96	[65.22]	2.89	[53.90]	5.18	[68.62]	4.32	[58.99]
1,3,4	0.00		0.00		0.00		0.00		0.00		0.00	
1,2,3,4	0.39		0.41		0.74		0.40		0.33		0.34	
Mannose												
1,4	0.60		0.95		1.12		0.20		0.60		0.94	
1,2,4	0.00	[0.61]	0.05	[1.83]	0.20	[3.91]	0.21	[1.00]	0.00	[0.92]	0.00	[1.82]
1,4,6	0.26		0.68		0.86		0.64		0.30		0.82	
Galactose												
Terminal	1.64		4.87		1.01		3.15		2.36		4.19	
1,2	1.26		5.35		0.95		2.93		1.93		1.28	
1,4	0.38		0.35		0.00		0.10		0.08		0.40	
1,6	0.34		0.40		0.22		0.45		0.82		3.29	
1,3,6	0.19	[3.92]	0.12	[10.02]	0.56	[1.88]	0.38	[8.13]	0.32	[4.02]	0.75	[13.23]
1,4,6	0.55		0.42		0.84		0.54		0.25		0.49	
Galactitol	0.31		0.00		0.52		1.02		0.26		0.40	
Glucose												
Terminal	1.17		1.01		0.99		1.80		2.01		1.23	
1,4	5.39		12.35		16.85		9.42		5.74		5.16	
1,4,6	6.17		31.60		4.75		15.82		9.65		6.19	
1,3	0.00	[10.79]	0.00	[40.43]	0.00	[26.20]	0.00	[29.30]	0.00	[20.71]	0.00	[13.76]
1,3,4	0.00		0.00		0.00		0.00		0.00		0.00	
1,2,4	0.00		0.00		0.00		0.00		0.00		0.00	
Glucitol	1.75		0.42		4.12		1.40		2.62		0.52	

the applied material and the carbohydrate composition suggested that it may be a xylan–pectic-polysaccharide complex low in uronic acid. Methylation analysis (Table 3, column 4) confirmed the presence of significant amount of (acidic) xylan, and small amounts of pectic polysaccharides and xyloglucans. The fourth fraction US80-D was very poor in carbohydrate, but linkage analysis (Table 3, column 5) showed that it contained significant amounts of acidic xylans and small amounts of pectic polysaccharides and xyloglucans. This fraction is probably a genuine complex containing polysaccharide and lignin initials and may be derived from tissues undergoing secondary thickening.

3.4. Lower stem: 40–60% EtOH fraction (LS60)

About 17% of this material was insoluble in phosphate buffer (LS60-Ins; Table 1) and carbohydrate accounted for only 40%. UV absorption studies indicated the presence of large amounts of phenolic material. Sugar analysis of the residue, particularly the ratio of xylose to glucose, suggested that acidic xylans and xyloglucans may be the major components (Table 1) and this was confirmed by methylation analysis (Table 4, column 1). The insolubility of this material is probably as a result of the acidic-xylan component and associated polyphenolic material.

The soluble fraction gave three subfractions on anion

Table 5
Glycosyl linkage composition of polysaccharides from 0.5 M KOH upper and lower stem fractions after gel permeation chromatography

