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HEMICELLULOSIC POLYMERS OF CABBAGE LEAVES

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Key Word Index—*Brassica oleracea*; Cruciferae; cabbage; cell-wall material; hemicelluloses; xyloglucan; arabinoxylan; pectic substances; protein; cellulose degradation; oligosaccharides; methylation analysis.

Abstract—The hemicellulosic polymers of depectinated cell-wall material of immature cabbage leaves have been extracted by alkali, fractionated by ion-exchange chromatography and their structural features studied. In the 1 M potassium hydroxide-soluble fraction the main polymers are arabinoxylan–xyloglucan–pectic, arabinoxylan–pectic–protein and arabinoxylan–xyloglucan–pectic–protein complexes. Small amounts of polysaccharide–protein–polyphenol complexes are also present. In the 4 M potassium hydroxide-soluble fraction the predominant polymers are two xyloglucans, the major one of which appears to have *ca* 10% of (1 → 4)-linked and 3% of (1 → 4, 6)-linked mannose residues associated with it, and both have terminal galactose, fucose and possibly arabinose residues in the side chains. Methylation analysis of the oligosaccharides, formed by degradation with cellulase, and partial hydrolysis of the methylated xyloglucan, followed by re-methylation with CD_3I, have enabled the formulation of a tentative structure which is similar to other plant galactoxyloglucans. It was not possible to establish whether the mannose residues are part of an associated glucomannan or whether they are an integral part of the glucan backbone. The general structural features of the hemicellulosic polymers are discussed in the light of these results.

INTRODUCTION

Xyloglucans are generally regarded as the main hemicellulosic polysaccharides of parenchymatous tissues of dicotyledonous plants [1–5]. They contain a (1 → 4)-β-D-glucan backbone with some units substituted at C-6 with D-xylose, singly, or in short chains terminated with D-galactose and, in some instances, with L-fucose and L-arabinose. Xyloglucans are strongly bound to the cellulose (probably by hydrogen bonding), and apparent covalent links with pectic polysaccharides have been found in some tissues (e.g. sycamore cells) but not in others [4–6]. In later work, however, large amounts of xyloglucan covalently linked to pectic polysaccharides could not be isolated from cultured sycamore cells [1]. A glucuronoarabinoxylan in small amount has been isolated from the hemicellulosic fraction of suspension cultured sycamore cells [7] and glucuronoxylans appear to be present in the cell walls of cambial tissues which are capable of differentiating into vascular tissues [8]. Studies with runner beans have shown that in addition to xyloglucans polysaccharide–protein–polyphenol complexes may also be present ([9]; O'Neill, M. A. and Selvendran, R. R., unpublished results).

Since little is known about the chemistry of alkali-soluble hemicellulosic polymers of primary cell walls of vegetables, the occurrence of these polymers in very immature cabbage leaves was investigated. The conducting elements of the leaves were barely stained by phloroglucinol/HCl, showing that they were not lignified. This paper reports the composition and structural features of the hemicellulosic polymers of cabbage with a tentative structure for the main xyloglucan.

RESULTS AND DISCUSSION

The composition of the cell-wall material (CWM) and that of fractions obtained by extraction of hot water and hot oxalate have been given in the previous paper [10]. The residue after these extractions was sequentially extracted with 1 M and 4 M potassium hydroxide containing 10 mM sodium borohydride.

1 M *Potassium hydroxide-soluble material*

The 1 M potassium hydroxide-soluble fraction, which constitutes 6.9% of the CWM [11], contained substantial amounts of arabinose, xylose, galactose and glucose, with some deoxyhexose and mannose (Table 1) and 6.4% protein (Table 2). This is rather different from the 1 M potassium hydroxide-soluble fraction from cabbage alcohol-insoluble residue which was obtained in greater yield and appeared to have a predominately pectic arabinogalactan type of composition [12]. The material from the fresh cabbage was dispersed in water and the soluble polymers were further fractionated on a column of DEAE-Sephacel (Cl^- form), using a Cl^- gradient; *ca* 20% of the starting material was insoluble in water and was removed prior to chromatography. Two fractions were obtained in the proportions of 2.5:1:A (Table 1) which was not retained on the column; and B which eluted with sodium chloride up to 0.6 M. Fraction B had a higher content of arabinose and xylose than the parent fraction and contained 15% protein (Table 2). Fraction A was further fractionated on DEAE-Sephacel (Ac^- form) and two main fractions were obtained in the proportions of 3:1: A1 (Table 1) not retained on the column and which contained the bulk of the mannose; and A2 (Table 1) which was eluted with up to 0.25 M potassium acetate and was rich in arabinose. In addition to these fractions, three further fractions, A3–A5 (Table 1), of low sugar content were obtained in low yield by elution with 5 M potassium acetate and then with 0.2 M sodium hydroxide.

