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Studies on Apple Protopectin. IV: Apple Xyloglucans and Influence of Pectin Extraction Treatments on their Solubility

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ABSTRACT

Hemicelluloses were extracted from apple cell walls with 1 M and 4 M sodium hydroxide and 8 M urea after depectinisation by a chelating agent, by a chelating agent and dilute sodium hydroxide or by a chelating agent and a pectin-lyase. The extracts were fractionated on Sephacryl S 500 and DEAE Sepharose CL-6B. The bulk of the hemicelluloses were solubilised by 4 M sodium hydroxide. The main hemicellulose was a fucogalactoxyloglucan. Some low-molecular-weight mannans were also present. Part of the xyloglucans could be extracted by urea after pectin extraction by a chelating agent or by pectin-lyase but not after pectin extraction by dilute sodium hydroxide. Dilute sodium hydroxide probably insolubilised some of the pectins and hemicelluloses.

INTRODUCTION

Primary plant cell walls are a complex structure which allow growth of the cell while acting as a skeleton for the plant. A lot of attention has been devoted to apple pectins (Barrett & Northcote, 1965; Knee, 1973*a*, *b*, 1978*a*, *b*; Knee *et al.*, 1975; de Vries *et al.*, 1981, 1982,

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1983*a*, *b*; de Vries, 1983; Aspinall & Fanous, 1984; Rouau & Thibault, 1984; Stevens & Selvendran, 1984; Renard *et al.*, 1990*a*, *b*, *c*, *d*). Less is known about apple hemicelluloses, and the main hemicellulose appears to be a xyloglucan, with traces of mannans, xylans and 1,3-glucans (Aspinall & Fanous, 1984; Stevens & Selvendran, 1984; Ruperez *et al.*, 1985; Voragen *et al.*, 1986). Xyloglucans are the main hemicellulosic polymers of the primary cell walls of Dicotyledons (Hayashi, 1989). They are heteropolymers with a β-(1→4) glucose backbone carrying α-xylosyl residues branched on the O-6 of the glucose, and galactose attached by a β-(1→2) link to the xylose. Except in Solanaceae, α-fucose residues are carried on the O-2 of the galactose units. Xyloglucans are found in close association with the cellulose as the backbone is a cellulose-like chain which binds specifically to cellulose by H-bonds. They coat microfibrils and are interwoven in the non-crystalline regions of the cellulose microfibrils. Xyloglucans are thought to have a major role in controlling cell elongation by allowing 'creep' of cellulose microfibrils (Hayashi, 1989).

Our previous work on enzymatic extraction of protopectin (Renard *et al.*, 1991*a*, *b*) showed that xyloglucans play a major role in connecting pectins to other cell wall polymers. The highest synergism for extraction of protopectin was obtained with combinations of pectolytic enzymes and an *endo*-glucanase. This *endo*-glucanase degraded the fucogalactoxyloglucan from apple cell walls, which led us to postulate the existence of a connection between the rhamnogalacturonan and the xyloglucan. In order to gain a better understanding of this interaction, we extracted apple xyloglucans with concentrated sodium hydroxide and urea at different stages of pectin extraction.

MATERIALS AND METHODS

Plant material

Apples (*Malus malus* L., Rosaceae, var. Golden delicious) were obtained from the Sprenger Institute (Wageningen, The Netherlands) as described elsewhere (Renard *et al.*, 1990).

Partially depectinated cell-wall material

Apple cell-wall material free from soluble pectins was prepared by extensive treatment of apple alcohol insoluble solids (AIS) by a chelating agent — cyclohexane diamino tetraacetic acid (CDTA) — as described by Renard *et al.* (1990), giving the CDTA insoluble residue (CDTA-IR).

Extraction of pectins (Fig. 1)

CDTA-IR (5 g) was treated with 500 ml NaOH 0·05 M containing $NaBH_4$ 0·026 M for 16 h at 0°C. The slurry was centrifuged (20 min, 23 300 × ***g***) and the residue was washed three times with distilled water. The extract was brought to pH 4·5 by HCl 1 M. CDTA-IR (5 g) was treated for 24 h with 66 000 nkat of pectin-lyase from *Aspergillus niger* (pectin-lyase type II) (van Houdenhoven, 1975) in 500 ml sodium succinate buffer 0·05 M,

