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ENZYMIC ANALYSIS OF CARROT CELL-WALL POLYSACCHARIDES*

PATRICE MASSIOT† AND JEAN-FRANÇOIS THIBAULT

Institut National de la Recherche Agronomique, Laboratoire de Biochimie et Technologie des Glucides, Rue de la Géraudière, B.P. 527, 44026 Nantes (France)

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ABSTRACT

The cell-wall polysaccharides of carrot roots were degraded by an endopectin lyase and an endopolygalacturonase from *Aspergillus niger*, a cellobiohydrolase from *Trichoderma reesei*, and an endoglucanase and a xylanase from *Dichomitus squalens* used individually, in combination, or in sequence. The endopectin lyase released >80% of the galacturonic acid, but no glucose, whereas a mixture of the cellulases removed ~40% of the glucans and ~15% of the pectic polysaccharides. The extraction of the pectic substances by the endopectin lyase greatly increased hydrolysis of the cellulose by the cellulases. The pectic polysaccharides were highly branched mainly through the rhamnose residues, but some parts of the homogalacturonans carried short chains of arabinose. The association of pectic polysaccharides and cellulose through ester linkages or *via* side chains of xylose is discussed.

INTRODUCTION

We have described[1,2] the preparation and fractionation of cell walls of the carrot (*Daucus carota* L.) and analysed the structure of the polysaccharide fractions. Chemical degradation partially revealed their structures.

Purified enzymes have been used to degrade the cell walls of suspension-cultured sycamore cells[3–5], apple fruit[6,7], and potato[8] to give fragments for structural study. Pectic polymers can be removed from carrot cell-walls by endopectate lyase[9] or exopolygalacturonase and endopectate lyase[10]. It was demonstrated that these polysaccharides comprise homogalacturonan ("smooth" regions) and branched fragments ("hairy" regions) composed of rhamnogalacturonan, arabinan, galactan, and arabinogalactan. The fragmentation of carrot cell-wall polysaccharides using pectinases and cellulases in sequence has not been reported hitherto and we now report such a study with endopectin lyase, endopolygalacturonase, cellobiohydrolase, endoglucanase, and xylanase used individually, in sequence, and in combination.

*Structural Study of the Cell Wall of Carrot (*Daucus carota* L.), Part III. For Part II, see ref. 2.
†Author for correspondence.

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EXPERIMENTAL

Cell-wall material (CWM). — CWM was prepared by Pronase-treatment of the alcohol-insoluble residue of carrot[1], washed with aqueous 96% ethanol, acetone, and ether, and air-dried. The particle size was <1 mm.

Enzymes. — Endopectin lyase [poly(methyl α-D-galactosiduronate) lyase, EC 4.2.2.10][11] and endopolygalacturonase [poly-(1→4)-α-D-galactosiduronate) glycanohydrolase, EC 3.2.1.15][12,13] were prepared from *Aspergillus niger*. Pectin methylesterase (pectin pectylhydrolase, EC 3.1.1.11) from *A. niger* was obtained from Rapidase CPE (Gist Brocades). CBH I [(1→4)-β-D-glucan cellobiohydrolase, EC 3.2.1.91] from *Trichoderma reesei* was purified from Celluclast[14,15]. An endoglucanase [(1→4)-β-D-glucan glucanohydrolase, EC 3.2.1.4] and a xylanase [(1→4)-β-D-xylan xylanohydrolase, EC 3.2.1.8] were purified from *Dichomitus squalens*[16,17]. β-D-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from almonds was a Sigma product.

The activities of enzymes are expressed in nkat; 1 nkat is the amount of enzyme which produces 1 nmol of reducing groups (or methanol for pectin methylesterase) per s, under standard conditions.

No polygalacturonase, endopectin lyase, and endopectate lyase activities were detected in the cellobiohydrolase, endoglucanase, and xylanase. These activities were determined after incubation for 30 min with 200 μg of protein, which was the amount added for the cell-wall degradations. Polygalacturonase activity was assayed[7] by measuring the increase in reducing sugars released from a 0.5% solution of poly(galacturonic acid) (ICN) in 0.05M sodium acetate buffer (pH 5.0) at 40°. Pectin lyase activity was measured[11] at 25° by the increase in absorbance at 235 nm of a solution containing 0.5% of 75% esterified apple pectin in McIlvaine buffer (μ 0.5; pH 6.0). Pectate-lyase activity was determined[18] using a 0.5% solution of poly(galacturonic acid) as substrate in 0.05M glycine–NaOH buffer (pH 9.4) containing 0.25mM $CaCl_2$.