Table 1. Sugar composition of fractions obtained from the 1 M potassium hydroxide-soluble fraction of cabbage cell-wall material

Fraction	Yield (%)	Sugar composition (μg/mg)*						
		Deoxyhexose	Ara	Xyl	Man	Gal	Glc	Uronic acid
1 M KOH-soluble	(100)†	18	90	150	28	79	127	91
Insoluble in water	22	18	133	79	10	75	72	99
A‡	35	41	95	165	63	120	241	74
B	14	23	116	315	28	69	62	84
A1§	14	56	54	178	100	128	362	57
A2	5	54	179	205	33	125	297	103
A3	3	4	30	92	14	13	39	22
A4	3	2	15	42	20	n.d.	27	n.d.
A5	4	6	40	38	3	21	18	18

*After Saeman hydrolysis.

†This accounts for 6.9% of the cell-wall material [11].

‡A and B are fractions obtained from chromatography of the water-soluble portion of the 1 M potassium hydroxide-soluble fraction on DEAE-Sephacel (Cl^- form).

§A1–5 are fractions from re-chromatography of fraction A on DEAE-Sephacel (OAc^- form).

n.d., Not detected.

Table 2. Amino acid composition of 1 M potassium hydroxide-soluble and 4 M potassium hydroxide-soluble fractions of cabbage cell-wall material

Amino acid	Amino acid composition (μg/mg)					
	Oxalate* soluble [10] insoluble residue	1 M KOH soluble	Fractions of 1 M KOH soluble			4 M KOH soluble
			Insoluble in water	A	B	
Ala	0.7	3.2	12.2	0.9	0.9	0.4
Gly	2.2	4.0	13.8	1.5	3.2	0.6
Val	1.8	4.1	14.1	1.6	1.3	0.5
Thr	5.4	2.8	10.2	1.8	2.9	0.3
Ser	5.6	3.9	12.8	2.9	7.2	0.8
Leu	5.9	4.9	19.1	2.8	5.9	0.6
Ile	1.9	2.6	8.9	0.3	2.9	0.3
Pro	7.2	3.1	11.6	1.9	5.5	0.5
Hyp	12.7	4.9	14.3	5.8	10.6	0.9
Asp	12.2	6.1	23.3	3.8	20.5	0.7
Phe	6.4	3.0	12.1	1.7	35.4†	0.5
Glu	16.0	8.0	29.2	5.7	28.8	1.6
Lys	15.7	4.8	17.1	0.4	9.5	0.8
Tyr	2.5	3.2	10.0	2.3	3.9	0.6
Arg	3.6	3.8	5.2	0.6	6.2	1.0
His	4.9	1.8	4.8	1.1	4.0	0.3
Total	104.7	64.2	218.7	35.1	148.7	10.5

*Sugar composition (μg/mg)—deoxyhex 43, Ara 179, Xyl 8, Man 3, Gal 55, Glc 12, uronic acid 672.

†Value probably too high due to interference from unidentified compound.

The 1 M potassium hydroxide-soluble fraction contained a substantial amount of phenolic material (27%, based on a $KMnO_4$ oxidation method) and *ca* 20% of the total remained in the water-insoluble residue (i.e. *ca* 25% w/w). However, based on UV absorption and recoveries of carbohydrate and protein, fractions A3, A4 and A5 contained mostly phenolic material although the small yields of these fractions did not permit quantification. Before discussing the structural features of the carbohydrate moieties of the polymers present in the above

fractions, the nature of the proteoglycan complexes, which are insoluble in water, will be considered.

Proteoglycan complexes

The water-insoluble material contained 22% protein, 45% carbohydrate and some phenolic material. Although the uronic acid content of this residue was lower and the protein content higher than the corresponding water-insoluble material isolated from the oxalate-soluble fraction (Table 2), they appeared to be comparable complexes. The proteins associated with both complexes were relatively rich in the hydroxyamino acids—hydroxyproline, serine and threonine. Similar proteoglycan complexes have been isolated from the leaves of *Phaseolus vulgaris* [14], *Vicia faba* [15], *Cannabis sativa* [16] and runner bean pods (O'Neill, M. A. and Selvendran, R. R., unpublished results). With *Phaseolus vulgaris*, pronase treatment of a hydroxyproline-poor fraction yielded glycopeptides rich in hydroxyproline and with increased levels of serine and threonine [14]. The polysaccharide–protein complexes of *Vicia faba*, some of which are rich in pectic material, could not be separated even by high voltage electrophoresis [15]. The detection of small but significant amounts of these complexes in a range of plant tissues suggests that they may be native to the walls, and are not artefacts of the extraction conditions. The work on the complexes isolated from the leaves of *Phaseolus vulgaris* suggests that the carbohydrate moieties may be linked to the proteins via hydroxyproline and serine, as in the case of hydroxyproline-rich glycoproteins of primary cell walls [17, 18]. Clearly more work is required to clarify the nature of the association between cell-wall proteins and pectic polysaccharides/hemicelluloses.