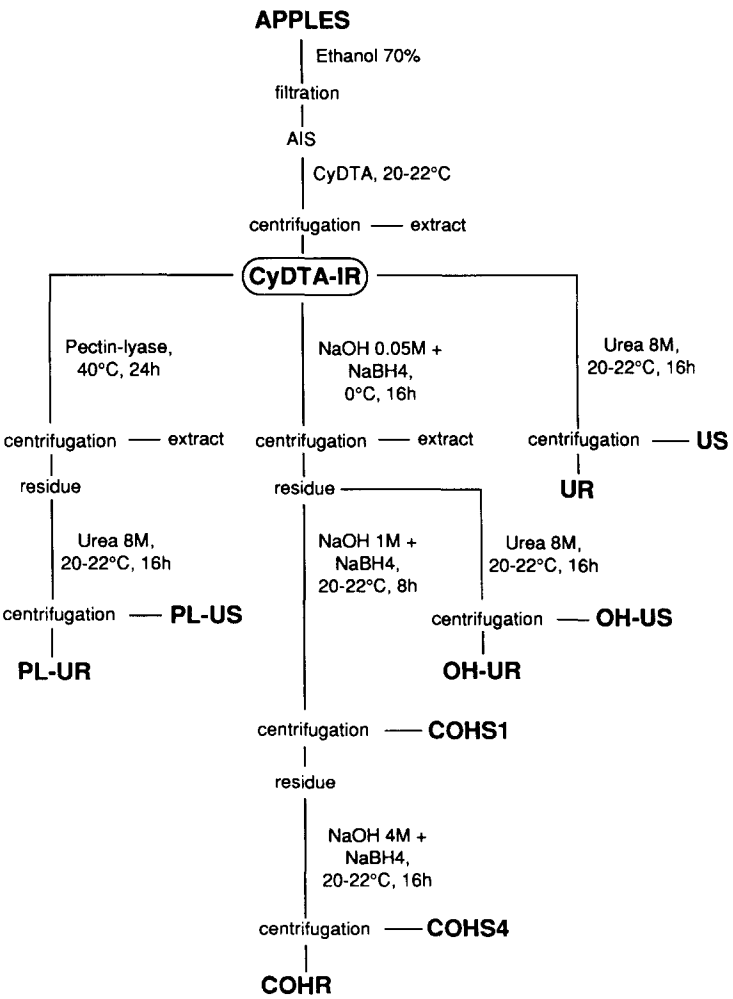

Fig. 1. Extraction scheme of the xyloglucans. CDTA-IR: CDTA insoluble material; US: urea soluble; UR: urea residue; PL-US: urea soluble after treatment by pectin-lyase; PL-UR: urea residue after treatment by pectin-lyase; OH-US: urea soluble after NaOH 0·05 M; OH-UR: urea residue after NaOH 0·05 M; COHS1: concentrated NaOH soluble (1 M); COHS4: concentrated NaOH soluble (4 M); COHR: concentrated NaOH residue.

pH 4·5 at 40°C. The slurry was centrifuged (20 min, 23 300 × ***g***) and the residue was washed three times with distilled water.

Extraction of xyloglucans (Fig. 1)

The residue from NaOH 0·05 M extraction was suspended in 500 ml NaOH 1 M (containing $NaBH_4$ 0·026 M) for 8 h at room temperature. The slurry was centrifuged (20 min, 23 300 × ***g***) and the extract was brought to pH 4·5 by HCl 1 M and dialysed against distilled water (theoretical cut-off of the tubing: 6000–8000 Da for oligopeptides). The residue was dispersed in 500 ml of NaOH 4 M (containing $NaBH_4$ 0·026 M) and further extracted for 16 h at room temperature. The slurry was centrifuged (20 min, 23 300 × ***g***). The extract was brought to pH 4·5 by HCl 1 M and dialysed against distilled water. The residue was washed with distilled water on a sintered glass filter (porosity 1) until washing water was at pH 6 and then freeze-dried.

Urea extractions were carried out on three different starting materials: CDTA-IR, the residue from NaOH 0·05 M treatment and the residue from pectin-lyase treatment. Five grams (or residues corresponding to five grams of starting material) (Fig. 1) of CDTA-IR were extracted for 16 h at room temperature by 500 ml of urea 8 M buffered to pH 4·5 by sodium succinate. The slurries were centrifuged (20 min, 23 300 × ***g***) and the extracts were dialysed against distilled water. The residues were washed extensively with distilled water on sintered glass filters (porosity 1). The following fractions were obtained: extract US and residue UR from CDTA-IR, extract OH-US and residue OH-UR from the residue of NaOH 0·05 M treatment and extract PL-US and residue PL-UR from the residue of pectin-lyase treatment. All extracts were concentrated with a rotary evaporator and kept frozen. Aliquots were thawed or freeze-dried as necessary.

Analytical

Galacturonic acid (GalA) and total neutral sugars (NS) concentrations were measured by automated *meta*-hydroxydiphenyl (Thibault, 1979) and orcinol assays (Tollier & Robin, 1979), respectively. Corrections were made for the mutual interferences. Individual NS were analysed as their alditol acetate derivatives by gas chromatography (Englyst & Cummings, 1984) after prehydrolysis by H_2SO_4 13 M for 1 h followed by hydrolysis by H_2SO_4 (1 M at 100°C) for 3 h for residues, and without prehydrolysis for soluble products and residues. Proteins were measured in the residues by semi-automatic micro-Kjeldahl (Roozen &

Ouwehand, 1978) using a conversion factor of 6·25, and in the extracts by the method of Lowry *et al.* (1951) and were detected in the chromatography fractions by the absorbance at 280 nm. Analyses were carried out in duplicate; the precision of the measurement was ± 5%.