Linkage types	Relative mol%							
	Upper and lower stem fractions				Gel-permeation column >			
	US60-A1		LS60-A1		LS60-A1X		LS60-A1P	
Rhamnose								
1,2	0.33		0.48		0.98		0.26	
1,3	0.00	[tr.]	0.12	[2.05]	0.75	[2.55]	0.08	[0.30]
1,2,4	0.58		1.02		0.68		0.12	
Fucose								
Terminal	4.29	[5.64]	4.82	[5.63]	5.46	[7.60]	4.97	[6.74]
Arabinose								
Terminal-f	1.00		0.54		0.56		0.47	
1,2	0.46		0.05		0.14		0.13	
1,3	0.26		0.23		0.43		0.10	
1,5	1.42	[0.60]	0.05	[0.59]	0.52	[0.96]	0.50	[0.80]
1,2,5	0.12		0.09		0.12		0.04	
1,3,5	0.68		0.13		0.10		0.14	
1,2,3,5	0.00		0.00		0.00		0.00	
Xylose								
Terminal	17.05		18.76		17.21		18.45	
1,2	7.37		9.12		7.72		8.48	
1,4	1.97		5.64		2.04		6.35	
1,2,4	0.15	[30.21]	2.14	[38.82]	0.29	[35.72]	1.92	[38.31]
1,3,4	0.00		0.00		0.00		0.00	
1,2,3,4	0.17		0.45		0.27		0.84	
Mannose								
1,4	1.86		0.24		1.27		0.50	
1,2,4	0.00	[2.84]	0.00	[1.02]	0.00	[2.28]	0.00	[1.64]
1,4,6	1.18		0.87		0.75		0.77	
Galactose								
Terminal	4.22		4.36		5.00		4.49	
1,2	5.20		5.32		6.41		5.43	
1,4	0.94		0.35		0.30		0.37	
1,6	0.48		0.32		0.40		0.14	
1,3,6	0.06	[10.67]	0.24	[8.50]	0.25	[9.25]	0.11	[8.57]
1,4,6	0.04		0.47		0.48		0.42	
Galactitol	0.62		0.00		0.00		0.00	
Glucose								
Terminal	1.17		1.25		2.10		1.41	
1,4	15.93		11.81		13.11		12.59	
1,4,6	31.51		30.24		32.10		26.73	
1,3	0.00	[50.05]	0.00	[43.40]	0.27	[41.65]	0.37	[43.64]
1,3,4	0.00		0.00		0.00		0.00	
1,2,4	0.00		0.00		0.00		0.00	
Glucitol	0.97		0.89		0.30		3.81	

exchange chromatography (Fig. 3). The ''neutral'' fraction A (LS60-A) was shown by sugar and methylation analysis (Tables 1–4) to consist of a xyloglucan with a small amount of acidic xylan and a trace amount of pectic polysaccharide. The structural features of the xyloglucan were comparable with those of the xyloglucan component of the US60-A and US80-B fractions. The second fraction LS60-B was rich in xylose and contained significant amounts of uronic acids and glucose. Methylation analysis suggested that this fraction contained an acidic xylan (major component), a slightly-branched xyloglucan (smaller component) and some pectic polysaccharides. It is probable that this fraction is a xylan–xyloglucan–pectic-polysaccharide complex. The third fraction LS60-C was relatively poor in carbohydrate and contained large quantities of associated phenolics (results not shown).

3.5. Lower stem: 60–80% EtOH fraction (LS80)

A major fraction, LS80-A, did not bind to the column (Fig. 4) and accounted for 93% of the material applied to the column. This fraction contained large quantities of xylose and glucose, together with appreciable amounts of uronic acids and galactose and also, small but significant levels of fucose, rhamnose, arabinose and mannose. From the carbohydrate and glycosidic linkage analysis (Tables 1 and 4) the ratio of xylans–xyloglucans–pectic-polysaccharides was computed to be 2:2:1. The non-carbohydrate

Table 6
Glycosyl linkage composition of polysaccharides from lower stem 80% supernatant (LS.SN) EtOH ppt. after gel filtration chromatography of native and digested fractions (values expressed as relative mol%)