Structural features of the carbohydrate moieties

Fractions A1, A2 and B were subjected to methylation analysis (Table 3). In fraction A1 the types and proportions of the main residues indicated that the polysaccharide was predominately a xyloglucan with *ca* 60% of the (1 → 4)-linked glucose residues substituted at C-6 with xylose residues, *ca* one-third of which possibly had terminal galactose residues linked to C-2, as in other galactoxyloglucans [1, 4, 5]. The proportions of terminal and (1 → 2)-linked xylose residues in Table 3 are low. This is probably due to poor recovery (67%) of the corresponding partially methylated alditol acetates (PMAA) of terminal- and (1 → 2)-linked xylose residues. Similar observations have been made with xylosylglucose (isoprimeverose) and other xyloglucans (Stevens, B. J. H. and Selvendran, R. R., unpublished results). With xylosylglucose the recovery of xylose as PMAA was only 65–70% of the glucose derivative (O'Neill, M. A. and Selvendran, R. R., unpublished results). The poor recovery of xylose derivatives may be due to manipulative losses occurring during their preparation, but it is more likely that the xylose derivatives are relatively unstable.

Fraction A2 appeared to be a xyloglucan of similar composition to that in A1 but possibly complexed with a pectic polysaccharide because (1 → 2)-linked, (1 → 2,4)-linked rhamnose residues and variously linked arabinose residues were present in addition to uronic acid. Polysaccharides of broadly similar composition and structure have been isolated from polygalacturonase-treated sycamore cell walls under similar chromatographic conditions [6, 19]. The arabinose residues, with the exception of those which are (1 → 3,5)-linked, were in similar proportions to those in the oxalate-soluble fraction [10]. The (1 → 4)-linked xylose residues probably came from an arabinoxylan.

Fraction B (Tables 1 and 3) also contained low levels of (1 → 2)-linked and (1 → 2,4)-linked rhamnose residues together with (1 → 5)- and (1 → 2)-linked arabinose residues and a small amount of uronic acid which suggested the presence of a small amount of a pectic polysaccharide. However, the high content of (1 → 4)-linked xylose residues with terminal arabinose and a substantial amount of (1 → 2,4)-linked xylose residues, coupled with the relatively high level of protein (Table 2), suggested that this polymer was mainly an acidic arabinoxylan–pectic–protein complex. There was insufficient material to determine the nature of the acidic groups but these could be glucuronic acid and/or 4-*O*-methyl glucuronic acid, and galacturonic acid. An arabinoxylan in the form of a glucuronoarabinoxylan has only recently been isolated from the primary cell walls of dicotyledons (suspension cultured sycamore cells) [7]. The total xylose content, of fraction B, determined by methylation analysis (Table 3) was much greater than that from direct analysis (Table 1). This could be due to the relative stability to acid hydrolysis of the aldobiouronic acid, Glc*p*A-(1 → 2)-Xyl*p*.

4 M *Potassium hydroxide material*

This fraction accounted for 13.6% of the CWM [11] and was rich in xylose and glucose with smaller amounts of other neutral sugars, uronic acid (Table 4) and a small amount of protein (Table 2). Oxidation with $KMnO_4$ showed that it contained *ca* 20% of phenolic material. On dissolution in water *ca* 1% of the starting material was insoluble and was removed prior to chromatography. On fractionation of the soluble material through a column of Sephadex A50 (Cl^- form), 60% of the starting material was not retained on the column but was still slightly acidic (C, Table 4). Elution with a linear gradient of sodium chloride produced two poorly defined fractions (D and E, Table 4) containing low levels of carbohydrate. Measurement of UV absorption indicated that these last two fractions were rich in phenolics. An attempt was made to purify C further by chromatography on DEAE-Sephacel (borate form). Two fractions were obtained: C1 (Table 4) eluting between 0.15 M and 0.40 M borate; and C2 (Table 4) eluting between 0.55 M and 1 M borate. Both fractions were rich in xylose and glucose but C2 also contained substantial amounts of mannose and galactose. The sugar composition (Table 4) and methylation analysis (Table 3) of C1 showed that it was a typical xyloglucan. Methylation analysis of C2 (Table 3) revealed that mannose was present as (1 → 4)- and (1 → 4,6)-linked residues. To obtain further information on the structure of C2 it was subjected to: (a) methylation followed by partial hydrolysis and re-methylation with CD_3I; and (b) the action of cellulase and separation and identification of the resultant oligosaccharides by GC/MS after suitable derivatization.

Products of partial hydrolysis

In order to determine the points of attachment of the terminal fucose and arabinose residues which are susceptible to mild acid hydrolysis, methylated C2 was

Table 3. Partially methylated alditol acetates derived from alkali-soluble fractions of cabbage cell wall