Chromatography

The concentrated extracts (1 ml, 5 to 10 mg carbohydrates) were applied to a 1·3 × 11-cm column of DEAE Sepharose CL-6B. The column was washed by 50 ml succinate buffer 0·005 M pH 4·8 and eluted by a succinate gradient (100 ml, from 0·005 to 0·4 M). Residual material was eluted by 30 ml of 0·4 M buffer followed by 30 ml of 1 M buffer and 50 ml of NaOH 0·25 M. Fractions (2 ml) were assayed for galacturonic acid and NS. Appropriate fractions were pooled, dialysed, freeze-dried and analysed for individual NS. The concentrated extracts (5 ml) were also applied to a 2·5 × 90 cm column of Sephacryl S 500 eluted by sodium succinate buffer 0·1 M pH 4·5. Fractions (5 ml) were assayed for galacturonic acid, NS and absorbance at 280 nm. Appropriate fractions were pooled, dialysed, freeze-dried and analysed for individual NS.

RESULTS

Yields and composition of the fractions

Results from the extraction of insoluble pectin with NaOH 0·05 M and pectin-lyase (Table 1) were in agreement with previously published work (Renard *et al.*, 1990, 1991*b*). Dilute sodium hydroxide and pectin-lyase extracted pectic material that was rich in galacturonic acid, arabinose and galactose. Pectin-lyase was more efficient than cold dilute sodium hydroxide for elimination of 'non-soluble' pectin.

NaOH 1 M and 4 M (after pretreatment with NaOH 0·05 M) extracted in total 14·8% of CDTA-IR (Table 1). The extract from NaOH 1 M was very rich in proteins (63·3%). The main sugars in these two extracts were glucose and xylose: glucose represented 43% of the sugars in the NaOH 1 M extract and 42% in the NaOH 4 M extract, and xylose represented 23% of the sugars in both extracts. NaOH 1 M extracted 6% of the xylose and 2% of the glucose from the CDTA-IR, and NaOH 4 M extracted 35% of the xylose and 11% of the glucose. Fucose was also solubilised to a large extent. NaOH 4 M extracted 75% of the mannose present in CDTA-IR. Both extracts were poor in galacturonic acids (up to 7·9% of the sugars in the NaOH 1 M extract and 2·9% in the NaOH 4 M extract) and

TABLE 1
Composition of the Pectin and Hemicellulose Extracts and Cell-Wall Residues

	Yields (% of CDTA-IR)	*Composition (weight %)*								
		GalA	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*Protein*
CDTA-IR[a]	100	19·8	2·6[b]		11·8	6·3	1·6	8·7	36·1 (1·3)[c]	5·7
Pretreatment with NaOH 0·05 M										
Pectin extract	11·4	53·4	0·7		8·1	1·1		4·0	0·3	32·4
Urea extract[d]	nd	28·9	2·6		42·0	5·3		18·4	2·6	nd
Urea residue	71	7·3	0·7	1·4	9·2	7·0	1·7	8·1	41·1 (1·6)	7·0
NaOH 1 M	4·3	2·9		2·5	2·5	8·3	0·7	4·0	15·8	63·3
NaOH 4 M	10·5	2·2		4·3	3·7	20·9	11·2	10·4	37·6	10·6
NaOH residue	43	8·3	0·7	0·7	10·9	4·6	0·2	7·9	53·5 (3·4)	0·5
Pretreatment with pectin-lyase										
Pectin extract	18·5	61·4	2·1		19·8	2·5	tr.	10·2	1·4	2·6
Urea extract	4·3	10·4	0·3	1·1	10·8	4·8		7·8	7·1	57·7
Residue	51	5·6	0·7	1·4	8·8	7·6	2·1	8·2	48·7 (1·7)	5·2
No pretreatment										
Urea extract	7·2	28·6	0·2	0·6	6·3	3·5	0·4	5·3	4·9	50·2
Residue	85	14·1	0·9	1·2	9·7	5·8	1·5	7·9	34·1 (1·2)	6·7

[a]Values of Renard *et al.* (1990).
[b]Rhamnose and fucose not separated.
[c]Values in parentheses are glucose contents measured in the residues without prehydrolysis.
[d]Sugar fraction only.
nd: Not determined.

in arabinose (up to 2·5% of the sugars in the NaOH 1 M extract and 3·7% in the NaOH 4 M extract), and were free from rhamnose, showing that the concentrated sodium hydroxide extracted little additional pectin. The α-cellulose residue (COHR) was almost devoid of proteins and mannose. It was enriched in glucose (cellulose), but still contained appreciable amounts of arabinose, galacturonic acid, galactose, xylose and rhamnose. The proportion of glucose that could be hydrolysed by H_2SO_4 1 M increased after treatment by NaOH 4 M.