Linkage types	Relative mol%											
	LS.SN-A1		LS.SN-A2		LS.SN-A1X1		LS.SN-A1X2		LS.SN-A1X3		LS.SN-A1P	
Rhamnose												
1,2	1.57		1.86		0.26		1.67		0.44		0.10	
1,3	0.25	[1.59]	0.45	[3.37]	0.61	[1.10]	2.41	[7.30]	0.35	[3.75]	0.00	[0.18]
1,2,4	0.65		1.12		1.08		2.65		0.71		0.05	
Fucose												
Terminal	2.28	[2.13]	1.47	[1.26]	2.93	[2.50]	1.94	[1.80]	0.30	[0.36]	2.40	[2.80]
Arabinose												
Terminal-f	1.19		1.79		1.22		1.07		1.43		0.78	
1,2	0.46		0.42		0.35		1.03		0.15		0.35	
1,3	0.66		0.23		0.21		0.96		0.26		0.10	
1,5	1.82	[2.05]	1.02	[2.78]	1.52	[2.70]	1.38	[3.37]	1.86	[3.54]	0.21	[1.25]
1,2,5	0.21		0.23		0.47		0.50		0.24		0.00	
1,3,5	0.60		0.51		0.73		0.45		1.18		0.08	
1,2,3,5	0.00		0.00		0.00		0.00		0.00		0.00	
Xylose												
Terminal	6.67		5.88		9.40		10.92		8.65		7.35	
1,2	4.45		3.21		6.05		3.85		2.15		4.61	
1,4	46.85		48.12		13.12		25.13		18.46		49.12	
1,2,4	5.50	[68.74]	5.93	[67.25]	4.45	[33.86]	7.14	[54.93]	2.68	[20.06]	5.96	[71.20]
1,3,4	0.00		0.00		0.00		0.00		0.00		0.00	
1,2,3,4	0.36		0.81		0.49		1.62		0.50		0.81	
Mannose												
1,4	0.00		0.90		1.02		1.02		2.47		0.00	
1,2,4	0.00	[0.07]	0.00	[3.07]	0.80	[2.10]	0.80	[2.90]	0.91	[4.88]	0.00	[0.05]
1,4,6	0.23		0.35		0.62		0.16		0.29		0.35	
Galactose												
Terminal	2.59		1.94		3.14		1.89		2.80		2.30	
1,2	1.70		1.27		3.72		0.86		1.08		1.81	
1,4	1.04		0.00		0.32		0.25		1.12		0.08	
1,6	0.20		0.50		1.26		1.05		0.48		0.10	
1,3,6	0.18	[6.30]	0.32	[3.46]	0.59	[9.40]	0.79	[5.47]	0.49	[7.16]	0.00	[5.05]
1,4,6	0.45		1.28		0.61		1.32		0.30		0.21	
Galactitol	0.60		0.56		0.62		4.38		1.52		0.56	
Glucose												
Terminal	1.20		3.18		2.33		3.04		23.26		2.60	
1,4	6.82		5.43		15.25		5.38		15.16		6.51	
1,4,6	10.70		7.45		22.05		4.39		5.71		10.35	
1,3	0.19	[19.13]	1.05	[18.81]	1.93	[48.42]	0.80	[24.24]	1.16	[60.24]	1.05	[19.47]
1,3,4	0.00		0.00		0.00		0.00		0.00		0.00	
1,2,4	0.00		0.00		0.00		0.00		0.00		0.00	
Glucitol	0.57		2.72		2.85		11.15		3.89		2.16	