Alditol acetates	Relative mol %: 1 M KOH soluble fractions A1	A2	B	4 M KOH soluble fractions C1	C2	C2P*	Ions in C2P m/z (rel. int.)
2,3,4 Me_3 Fuc†	2.2	tr	0.3	tr	2.8	—	
3,4 Me_2 Rha	—	0.6	0.9	—	—	—	
3 Me Rha	—	0.5	0.7	—	—	—	
2,3,5 Me_3 Ara	1.0	7.1	5.8	0.9	0.8	—	
2,3 Me_2 Ara	1.2	6.3	3.9	—	—	—	
3,5 Me_2 Ara	—	0.6	0.5	—	—	—	
2 Me Ara	—	0.5	—	—	—	—	
Arabinitol	—	2.2	1.9	—	—	—	
2,3,4 Me_3 Xyl	9.4	8.9‡	4.2	17.2‡	11.3	12.4D	101 (29), 102 (21), 103 (6), 117 (28), 118 (21), 121 (6), 161 (5), 162 (4), 165 (1)
2,3 Me_2 Xyl	0.8	14.9	54.6	3.3	1.0	—	
3,4 Me_2 Xyl	4.8	5.4	5.1	10.0	7.1	5.5	
3 Me Xyl	0.5	2.1	9.4	—	—	—	
Xylitol	—	—	1.0	—	—	—	
2,3,4,6 Me_4 Man§	0.6	—	—	—	—	8.3D	101 (9), 104 (14), 129 (12), 132 (19), 145 (1), 148 (13), 161 (1), 164 (6)
2,3,6 Me_3 Man	9.1	—	0.7	—	10.6	—	
2,3 Me_2 Man	3.5	tr	—	—	3.3	—	
2,3,4,6 Me_4 Gal	6.9	3.9	—	6.0	6.7	8.4D	102 (8), 105 (8), 118 (9), 121 (9), 162 (2), 165 (2)
2,3,6 Me_3 Gal	6.0	6.5	3.7	1.7	tr	—	
2,4,6 Me_3 Gal	—	2.0‖	—	—	—	—	
2,3 Me_2 Gal	—	0.7	—	—	—		
2,3,6 Me_3 Glc	22.3	12.6	3.1	24.7	26.2	36.8D	173 (4), 176 (4), 233 (7), 236 (7)
3,4,6 Me_3 Glc	—	—	0.7‖	—	—	—	
2,3 Me_2 Glc	29.8	22.2	1.9	36.2	30.2	27.2	
Hexitol	1.9	3.7	0.9	tr	tr	1.4	

*Methylated C2 after partial hydrolysis, re-methylation with CD_3I.
†2,3,4 Me_3 Fuc = 1, 5-di-*O*-acetyl-2,3,4-tri-*O*-methyl fucitol, etc.
‡Includes small amount of 2,3,4 Me_3 Fuc.
§Assumed identity since this compound elutes with 2,3,4,6 Me_4 Glc.
‖Not positively identified.
D, Fragment ions containing deuterium are present. tr, Trace.

partially hydrolysed, re-methylated using CD_3I and then completely hydrolysed and converted to PMAA. GC/MS (Table 3) showed the incorporation of deuterium atoms into the 2,3,4-tri-*O*-methyl xylose, 2,3,4,6-tetra-*O*-methyl hexose and 2,3,6-tri-*O*-methyl glucose derivatives. In the xylose derivative the presence of the ion of *m/z* 121 indicated incorporation of CD_3 at C-2 showing that xylose was linked through this group before partial hydrolysis. Likewise, ions of *m/z* 121 from the 2,3,4,6-tetra-*O*-methyl galactose derivative indicated linkage through C-2. GC/MS with an ECNSS-M column confirmed that the D atoms were in the galactose derivative and not in the 3,4-di-*O*-methyl xylose derivative, which on OV-225 co-chromatographs with the galactose derivative The 3,4,6-tri-*O*-methyl galactose derivative from (1 → 2)-linked galactose was not detected in the methylated xyloglucan which had not been partially hydrolysed but a low level could remain undetected since it elutes with the 2,3,6-tri-*O*-methyl hexose derivatives and has a mass spectrum which is too similar to allow positive identification.

The presence of ions of *m/z* 264 and 117 from the 2,3,4,6-tetra-*O*-methyl glucose/mannose derivative indicated incorporation of D atoms at C-4 showing degradation of the (1 → 4)-linked glucan (or mannan) backbone during the partial hydrolysis. The glucose and mannose derivatives co-chromatographed on the GC phase used but the disappearance of the 2,3,6-tri-*O*-methyl mannose derivative after partial hydrolysis indicated that the mannan was preferentially degraded. A previous study has shown that the glucan backbone is only slightly degraded under these conditions [4]. Incorporation of CD_3 on C-6

Table 4. Sugar composition of fractions obtained from the 4 M potassium hydroxide-soluble fraction of cabbage cell-wall material

Fraction	Yield (%)	Sugar composition (μg/mg)*						
		Deoxyhexose	Ara	Xyl	Man	Gal	Glc	Uronic acid
4 M KOH soluble	(100)†	50	30	192	81	96	297	79
C‡	60	52	17	216	97	108	365	104
D	9	6	7	38	8	12	33	6
E	11	6	17	12	—	13	17	4
C1§	10	28	8	171	15	54	284	62
C2	42	54	5	187	104	102	368	56

*After Saeman hydrolysis.
†This accounts for 13.6% of the cell-wall material [11].
‡C, D and E are fractions from chromatography on DEAE-Sephadex (Cl^- form).
§C1 and C2 are fractions from C on DEAE-Sephacel (borate form).

of the 2,3,6-tri-*O*-methyl glucose derivative, as shown by the presence of ions of *m/z* 176 and 236, suggested the removal of side chains during partial hydrolysis.