Urea extractions were carried out on three different cell-wall materials (Fig. 1) differing by preceding extractions of pectins: (i) CDTA-IR; (ii) CDTA-IR treated by a pectin-lyase, which extracted 57% of the residual galacturonic acids; and (iii) CDTA-IR treated by cold NaOH 0·05 M, which extracted 31% of the residual galacturonic acids. Urea solubilised low amounts of material of varied composition, characterised by low proportions of sugars and high proportions of proteins (Table 1). Urea extracts of CDTA-IR and after pectin-lyase action were rich in galacturonic acid and arabinose, and relatively poor in xylose, glucose and fucose. The proportions of NS were higher after treatment by pectin-lyase. Yields of xylose and glucose were similar in those two extracts: urea extracted 4% of the xylose and 1% of the glucose from CDTA-IR, and after pectin-lyase the yields were 3% and 1%, respectively. The yield calculated for the sugar fraction of the urea extract after NaOH 0·05 M treatment was very low: 0·7% of the sugars of CDTA-IR, versus 2·2% for the urea extract after pectin-lyase and 4·3% for the urea extract of CDTA-IR. The urea extract after NaOH 0·05 M also differs by its composition: it contained no fucose and low proportions of xylose and glucose. The urea residues were little modified compared to the CDTA-IR, residues obtained from treatment with dilute sodium hydroxide (Renard *et al.*, 1990) or with PL (Renard *et al.*, 1991*a*). The high nitrogen content was probably due to persistence of urea in the residues even after extensive washing.

Yields of extracts and residue did not add up to 100% due to losses during dialysis and to difficulties in quantitatively recovering the residues.

Gel-filtration chromatography on Sephacryl S 500

The NaOH 1 M extract eluted as a single symmetric peak (Fig. 2) at $K_{av} = 0·7$. However, its composition was not homogeneous (Table 2): the high-molecular-weight part of that peak was poorer in arabinose; and the low-molecular-weight part was poorer in fucose, xylose and glucose, and richer in mannose and arabinose. No proteins could be detected in the

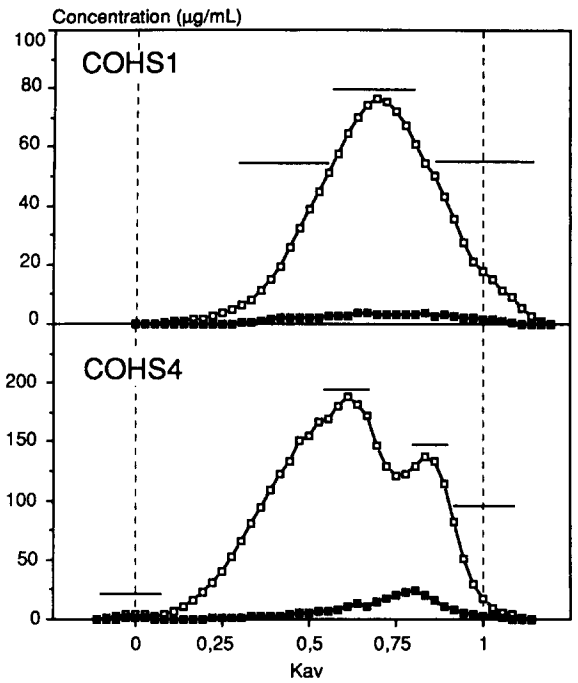

Fig. 2. Gel-filtration chromatography of the NaOH 4 M and 1 M extracts on Sephacryl S 500 eluted with sodium succinate buffer 0·1 M pH 4·5. The bars indicate fractions that were pooled for sugars analysis. ■: Neutral sugars; □: galacturonic acid.

eluant: they were probably eliminated with the precipitate (poor in sugars) formed on thawing the extract and removed by centrifugation prior to injection. The NaOH 4 M extracted showed two peaks, one at $K_{av} = 0{\cdot}6$, and the other at $K_{av} = 0{\cdot}8$. The peak at $K_{av} = 0{\cdot}6$ had almost the same composition as the NaOH 1 M extract. It was poorer in arabinose and had a slightly higher molecular weight. The low-molecular-weight material contained arabinose and mannose-rich polymers. A minor fraction eluted at the void volume. This fraction contained galacturonic acid (54 mol%), fucose, xylose, galactose and glucose in the molar ratios 1:4:2:7 and some mannose. In this extract also no proteins were detected in the eluant and were probably eliminated in the precipitate formed on thawing.

The urea extract of CDTA-IR showed distinct elution patterns for proteins, galacturonic acid and NS (Fig. 3), suggesting separate populations. The low-molecular-weight fraction was enriched in fucose, xylose and glucose (Table 3), suggesting the presence of xyloglucan, and the high-molecular-weight material was apparently of pectic nature. Four

TABLE 2

Composition (mol%) of Gel-filtration Chromatography Fractions of Concentrated NaOH Extracts[a]

	NaOH 1 M			*NaOH 4 M*			
	$K_{av} = 0{\cdot}3$–$0{\cdot}5$	$K_{av} = 0{\cdot}5$–$0{\cdot}8$	$K_{av} > 0{\cdot}8$	$K_{av} < 0{\cdot}1$	$K_{av} = 0{\cdot}5$–$0{\cdot}7$	$K_{av} = 0{\cdot}8$–$0{\cdot}9$	$K_{av} = 1$
Rha	0·1	0·4	0·6	0	tr.	0·4	
Fuc	7·9	7·1	3·6	3·2	6·5	0·5	0·7
Ara	3·9	8·0	11·5	0	1·9	31·4	11·6
Xyl	28·3	29·5	25·3	11·9	31·5	10·8	8·7
Man	0·5	0·3	12·9	3·5	0·1	26·8	41·3
Gal	11·8	12·5	12·4	6·7	11·2	8·0	11·8
Glc	43·9	42·2	32·3	20·8	46·6	15·8	23·9
GalA	3·6	tr.[b]	1·4	53·9	2·1	6·4	2·0

[a]Gel-filtration chromatography was carried out on a Sephacryl S 500 column eluted with succinate buffer 0·1 M pH 4·5.
[b]tr: Traces.