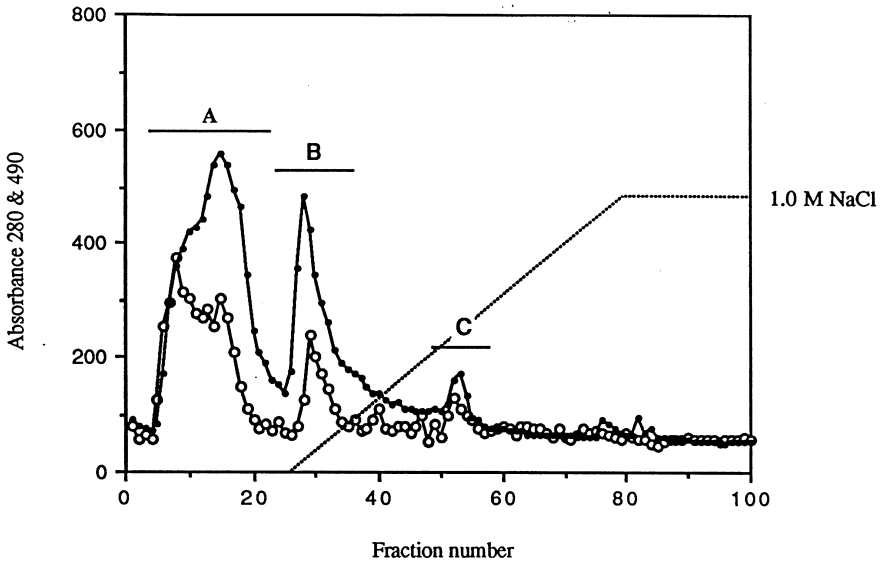


Fig. 5. Ion-exchange chromatography of fraction LS.SN on DEAE-Trisacryl M. Symbols: ∘—∘, OD 280 nm (carbohydrate); · — · , OD 490 nm (phenolics).

component of this fraction (about 20%) was probably phenolic material as indicated by the absorbance at 280 nm (Fig. 4). Fraction LS80-B was a minor fraction which, from the carbohydrate analysis, contained high amounts of pectic material in association with xyloglucans.

3.6. Lower stem: 80% ethanol supernatant (LS.SN)

Anion exchange chromatography gave three fractions (Fig. 5). The first fraction LS.SN-A exhibited UV absorbtion, and was relatively rich in xylose and glucose, and contained smaller amounts of sugars usually associated with pectic polysaccharides (Table 1). Methylation analysis results (Table 4, column 5) suggested the presence of acidic xylans, highly-branched xyloglucans, and some pectic polysaccharides. It is probably a complex containing the aforementioned polymers. The second fraction LS.SN-B also exhibited UV absorption, and was relatively rich in xylose, but contained less glucose and more sugars usually associated with pectic polysaccharides. Methylation analysis suggested the presence of acidic xylans, and small amounts of xyloglucan and pectic polysaccharides. Like fraction LS.SN-A, this fraction was probably also a complex containing phenolic material, xylan, xyloglucan and pectic polysaccharides. Fraction LS.SN-C was a minor fraction which, from the carbohydrate analysis, probably contained xyloglucans, xylans and pectic polysaccharides in conjunction with phenolic moieties (Fig. 5).

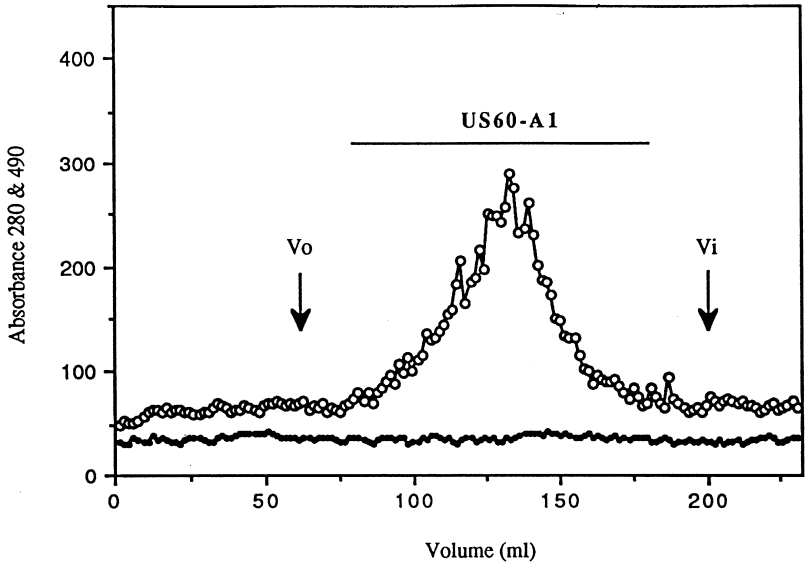


Fig. 6. Gel permeation chromatography on Sephacryl S-400-HR of fraction US60-A. Symbols: ∘—∘, OD 280 nm (carbohydrate); · — · , OD 490 nm (phenolics).