From the disappearance of the 2,3,4-tri-*O*-methyl fucose and 2,3,5-tri-*O*-methyl arabinose derivatives after partial hydrolysis, together with the above results, and by analogy with the structures proposed for other plant xyloglucans, it can be assumed that the terminal fucose residues are linked to C-2 of galactose and that the terminal arabinose groups are linked to C-2 of xylose.

Characterization of oligosaccharides produced on digestion with cellulase

The oligosaccharides produced on enzymic hydrolysis were reduced with $NaBD_4$ and the resulting alditols were methylated and examined by GC on OV-1. Six peaks were detected but two of them were shown by mass spectrometry to arise from butyl-phthalate and di-octylphthalate, which were present as contaminants. The peaks from oligosaccharide derivatives (peaks 1–4) were obtained in the ratio 50:8:1:2. These compounds represent only a proportion of the total oligosaccharide derivatives produced, the higher ones not being amenable to study by GC/MS under the conditions used. The following data were used to identify the oligosaccharide derivatives: (a) RR_t with reference to methylated cellobiitol; (b) the oligosaccharides that can be inferred to be present from the results of methylation analysis of the native and partially hydrolysed xyloglucan; and (c) the diagnostic fragment ions in the mass spectrum using established principles as applied to methylated oligosaccharides from plant cell walls [4, 20–24]. The relative abundance of the pertinent ions in the mass spectrum of the peaks are given in Table 5. The nomenclature for the degradation of methylated oligosaccharide alditols and the symbols employed correspond to those of Kochetkov and Chizhov [25].

Peak 1 (RR_t 0.83) was eluted in the permethylated disaccharide alditol region. EIMS gave ions of the aA (*m/z* at 175, 143 and 111) and bA (*m/z* at 236, 204 and 172) series indicating the presence of a pentosylhexitol derivative. The nature of the linkage was deduced from the ions produced by cleavage of the carbon–carbon bonds of the methylated hexitol (Fig. 1). The relative abundance of the ions at *m/z* 178 and 222 showed that the hexitol was substituted at position 6, and hence a (1 → 6)-linkage. This inference was confirmed by the ions at *m/z* 296 (abJ_1), 337 $[M-90]^+$, 261 $[M-166]^+$ and 249 $[M-178]^+$. From these results and those of the preceding section the parent oligosaccharide of the compound in this peak could be inferred to be Xyl*p* (1 → 6)-Glc*p*.

Peak 2 (RR_t 1.00) also eluted in the permethylated disaccharide alditol region. EIMS gave ions of the aA (*m/z* at 219, 187 and 155) and bA (*m/z* at 236, 204 and 172) series (Fig. 2) indicating the presence of a hexosyl hexitol derivative. The nature of the linkage was deduced from the relative abundance of the ions at *m/z* 134, 296 (abJ_1), 337 $[M-134]^+$, 381 $[M-90]^+$, 425 $[M-46]^+$ and 426 $[M-45]^+$, which showed that the hexitol was substituted at C-4. The relatively small amounts of the ions at *m/z* 178 and 222 could have arisen from the component present in peak 1. The combined data suggested that the major component of peak 2 was derived from cellobiose, Glc*p*-(1 → 4)Glc*p*.

The components of peak 3 (RR_t 2.34) eluted as a multiple peak in the permethylated trisaccharide alditol region. Selected ion monitoring across the area of the peak for the ions at *m/z* 175, 219 and 236 clearly showed that the peak was a composite containing at least two compounds (Pk $3_{1\text{-}2}$). As the compounds were not fully resolved, EIMS across the peak gave ions from the overlapping components. However, for clarity, when outlining the evidence for the structure of a particular component only the ions which helped to resolve its structure will be described.

EIMS of subfraction $Pk3_1$ gave ions of the aA (*m/z* 175, 143 and 111) and cA (*m/z* 236, 204 and 172) series indicating the presence of a trisaccharide derivative containing terminal non-reducing pentose and hexitol residues, respectively (Fig. 3). The hexitol was inferred to be linked through C-4 to the internal sugar residue from the relative abundance of the ion at *m/z* 134. The internal residue was deduced from the ions at *m/z* 296 (bcJ_1), 440 (bcA_1), 408 (bcA_2), 500 ($abcJ_1$) 379 (baA_1) and 347 (baA_2) to be a hexose. The combined data were consistent with the structure Xyl*p*-(1 → 6)-Glc*p*-(1 → 4)-Glc*p* for the parent trisaccharide, which is xylosylcellobiose.