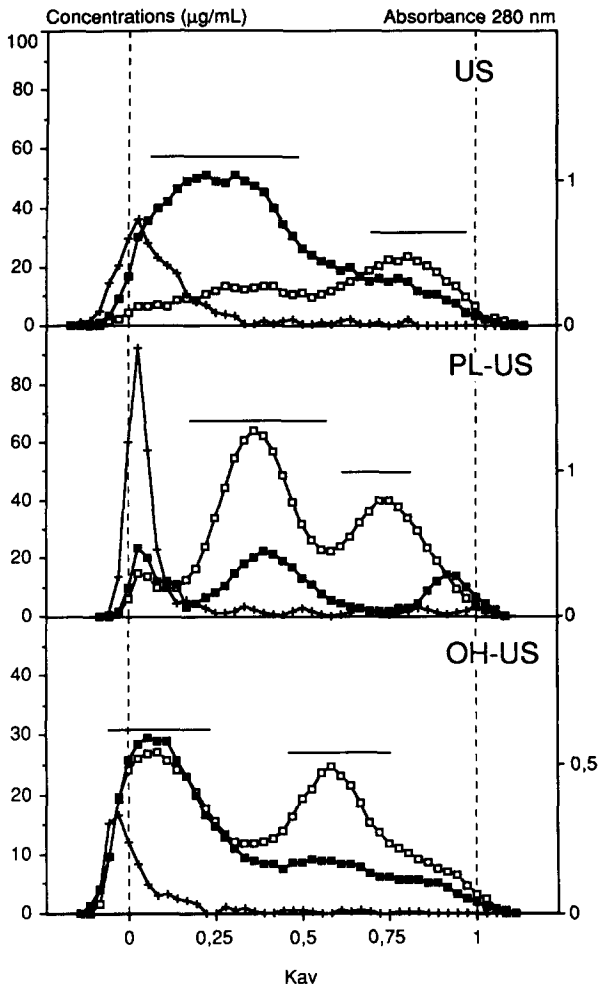

Fig. 3. Gel-filtration chromatography of the urea extracts on Sephacryl S 500 eluted with sodium succinate buffer 0·1 M pH 4·5. The bars indicate fractions that were pooled for sugars analysis. ■: Neutral sugars; □: galacturonic acid; +: absorbance 280 nm.

different fractions could be distinguished in the urea extract after pectin-lyase. Proteins eluted near the void volume, as in all urea extracts. The fraction at $K_{av} = 0{\cdot}4–0{\cdot}5$ had a composition similar to that of the fraction eluting at the same K_{av} for the PL extract (Renard *et al.*, 1991*c*). The fraction eluting at $K_{av} \approx 0{\cdot}8$ was rich in fucose, xylose, galactose and fucose, suggesting the presence of a fucogalactoxyloglucan. Low-molecular-weight uronides eluted at $K_{av} \approx 0{\cdot}9$. Both sugar peaks of the urea extract after NaOH 0·05 M contained pectic material, rich in

TABLE 3
Composition (mol%) of Gel-filtration Chromatography Fractions of the Urea Extracts[a]

	CDTA-IR		*Pectin-lyase*		*NaOH 0·05 M*	
	K_{av} = *0·1–0·5*	K_{av} = *0·7–1*	K_{av} = *0·2–0·6*	K_{av} = *0·6–0·8*	K_{av} = *0–0·2*	K_{av} = *0·5–0·7*
Rha	0·9	1·1	4·7	0·3	4·1	3·3
Fuc		3·2		6·5		tr.[b]
Ara	16·0	14·0	50·0	6·2	42·8	48·1
Xyl	2·9	17·1	8·0	28·4	6·2	4·4
Man	1·0	2·5	0·3	0·5	0·4	0·5
Gal	4·6	13·8	24·0	10·6	14·4	22·3
Glc	2·8	23·0	3·8	41·8	1·9	3·8
GalA	71·8	25·3	14·2	2·9	30·2	17·6

[a]Gel-filtration chromatography was carried out on a Sephacryl S 500 column eluted with succinate buffer 0·1 M pH 4·5.
[b]tr: Traces.

arabinose, galactose and galacturonic acid. No xyloglucan-containing fraction could be detected for this extract, as could be expected from its composition.