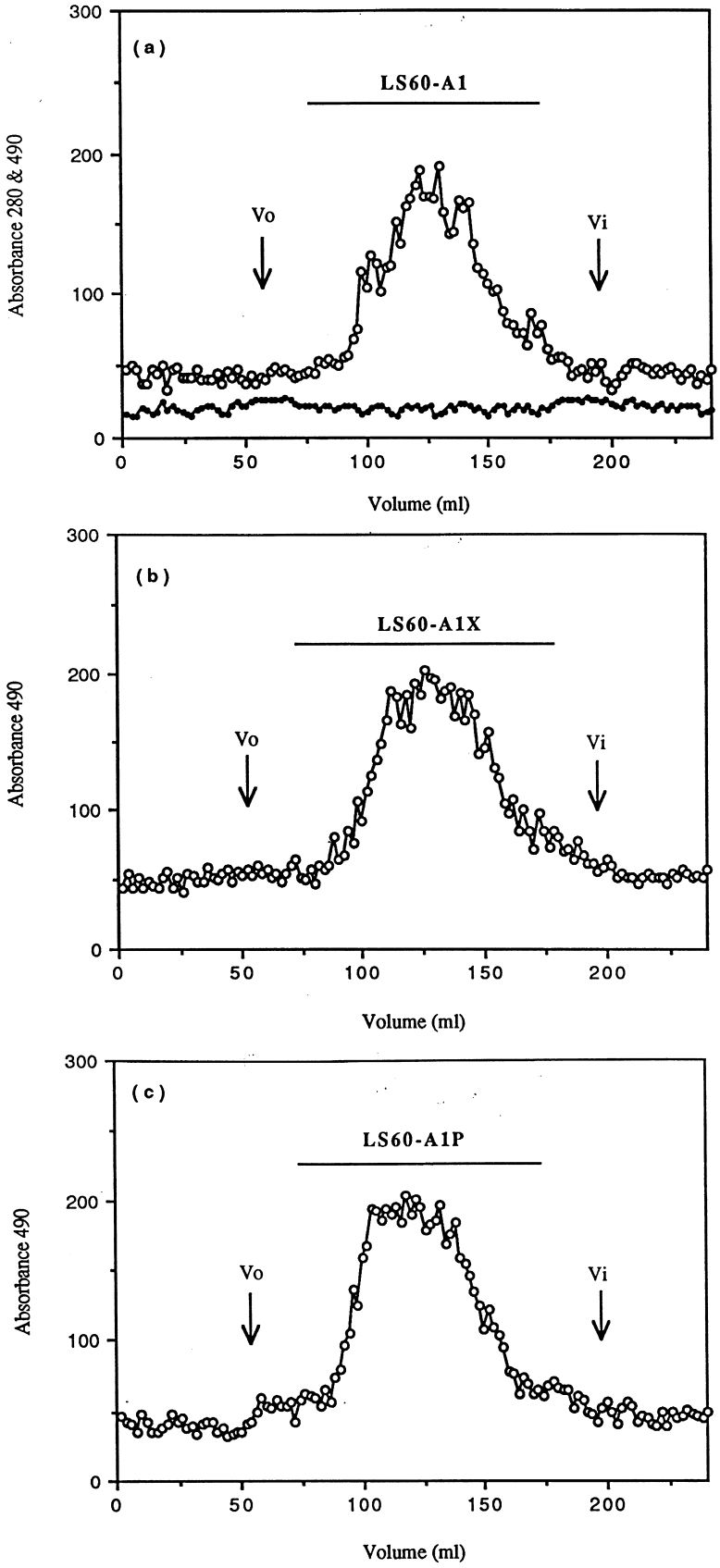
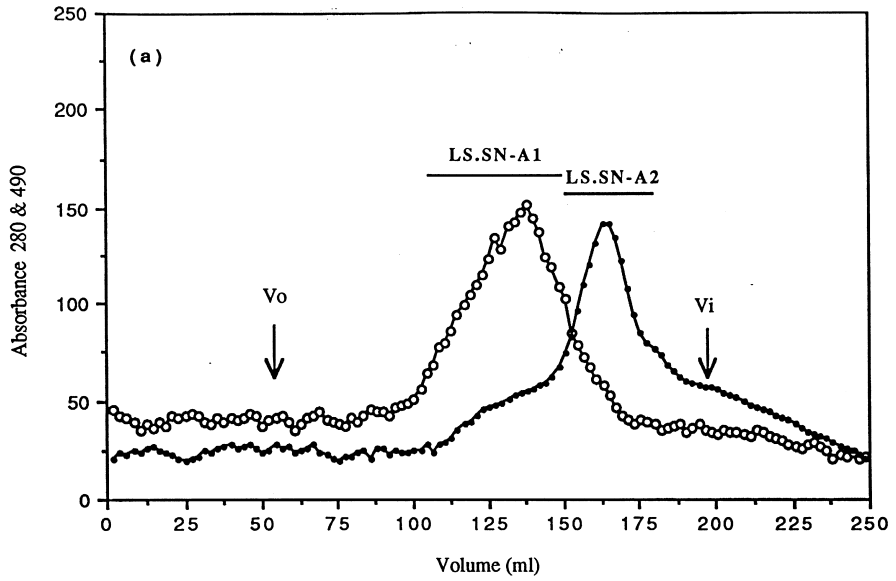