Enzymic degradation. — The scheme for degradation of CWM and fractionation of the enzyme-soluble extracts is shown in Fig. 1. Endopolygalacturonase (10 nkat), endopectin lyase (200 nkat), pectin methylesterase (150 nkat), cellobiohydrolase (1 nkat), endoglucanase (280 nkat), and xylanase (200 nkat) were added individually or in combination to 100 mg (dry wt.) of CWM suspended in 20 mL of 0.05M sodium acetate buffer (pH 4.5) containing 0.02% of sodium azide. For endopectin lyase, the medium also contained 2mM $CaCl_2$. Each suspension was stirred at 30° for 48 h and then filtered through a G_4 sintered-glass filter (pore size, 10–15 μm). The residue was dried by solvent exchange and then in the air. Each experiment was duplicated and enzyme was omitted from the controls.

For each sequential degradation, CWM (250 mg) was treated with endopectin lyase (500 nkat) as described above, the insoluble material (CWM1) was collected, washed extensively with water, suspended in 20 mL of the same buffer, and treated for 48 h with cellobiohydrolase (2.5 nkat) and endoglucanase (700 nkat) to give

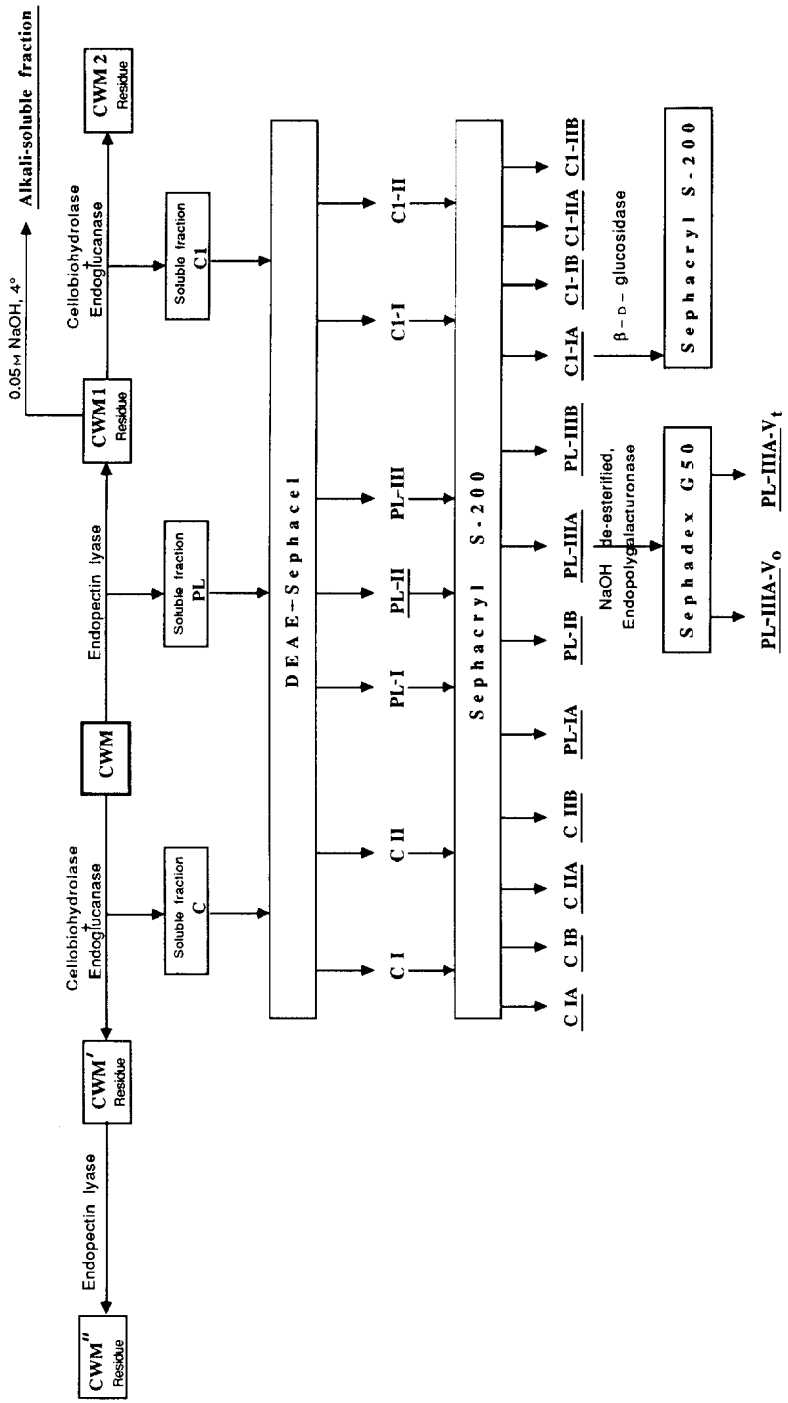

Fig. 1. Degradation scheme of carrot cell-wall material (CWM) with endopectin lyase, cellobiohydrolase, and endoglucanase, and fractionation of the fragments released.