Ion-exchange chromatography

The NaOH 1 M extract and the urea extracts after CDTA and after pectin-lyase were fractionated by ion-exchange chromatography in order to separate xyloglucan and pectic material. The NaOH 1 M extract gave a major fraction eluting with the starting buffer (chromatogram not shown) with only traces of acidic material eluted by the gradient. The first fraction contained 80% of the NS. The urea extracts also presented important neutral fractions (not bound on the DEAE Sepharose CL-6B gel) (Fig. 4) rich in glucose, xylose and fucose (Table 4). Molar ratios fucose/xylose/galactose/glucose were similar: 1:3·5:1·5:6 for the urea extract of CDTA-IR, 1:5·5:2:8 for the urea extract after pectin-lyase action and 1:4:1·5:6 in the NaOH 1 M extract. The bound fraction of the urea extract of CDTA-IR was rich in galacturonic acid, and the

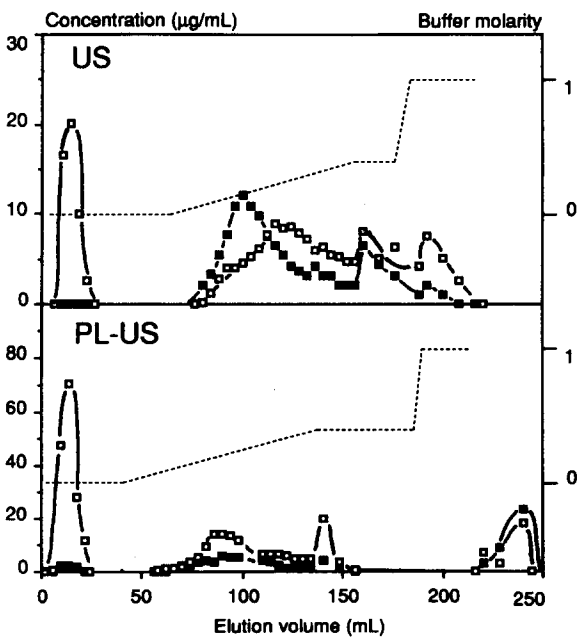

Fig. 4. Ion-exchange chromatography of the urea extracts of CDTA-IR and after pectin-lyase on DEAE Sepharose CL-6B eluted with sodium succinate buffer pH 4·8. The bars indicate fractions that were pooled for sugars analysis. ■: Neutral sugars; □: galacturonic acid; – – –: buffer molarity.

TABLE 4
Composition (mol%) of Ion-exchange Chromatography Fractions of NaOH 1 M Extract, Urea Extract of CDTA-IR and after Pectin-lyase[a,b]

	NaOH 1 M	*Urea on CDTA-IR*		*Urea after pectin-lyase*	
	Not bound	*Not bound*	*Bound*	*Not bound*	*Bound*
Rha			tr.[c]		tr.
Fuc	7·8	7·2		5·3	tr.
Ara	1·6	3·0	21·3	2·5	40·7
Xyl	29·6	26·3	3·7	29·2	10·9
Man	1·5	5·3		3·0	5·6
Gal	11·6	12·3	9·7	12·2	21·0
Glc	47·8	43·8	9·2	43·8	4·6
GalA	0·1	2·2	56·1	4·1	17·1

[a]Ion-exchange chromatography was carried out on a Sepharose CL-6B column eluted with an ionic strength gradient of succinate buffer pH 4·8.
[b]Sugar compositions are expressed in mol% of the sugars.
[c]tr: Traces.

bound fraction of the urea extract after pectin-lyase was rich in arabinose and galactose. These fractions were similar for the urea extract of CDTA-IR to the extract obtained with buffer alone in similar conditions and for the urea extract after pectin-lyase to the pectin-lyase extract (minus the low-molecular-weight uronides fraction) Renard *et al.* (1991*c*), though they contained remarkably little rhamnose.

DISCUSSION

Extractions with concentrated sodium hydroxide

Xyloglucans have previously been extracted from apple cell-walls by concentrated NaOH (Aspinall & Fanous, 1984; Voragen *et al.*, 1986) or KOH (Stevens & Selvendran, 1984; Ruperez *et al.*, 1985) after extraction of pectins by cold dilute NaOH (Ruperez *et al.*, 1985; Voragen *et al.*, 1986) or hot ammonium oxalate (Aspinall & Fanous, 1984; Stevens & Selvendran, 1984). The total yield with NaOH 1 M and 4 M obtained here (14·8%) was slightly lower than the 17% yield obtained by Voragen *et al.* (1986) with NaOH 4 M. Stevens and Selvendran (1984) and Ruperez *et al.* (1985) extracted 18·2% and 13·2%, respectively, with an additional extraction step by cold KOH 1 M. Extraction of 14% of the cell wall by NaOH 1 M by Aspinall and Fanous (1984) was probably due to chlorite

pretreatment. NaOH 1 M extracted mostly proteins, some hemicelluloses and a low proportion of pectins, as noted by Stevens and Selvendran (1984) and Ruperez *et al.* (1985) for the KOH 1 M extracts. NaOH 4 M extracted the bulk of apple hemicelluloses, in agreement with the results of Stevens and Selvendran (1984) and Ruperez *et al.* (1985). NaOH 4 M causes the swelling and loss of crystallinity of the cellulose microfibrils that is necessary for extraction of xyloglucan (Hayashi, 1989). This disruption of cellulose also increased its hydrolysis by 1 M sulfuric acid. Galacturonic acid, arabinose and rhamnose contents were lower than those reported by Stevens and Selvendran (1984), Aspinall and Fanous (1984), Ruperez *et al.* (1985) and Voragen *et al.* (1986). However, Stevens and Selvendran (1984) and Aspinall and Fanous (1984) had extracted pectins with hot ammonium oxalate, so that the level of residual pectin was higher than after treatment with dilute sodium hydroxide.