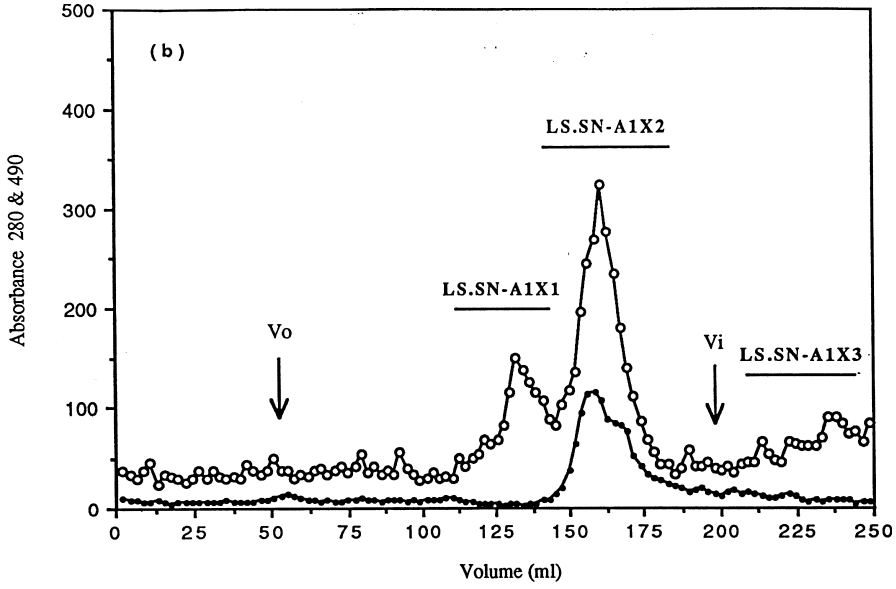
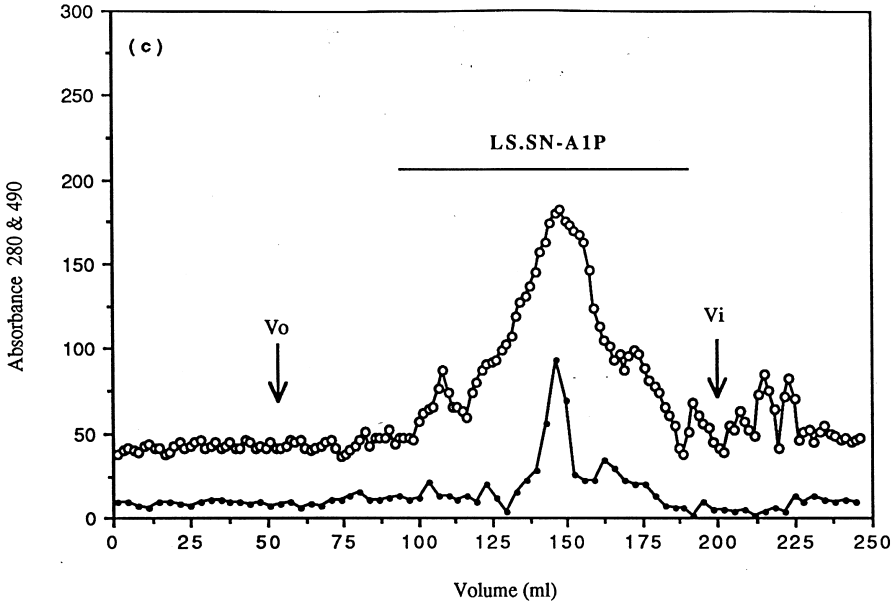