The relative abundance in subfraction $Pk3_2$ of the baA series of ions at *m/z* 335 and 303 coupled with the ions at

Table 5. Diagnostic ions obtained from GC/MS of permethylated oligosaccharide alditols derived from the cellulase digest of cabbage xyloglucan

Diagnostic ions (*m/z*)	Relative abundance				Symbol*
	Peak 1	Peak 2	Peak 3	Peak 4	
88	67.0	100.0	100.0	100.0	
89	19.8	28.9	26.2	27.5	
90	35.8	10.2	8.6	8.2	
101	100	74.7	78.8	71.1	
111	11.6	37.1	25.3	36.6	
134	13.8	9.1	8.3	8.0	
143	71.3	8.8	71.5	16.3	
155	0.6	9.9	8.3	12.8	
157	2.0	5.0	9.2	8.4	
172	11.3	15.4	14.7	14.7	
175	54.7	4.1	42.6	8.8	aA_1 (Pk1; Pk3)
178	16.7	1.4	3.3	2.7	
187	1.1	51.7	8.2	36.1	aA_2 (Pk2; Pk_4)
189	1.4	2.6	6.9	5.9	
204	3.3	3.3	4.7	3.6	
219	0.5	35.7	4.1	15.1	aA_1 (Pk2; Pk4)
222	2.1	0.8	2.0	3.2	
236	25.1	42.2	37.1	32.3	Hexitol
249	0.5	1.3	3.5	4.1	
261	1.9	0.9	1.5	—	
296	7.2	4.4	4.7	4.2	abJ_1 (Pk1,2); bcJ_1 (Pk3,4)
303	0.1	0.3	1.5	0.9	baA_2 (Pk3)
335	0.0	0.1	0.3	0.1	baA_1 (Pk3)
337	0.1	0.2	—	—	
347	—	—	2.1	1.5	baA_2 (Pk4)
379	—	—	—	0.2	baA_1 (Pk4)
381	—	1.3	—	—	
396	—	—	2.3	1.2	bcA_1 (Pks 3 and 4)
408	—	—	2.4	1.6	
425	—	0.3	—	—	
426	—	0.2	—	—	
440	—	—	1.2	5.8	
454	—	—	—	—	
500	—	—	1.0	1.2	$abcJ_1$ (Pk3)

*Symbols correspond to those used in ref. [25].

m/z 396 (bcA_1), 236, 222 and 178 suggested the occurrence of a trisaccharide alditol derivative, the parent compound of which has the empirical structure non-reducing pentose-(1 → 2)-pentose-(1 → 6)-hexitol. The combined data were consistent with the occurrence of a small amount of the parent trisaccharide Araf-(1 → 2)-Xylp-(1 → 6)-Glcp.

Peak 4 (RR_t 2.64) was also eluted in the permethylated trisaccharide alditol region as a slightly diffuse peak. Selected ion monitoring over the area of the peak for the ions at *m/z* 175, 219 and 236 showed that it contained more than one component. The relative abundance of the ions at *m/z* 219 and 236 showed that the major components were derived from trisaccharide alditols containing non-reducing hexose and hexitol residues. The ions at *m/z* 296 (bcJ_1), 396 (bcA_1) and 454 ($abcJ_1$) showed that the sugar residue internal to one of the trisaccharide alditols was a pentose (Fig. 4). This inference was supported by the ions at *m/z* 379 (baA_1) and 347 (baA_2), and the relative abundance of the ions suggested that the internal pentose was linked through C-2 to the terminal non-reducing hexose. The relative abundance of the ions at *m/z* 178 and 222 showed the presence of 6-linked hexitol in the parent oligosaccharide alditol. The combined data were consistent with the structure Galp-(1 → 2)-Xylp-(1 → 6)-Glcp for the parent trisaccharide.

The ions at *m/z* 440 (bcA_1), 408 (bcA_2) and 500 ($abcJ_1$) showed that one of the trisaccharide alditol derivatives in peak 4 contained a hexose as the internal residue. The combined data were consistent with the occurrence of a parent trisaccharide alditol having the empirical structure hexose → hexose → hexitol. This compound is more likely to have arisen from a mannose containing trisaccharide rather than cellotriose. The presence of a small but significant amount of (1 → 4)-linked mannose residues in the 'purified' xyloglucan would account for the origin of the above trisaccharide.

Based on the results of the above experiments a

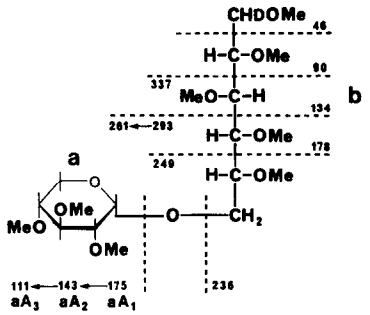

Fig. 1. Mass spectral fragmentation of methylated xylosyl-glucose (peak 1) after reduction with $NaBD_4$.

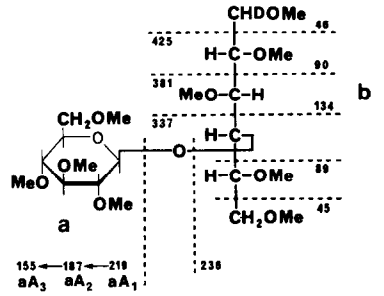

Fig. 2. Mass spectral fragmentation of methylated disaccharide (peak 2) after reduction with $NaBD_4$.