Sugar composition of the extracts indicated that the main hemicellulose was a fucogalactoxyloglucan, which could be purified by ion-exchange on DEAE Sepharose CL-6B. The composition of the fucogalactoxyloglucan was very similar to compositions reported by Aspinall and Fanous (1984) and Stevens and Selvendran (1984). The xyloglucans from the NaOH 1 M and 4 M extracts eluted at K_{av} values similar to those reported by Voragen *et al.* (1986). A low-molecular-weight mannan, such as that detected by Voragen *et al.* (1986), was extracted by NaOH 4 M, and traces of this mannan were present in the NaOH 1 M extract. Other types of hemicelluloses, such as (arabino)-xylans (Stevens & Selvendran, 1984; Voragen *et al.*, 1986), were present in very low amounts and did not appear as distinct fractions. The α-cellulose residue (COHR) still contained significant amounts of galacturonic acid and arabinose but also of xylose, galactose and fucose, as reported by Stevens and Selvendran (1984) and Ruperez *et al.* (1985). The concentrated sodium hydroxide was not able to extract the remaining pectic material. It seemed that some xyloglucan also resisted extraction. In most cases xyloglucan and pectic material could be obtained in distinct fractions by ion-exchange or gel-filtration chromatography. A (minor) fraction containing both some fucogalactoxyloglucan and an homogalacturonan devoid of arabinose and rhamnose was present in the NaOH 4 M extract, but it is not possible to affirm that they are covalently linked.

Urea extracts

Urea extracted more proteins than polysaccharides from apple cell-walls. The bulk of these proteins probably arose from co-precipitated

intracellular proteins. Urea extracted some xyloglucans from CDTA-IR and after pectin-lyase treatment. These xyloglucans have compositions and molecular weight similar to those of the NaOH 1 M extract, though they represented only about half of the xyloglucans extracted by NaOH 1 M. The urea extracts of CDTA-IR and after pectin-lyase also contained some pectic materials different in nature. In the urea extract of CDTA-IR, the pectic material was comparable to that extracted by the buffer alone, and in the urea extract after pectin-lyase it was similar to the high-molecular-weight material extracted by the pectin-lyase (Renard *et al.*, 1991*c*). These polysaccharides had been retained in the cell wall after the pectin-lyase treatment either because of limited diffusion or because of H-bonds with other cell-wall components. The urea extracts thus contained material extracted by the urea itself, i.e. the proteins and xyloglucans, and material arising from the pretreatment.

The urea extract after dilute sodium hydroxide differed from the other two extracts: it contained no xyloglucan, only pectin-like material rich in rhamnose. The dilute sodium hydroxide thus seems to have changed the interactions in the cell walls: a fraction of the xyloglucans, previously extractable by urea, has become unextractable unless harsher treatments (like NaOH 1 M) are used. This might also influence extraction with concentrated sodium hydroxide. The cold dilute NaOH treatment solubilised only 30% of the galacturonic acid, versus 57% for the pectin-lyase treatment. However, 27% of the uronides could not then be extracted even with NaOH 4 M. These pectins, though originally held in the cell wall only by being part of larger molecules, became strongly bound to the cellulose because of the dilute sodium hydroxide treatment. Dilute sodium hydroxide thus also insolubilises the pectic material. These insolubilising effects should be taken into account when using extraction results to understand interactions in cell walls.

Xyloglucans could be obtained from the cell walls in three ways: xyloglucans extractable by urea (in the absence of artefacts arising from alkaline treatments); xyloglucans extractable by NaOH 1 M; and xyloglucans extractable by NaOH 4 M. Those populations, however, had similar compositions and molecular weights, so that apple fucogalactoxyloglucans appeared to be quite homogeneous. No link with pectins could be detected, except perhaps in the fraction of the NaOH 4 M extract eluting at the void volume on Sephacryl S 500. However, not all the xyloglucans were extracted, even by NaOH 4 M, which might explain why, though enzymatic extraction showed the existence of a connection between xyloglucan and rhamnogalacturonan (Renard *et al.*, 1991*a*, *b*), no fraction corresponding to that connection was isolated in significant amounts. This also indicates that rhamnogalacturonan is further anchored in the wall by other linkages than hydrogen bonding (split by

urea and sodium hydroxide), ester bonds (split by sodium hydroxide) or mechanical enmeshment. Further studies will be concerned with elucidation of the nature of that connection.