CWM2. CWM was also treated first with the cellulases in combination to give CWM′, then with endopectin lyase to give CWM″. All the soluble extracts were collected and concentrated under reduced pressure at 40°.

The soluble polysaccharides (10 mg) isolated by chromatography were treated with β-D-glucosidase (30 nkat) for 8 h in 2 mL of 0.05M sodium acetate (pH 5.0) at 40° or de-esterified and treated with endopolygalacturonase (2 nkat) for 24 h at 30°.

De-esterification. — CWM1 (50 mg) was treated with 50 mL of 0.05M NaOH at 4° for 12 h. The soluble polysaccharides, isolated by chromatography, were treated with 0.05M sodium hydroxide at 4° for 5 h, then acidified to pH 5.0 with 17.5M acetic acid.

Chromatography. — The extract from CWM or from CWM1 (~5 mg of polysaccharides) were each loaded onto a column (15 × 1.6 cm) of DEAE-Sephacel (Pharmacia) and eluted first with 0.05M sodium acetate buffer (100 mL, pH 4.8) at 50 mL/h. Bound material was then eluted by a linear gradient 0.05→0.5M (100 mL) of sodium acetate (pH 4.8). Fractions (5 mL) were assayed for galacturonic acid and neutral sugars. Appropriate fractions were combined, concentrated under reduced pressure (at 40°), desalted on Sephadex G-25 (Pharmacia) with water as eluant, concentrated, and freeze-dried.

Gel-permeation chromatography was performed on a column (79 × 2.1 cm) of Sephacryl S-200 (Pharmacia) by ascending elution with 0.1M sodium acetate buffer (pH 4.0) at 20 mL/h on samples (5–15 mg of polysaccharides) fractionated by ion-exchange chromatography. Fractions (4 mL) were analysed and treated as above. A column (2 × 84 cm) of Sephadex G-50 (Pharmacia) was loaded with 7.5 mg of polysaccharides and then eluted with 0.1M sodium acetate buffer (pH 4.0) at 20 mL/h. Fractions (4 mL) were treated as above. Results are expressed as a function of $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e, V_o, and V_t are the elution volume of the fraction, and the void and total volumes of the column, respectively.

Analytical methods. — All the data were on a moisture-free basis. Galacturonic acid and neutral sugars were determined by the automated 3-hydroxybiphenyl[19] and orcinol[20] methods, respectively, the latter being corrected for interfering galacturonic acid. The galacturonide content of the insoluble fractions was estimated colorimetrically with 3-hydroxybiphenyl[21] after grinding (3 min) in liquid nitrogen, using a Spex 6 700 freeze-mill, and hydrolysis with sulphuric acid[22]. The neutral sugar composition was determined by g.l.c. (capillary column of 30 m × 0.32 mm i.d. with DB 225, 0.15-μm film thickness; J & W Scientific; at 220°), using hydrogen as carrier gas, after Saeman hydrolysis[22] of the insoluble fractions or hydrolysis (100°, 2 h) with M H_2SO_4 of the soluble fractions and conversion of the products into alditol acetates[23]. *myo*-Inositol was used as the internal standard.

Uronic acids were identified after hydrolysis (M H_2SO_4, 100°, 2 h) by h.p.l.c. using a Waters Model 590 instrument equipped with a column (30 × 0.78 cm i.d.) of Aminex HPX 87H (9 μm, Bio-Rad) at 50° and elution with 5mM H_2SO_4 at 0.6 mL/min. Refractive index detection was used.

TABLE I

WEIGHT LOSS AND SUGAR SOLUBILISATION OF CELL-WALL MATERIAL TREATED WITH DIFFERENT ENZYMES

Enzyme treatment	*Weight loss*[a]	*% Solubilisation of*[b]	
		Galacturonic acid	*Neutral sugars*
Control	3.0	1.2	0.5
Endopolygalacturonase (PG)	33.9	76.4	12.3
Pectin methylesterase (PE)	7.5	1.5	10.8
PG + PE	45.6	90.3	28.9
Endopectin lyase	44.1	81.7	23.2
Cellobiohydrolase (CBH I)	9.0	7.5	11.0
Endoglucanase (En I)	10.6	11.1	2.4
Xylanase (Ex II)	6.4	9.0	2.3
CBH I + En I	23.2	15.2	27.7
CBH I + En I + Ex II	26.3	19.4	28.8

[a]% of CWM. [b]% of initial sugars, estimated by 3-hydroxybiphenyl[19] and orcinol[20] methods.