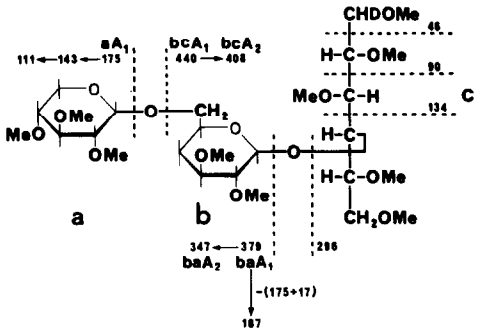

Fig. 3. Mass spectral fragmentation of methylated trisaccharide (peak 3_1) after reduction with $NaBD_4$.

tentative structure is given in Fig. 5 for a portion of the xyloglucan from the 4 M potassium hydroxide-soluble fraction of cabbage cell walls.

General discussion

The results of this investigation show that the hemicellulosic polymers of immature cabbage leaves consist of a range of carbohydrate conjugates, the major components of which are polysaccharides (xyloglucans). However, the mixture also contains small but significant amounts of polysaccharide–protein–polyphenol complexes in which the carbohydrate moieties could be pectic

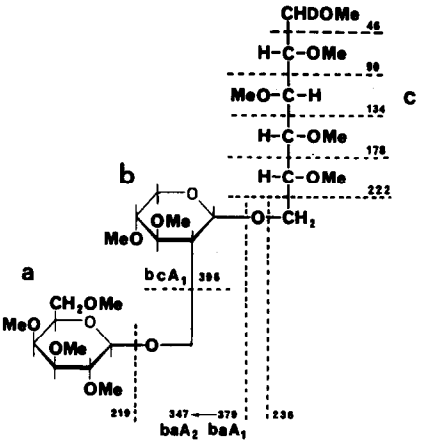

Fig. 4. Mass spectral fragmentation of methylated trisaccharide (peak 4) after reduction with $NaBD_4$.

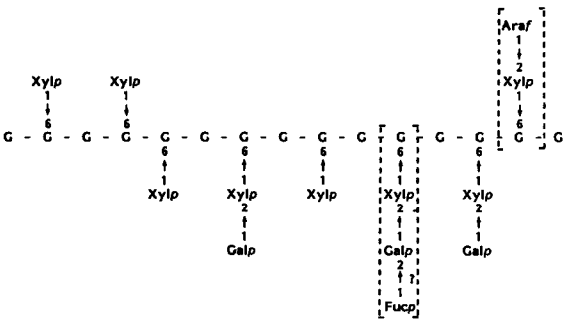

Fig. 5. Tentative structure for a portion of the xyloglucan from cabbage cell walls; the order of the side-chain substituents is arbitrary. The carbohydrate moieties within the brackets have not been positively characterized.

substances, hemicelluloses or both. Similar proteoglycan complexes have been isolated by other workers from various plant sources [14–16, 18], but so far these compounds have received little attention. Monro *et al.* [26–28] have isolated hemicellulosic fractions containing hydroxyproline from lupin hypocotyls but have not fractionated these complexes further. Since the cell-wall preparation used in this study was virtually free of contamination with intracellular proteins, the isolation of these complexes suggests that they are native to the walls and are not artefacts. Some of these complexes may be cell-wall enzymes which are located at specific sites within the wall and the others may have a role as linking compounds in the cell-wall matrix. In cabbage, as in some other tissues [15, 18, 29], the hydroxyproline-rich glycoprotein is closely associated with the pectic substances, in contrast to tissues such as sycamore callus [30] and runner bean parenchyma [5, 31] in which it is associated with the cellulose. Further, recent work on the cell-wall polymers of barley has provided good evidence that some of the β-D-glucans are, in fact, proteoglycans [32–34]; the

available evidence to date has been summarized in ref. [35].

These studies show that proteoglycan complexes are fairly widespread in higher plant cell walls. In immature cabbage, as with other parenchymatous tissues, xyloglucans are the main structural hemicellulosic polysaccharides. Although most of the xyloglucan is strongly bound in the cell wall to cellulose and requires 4 M potassium hydroxide to release it, two xyloglucans less strongly bound are extractable with 1 M potassium hydroxide. The 4 M potassium hydroxide-extractable xyloglucan has the structural features that seem to be common to most cell-wall xyloglucans but in addition to fucose and galactose probably contains terminal arabinose groups as in the xyloglucan from potato tubers [4]. Although the mannose residues could arise from a (galacto) glucomannan closely associated with the xyloglucan, it is equally conceivable that they are an integral part of the glucan backbone. The presence of glucomannans in dicotyledonous leafy tissues has not been reported but (1 → 4)-linked and (1 → 4, 6)-linked mannose residues, together with galactose residues, have been found in a xyloglucan-containing fraction of the extracellular polysaccharides from suspension cultured tobacco cells [36].