Fig. 7. (a) Gel permeation chromatography on Sephacryl S-400-HR of fraction LS60-A (native). Symbols: ∘—∘, OD 280 nm (carbohydrate); ・—・, OD 490 nm (phenolics); (b) Gel permeation chromatography on Sephacryl S-400-HR of fraction LS60-A (*endo*-xylanase digested). Symbols: ∘—∘, OD 280 nm (carbohydrate); ・—・, OD 490 nm (phenolics); (c) Gel permeation chromatography on Sephacryl S-400-HR of fraction LS60-A (*endo*-polygalacturonase digested). Symbols: ∘—∘, OD 280 nm (carbohydrate); ・—・, OD 490 nm (phenolics).


(a)
LS.SN-A1
LS.SN-A2
Vo
Vi
Absorbance 280 & 490
Volume (ml)


(b)
LS.SN-A1X2
LS.SN-A1X1
LS.SN-A1X3
Vo
Vi
Absorbance 280 & 490
Volume (ml)


(c)
LS.SN-A1P
Vo
Vi
Absorbance 280 & 490
Volume (ml)

3.7. Gel-permeation chromatography

The fractions US60-A and LS60-A which were rich in xyloglucans, and the neutral fraction from LS.SN (LS.SN-A) were submitted to gel filtration chromatography on Sephacryl S-400-HR, and the elution profiles of the applied materials are shown in Figs. 6–8.

3.8. Fractions US60-A and LS60-A

The fractions US60-A and LS60-A gave the symmetrical peaks US60-A1 (Fig. 6) and LS60-A1 (Fig. 7(a)) respectively, and their sugar compositions are given in Table 2; these compositions were highly comparable with those of the fractions before passing through the column. The xyloglucans present in these fractions, which were free of phenolics, were found to have a MW of 50–60 kDa, using low molecular weight dextran standards.

Treatment of LS60-A1 with highly purified xylanase and re-chromatography on Sephacryl S-400-HR did not significantly alter either its elution profile (Fig. 7(b)) or its carbohydrate composition and linkage profile (LS60-A1X, Tables 2 and 5). Also, treatment of LS60-A1 with highly pure polygalacturonase and re-chromatography did not significantly modified its elution profile (Fig. 7(c)) or its carbohydrate content and linkage profile (LS60-A1P, Tables 2 and 5). This clearly showed that the purified xylanase and polygalacturonase were free of xyloglucanase activity.

The absence of xyloglucanase activity in the aforementioned enzymes was of particular importance as these enzymes were used in the following studies to confirm the complexing of xylans, xyloglucans and pectic polysaccharides in LS.SN-A fraction.

3.9. Fraction LS.SN-A

Fraction LS.SN-A was resolved into two slightly overlapping peaks by chromatography on Sephacryl S-400-HR (LS.SN-A1 and LS.SN-A2; Fig. 8(a)). To minimise contamination between the peaks, the fraction 75–135, and 145–200 were pooled, dialysed and freeze-dried. Their carbohydrate compositions and linkage profiles are given in Tables 2 and 6. Although the mol% composition of fractions LS.SN-A1 and LS.SN-A2 was comparable, the carbohydrate recovery from LS.SN-A2 was much lower confirming the presence of large amounts of phenolics (Fig. 8(a)). The molecular weights of the fractions LS.SN-A1 and LS.SN-A2 was computed to be 70 and 10 KDa, respectively.