REFERENCES

Aspinall, G. O. & Fanous, H. K. (1984). Structural investigation on the non-starchy polysaccharides of apples. *Carbohydr. Polymers*, **4**, 193–214.

Barrett, A. J. & Northcote, D. H. (1965). Apple fruit pectic substances. *Biochem. J.*, **94**, 617–27.

Englyst, H. N. & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst*, **109**, 937–42.

Hayashi, T. (1989). Xyloglucans in the primary cell wall. *Ann. Rev. Plant Physiol. Mol. Biol.*, **4**, 139–68.

Houdenhoven, F. E. A. van (1975). *Studies on pectin-lyase.* PhD thesis, Agricultural University, Wageningen, The Netherlands.

Knee, M. (1973*a*). Polysaccharides and glycoproteins of apple fruit cell walls. *Phytochem.*, **12**, 637–53.

Knee, M. (1973*b*). Polysaccharide changes in cell walls of ripening apples. *Phytochem.*, **12**, 1543–9.

Knee, M. (1978*a*). Properties of polygalacturonate and cell cohesion in apple fruit cortical tissues. *Phytochem.*, **17**, 1257–60.

Knee, M. (1978*b*). Metabolism of polymethylgalacturonate in apple fruit cortical tissues during ripening. *Phytochem.*, **17**, 1261–4.

Knee, M., Fielding, A. H., Archer, S. H. & Laborda, F. (1975). Enzymic analysis of cell wall structure in apple fruit cortical tissue. *Phytochem.*, **14**, 2213–22.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 266–75.

Renard, C. M. G. C., Voragen, A. G. J., Thibault, J. F. & Pilnik, W. (1990). Studies on apple protopectin. I: Extraction of insoluble pectin by chemical means. *Carbohydr. Polymers*, **12**, 9–25.

Renard, C. M. G. C., Searle-van Leeuwen, M. J. F., Voragen, A. G. J., Thibault, J. F. & Pilnik, W. (1991*a*). Studies on apple protopectin. II: Apple cell walls degradation by pure polysaccharidases and their combinations. *Carbohydr. Polymers*, **14** (3), 295–314.

Renard, C. M. G. C., Schols, H. A., Voragen, A. G. J., Thibault, J. F. & Pilnik, W. (1991*b*). Studies on apple protopectin. III. Characterization of the material extracted by pure polysaccharidases from apple cell walls. *Carbohydr. Polymers*, **15** (1), 13–32.

Renard, C. M. G. C., Voragen, A. G. J., Thibault, J. F. & Pilnik, W. (1991*c*). Comparison between enzymatically- and chemically-extracted pectins from apple cell walls. *Animal Feed Science and Technology.*, **32**, 69–76.

Roozen, J. P. & Ouwehand, M. (1978). Half-automatische bepaling van stikstoff in droge levensmiddelen. *Voedingsmiddelentechnologie*, **11**, 23–5.

Rouau, X. & Thibault, J. F. (1984). Apple juice pectic substances. *Carbohydr. Polymers*, **4**, 111–25.

Ruperez, P., Selvendran, R. R. & Stevens, B. J. H. (1985). Investigation of the heterogeneity of xyloglucans from the cell walls of apple. *Carbohydr. Res.*, **142**, 107–13.

Stevens, B. J. H. & Selvendran, R. R. (1984). Structural features of cell wall polymers of the apple. *Carbohydr. Res.*, **135**, 155–66.

Thibault, J.-F. (1979). Automatisation du dosage des substances pectiques par la méthode au méta-hydroxydiphényl. *Lebensm.-Wiss. u.-Technol.*, **12**, 247–51.

Tollier, M. T. & Robin, J. P. (1979). Adaptation de la méthode à l'orcinol sulfurique au dosage automatique des oses neutres et totaux. *Ann. Technol. Agric.*, **28**, 1–15.

Voragen, A. G. J., Schols, H. A. & Pilnik, W. (1986). Structural features of the hemicellulose polymers of apples. *Z. Lebensm. Unters. Forsch.*, **183**, 105–10.

Vries, J. A. de (1983). *Structural Features of Apple Pectic Substances.* PhD thesis, Agricultural University, Wageningen, The Netherlands.

Vries, J. A. de, Voragen, A. G. J., Rombouts, F. M. & Pilnik, W. (1981). Extraction and purification of pectins from alcohol insoluble solids from ripe and unripe apples. *Carbohydr. Polymers*, **1**, 117–27.

Vries, J. A. de, Rombouts, F. M., Voragen, A. G. J. & Pilnik, W. (1982). Enzymic degradation of apple pectins. *Carbohydr. Polymers*, **2**, 25–33.

Vries, J. A. de, Rombouts, F. M., Voragen, A. G. J. & Pilnik, W. (1983*a*). Distribution of methoxyl groups in apple pectic substances. *Carbohydr. Polymers*, **3**, 245–58.

Vries, J. A. de, Uijl, C. H. den, Voragen, A. G. J., Rombouts, F. M. & Pilnik, W. (1983*b*). Structural features of the neutral side chains of apple pectic substances. *Carbohydr. Polymers*, **3**, 193–205.