The xyloglucans extracted with 1 M potassium hydroxide differ in their associated polysaccharides. Both contain an appreciable level of (1 → 4)-linked galactose residues but that from sub-fraction A2 contains no mannose and appears to be closely associated with a pectic polysaccharide and with an arabinoxylan. Apparent covalent linkages between xyloglucans and pectic polysaccharides have been reported in suspension cultured cells [20], and, in the past, have led to the conclusion that pectic polysaccharides in the cell wall are linked to xyloglucan. Owing to the failure to isolate large amounts of pectic–xyloglucan complexes, this view has been modified and it appears that the bulk of the xyloglucan is not linked in this way. However, the present study confirms that small amounts of closely associated xyloglucans and pectic polysaccharides can be isolated though the nature of the bonding is uncertain.

It is possible that pectic–xyloglucan–arabinoxylan–protein complexes can exist in the cell wall. Complexes of this type have been isolated from rice endosperm cell walls [37, 38]. Glucuronoarabinoxylans have only recently been isolated from the primary cell walls of dicotyledons [7]. It is not clear yet whether the presence of these is a general feature of primary cell walls or whether they are confined to tissues which will eventually differentiate into specialized structures such as xylem. The cabbage used in the present study would contain such tissues.

EXPERIMENTAL

Sources of chemicals and plant material and general methods of analysis were as described previously [10], as were preparation of CWM and sequential extraction with aqueous inorganic solvents [12, 39].

Fractionation of 1 M KOH-*soluble material.* 1 M KOH soluble material (140 mg) was added to H_2O (15 ml) and kept overnight at 1°. An insoluble residue was removed by centrifugation and freeze-dried (31 mg). The supernatant was adjusted to 10 mM Pi (pH 6.4) and applied to a column (11.5 cm × 1.5 cm) of DEAE-Sephacel (Cl^- form). Elution was with 10 mM K-Pi buffer (pH 6.4), alone initially (30 ml), then in a linear gradient of NaCl up to 1 M (50 ml) followed by 1 M NaCl (25 ml). Fractions (2 ml) were collected and after monitoring by reaction with PhOH–H_2SO_4 [40], and for UV absorption at 280 nm, appropriate fractions were combined, dialysed and freeze-dried to yield two polysaccharide fractions, A (50 mg) and B (20 mg).

Fraction A (33 mg) in H_2O (7 ml) was applied to a column (31.5 cm × 1 cm) of DEAE-Sephacel (Ac^- form) and eluted with H_2O (65 ml) followed by a linear gradient of KOAc (0–1 M, 150 ml) then with 5 M KOAc (55 ml) and finally with 0.2 M NaOH (50 ml). Fractions (2 ml) were collected, monitored by reaction with PhOH–H_2SO_4 and appropriate ones combined, dialysed and freeze-dried to yield five fractions: A1 (13 mg), A2 (5 mg), A3 (3 mg), A4 (3 mg) and A5 (4 mg).

Fractionation of 4 M KOH-*soluble material.* 4 M KOH soluble material (70 mg) was dissolved in 10 mM K-Pi buffer (pH 6.4), a slight ppt. (1 mg dry wt) was removed by centrifugation, and the supernatant applied to a column (7 cm × 1 cm) of DEAE-Sephadex A50 (Cl^- form). Elution was with 10 mM K-Pi (30 ml) initially, then with a linear gradient of NaCl up to 0.7 M (150 ml). Fractions (2 ml) were collected and monitored by reaction with PhOH–H_2SO_4. Appropriate fractions were combined, dialysed and freeze-dried to yield three polysaccharide fractions: C (42 mg), D (6 mg) and E (8 mg). C (30 mg) in 5 mM Na borate buffer (pH 8.0) was rechromatographed on a column (23 cm × 1 cm) of DEAE-Sephacel (borate form). Elution was initially with 5 mM Na borate (pH 8.0, 60 ml), then with a linear gradient of Na borate (pH 8.0, 5 mM–1 M, 140 ml) and finally with 1 M Na borate (5 ml). Fractions (3 ml) were collected and monitored by reaction with PhOH–H_2SO_4. Appropriate fractions were combined, dialysed and freeze-dried to yield two xyloglucans, C1 (5 mg) and C2 (21 mg).

Estimation of phenolic content. This was carried out by titrimetric determination of $KMnO_4$ consumed in oxidizing the phenolics [13].

Methylation analysis and GC/MS. Methylation by a modification of the Hakomori method, conversion to PMAA and GC on columns of OV-225 and ECNSS-M, and GC/EIMS was carried out as described in ref. [41]. Methylated oligosaccharides were separated by GC on a 4% OV-1 column and subjected to GC/MS as described previously [10].

Cellulase degradation of xyloglucan. Xyloglucan C2 (10 mg) in acetate buffer (0.1 M, pH 4.7) was treated with *Trichoderma viride* CS12 cellulase as described in ref. [4]. The products were reduced with $NaBD_4$, methylated and extracted into CH_2Cl_2 [42] for GC/MS (OV-1 column).

Partial acid hydrolsis. Xyloglucan C2 (7 mg) was methylated, one-third taken for conversion to PMAA, and the remainder partially hydrolysed with HCO_2H (5 ml) at 70° for 40 min, reduced with $LiBH_4$ and re-methylated using CD_3I, as described in ref. [4], and then converted to PMAA.

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