3.10. Treatment of LS-SN-A1 with xylanase

Treatment of fraction LS-SN-A1 with xylanase produced 3 separate sub-fractions on re-chromatography on Sephacryl S-400 (Fig. 8(b)). The first, LS.SN-A1X1, exhibited similar size-exclusion properties to the parent compound. However, the other two (LS.SN-A1X2 and 3) were of a lower molecular weight. Compared to LS-SN-A1, all three fractions contained much less xylose (Table 2), particularly (1 → 4)-linked xylose (Table 6). Further, the remaining xylan moieties were more highly branched than the parent compound as indicated by the significantly lower ratios of (1 → 4)- to (1 → 2,4)-linked xylose residues (Table 6). Linkage analysis also revealed the presence of xyloglucans and small amounts of pectic polysaccharide moieties in the LS.SN-A1X1,2 and 3 fractions. The total material recovered from the three digested fractions accounted for only 50% of the applied material. However, over 95% of the material not recovered could be accounted for by the loss of (1 → 4)-linked xylose residues. These were detected in the dialysate of the digested material (results not shown). These results demonstrate that specific degradation of the xylan moiety in the parent LS-SN-A1 compound will result in a significant decrease in the molecular weights of pectic-xyloglucan-containing moieties.

3.11. Treatment of fraction LS.SN-A1 with polygalacturonase

Treatment of fraction LS.SN-A1 with polygalacturonase and re-chromatography on SephacrylS-400-HR gave rise to a single peak of lower molecular weight (40–45 kDa; Fig. 8(c)). Carbohydrate analysis (Table 2) showed that the uronic acid component was considerably reduced, and that there was a correspondingly higher level (on a mol% basis) of the other prominent sugars, xylose and glucose. The specificity of the polygalacturonase was highlighted by the observation that the linkages of the main neutral sugars of LS.SN-A1P are highly comparable to those of the parent compound. These results show that specific degradation of the uronide moiety in the parent LS-SN-A1 compound result in the decrease in the molecular weight of other xylan and xyloglucan components.

4. Conclusions

The results of this investigation on the 0.5 M KOH extracts of cauliflower cell walls, containing xylose, glucose, arabinose and uronic acid, their fractionation by graded ethanol precipitation and ion-exchange chromatography provided good evidence for the occurrence of a range of closely associated acidic xylans, xyloglucans and pectic polysaccharides, possibly in covalent association.

Fig. 8. (a) Gel permeation chromatography on Sephacryl S-400-HR of fraction LS.SN-A (native). Symbols: ◦—◦, OD 280 nm (carbohydrate); · — ·, OD 490 nm (phenolics); (b) Gel permeation chromatography on Sephacryl S-400-HR of fraction LS.SN-A (*endo*-xylanase digested). Symbols: ◦—◦, OD 280 nm (carbohydrate); · — ·, OD 490 nm (phenolics); (c) Gel permeation chromatography on Sephacryl S-400-HR of fraction LS.SN-A (*endo*-polygalacturonase digested). Symbols: ◦—◦, OD 280 nm (carbohydrate); · — ·, OD 490 nm (phenolics).

Further chromatography of a specific fraction (LS.SN-A1) by gel filtration, and subsequent methylation analysis, confirmed the heterogeneous composition of the complexes (which gave symmetrical peaks on elution) and indicated molecular weights of 70 kDa. Treatment of the pectic-xylan–xyloglucan moiety with purified specific xylanase or polygalacturonase enzymes resulted in the degradation of only the xylan and pectic components respectively, and an accompanying decrease in the molecular weight of the remaining xyloglucan-rich moiety. The xyloglucan component was not degraded.

These results clearly show that the fraction LS.SN-A1 is a genuine complex containing acidic xylans, xyloglucan and pectic polysaccharides. A similar approach, using polysaccharide degrading enzymes, has been used to demonstrate the presence of xylan–xyloglucan complexes in the cell walls of olive pulp (Coimbra et al., 1995). The aforementioned work, in conjunction with the results reported by Femenia et al. (1999) gives direct evidence for the maturation-related increase in cell-wall cross-linking in cauliflower stems.

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