Glucose and cellobiose were measured by h.p.l.c. on a column (25 × 0.46 cm i.d.) of RSil C18 HL (5 μm, RSL) eluted with water at 10 mL/min. Refractive index detection was used.

Cell-wall polysaccharides were methylated[1] by the Hakomori technique[24] as adapted by Lomax *et al.*[25] and Harris *et al.*[26]. Soluble fractions were concentrated and dried overnight at 40° *in vacuo* before methylation. Partially methylated alditol acetates were analysed[1] by g.l.c. on OV-1 and CP Sil88 capillary columns, using hydrogen as carrier gas.

RESULTS

Degradation of CWM by various purified enzymes. — Table I shows the sugar solubilisation and the weight loss of CWM treated with an endopolygalacturonase, a pectin methylesterase, an endopectin lyase, a cellobiohydrolase, an endoglucanase, or a xylanase, individually, or in combination. Under the conditions of reaction (pH 4.5, 30°), the limit of degradation was attained for each enzyme in 48 h. The endopolygalacturonase removed 75.2% of the galacturonic acid and 11.8% of the neutral sugars. Pectin methylesterase released 0.3% of the galacturonic acid and 10.3% of the neutral sugars (including 56% of galactose and 28% of arabinose, indicating a contamination with galactanase and arabinanase). Endopolygalacturonase and pectin methylesterase in combination solubilised 55% of the CWM polysaccharides, especially the galacturonans. Endopectin lyase solubilised 42.1% of CWM, of which 91.5% was polysaccharide.

Degradations of CWM with cellobiohydrolase, endoglucanase, or xylanase released 2–11% of neutral sugars and 6.3, 9.9, and 7.8%, respectively, of the

TABLE II

SUGAR COMPOSITION OF CELL-WALL MATERIAL (CWM) AND OF INSOLUBLE FRACTIONS OBTAINED BY SEQUENTIAL ENZYMIC TREATMENT WITH ENDOPECTIN LYASE (EnPL), CELLOBIOHYDROLASE (CBH I), AND ENDOGLUCANASE (En I)

Fraction	*Pretreatment*	*Treatment*	*%*	*Weight of sugar (g/100 g of dry fraction)*							
				GalA	*Rha*	*Ara*	*Gal*	*Xyl*	*Man*	*Glc*	*Total sugars*
CWM[a]			100	34.3	1.8	4.6	7.2	2.0	2.0	29.5	81.7
CWM1		EnPL	55.9	11.2 (18.3)[b]	1.7 (52.8)	2.7 (32.8)	4.0 (31.1)	2.7 (75.5)	2.3 (64.3)	51.7 (98.0)	76.3 (52.2)
CWM2	EnPL	CBH I + En I	21.1	8.9 (5.5)	0.5 (5.9)	1.5 (6.9)	1.7 (5.0)	1.8 (19.0)	1.0 (10.6)	18.7 (13.4)	34.1 (8.8)
CWM′		CBH + En I	76.8	37.5 (85.7)	2.2 (93.9)	5.5 (91.8)	8.3 (88.5)	2.2 (84.5)	1.7 (65.3)	22.9 (59.6)	80.3 (75.5)
CWM″	CBH I + En I	EnPL	40.9	16.1 (19.2)	1.2 (27.3)	3.4 (30.2)	4.7 (26.7)	3.5 (71.6)	2.6 (53.2)	39.9 (55.3)	71.4 (35.7)

[a]Values from ref. 1; fucose (0.2%) and ribose (0.1%) were not detected in other fractions. [b]Values in parentheses are % of initial sugar content in CWM.

galacturonic acid. Cellobiohydrolase with endoglucanase, or cellobiohydrolase with endoglucanase and xylanase, removed up to 29% of the neutral sugars.

Sequential degradation of CWM. — The CWM was degraded sequentially with endopectin lyase and a combination of cellobiohydrolase and endoglucanase (Fig. 1). The sugar compositions of the residual insoluble materials are shown in Table II. Endopectin lyase removed >80% of the initial galacturonic acid together with arabinose (67.2%), galactose (68.9%), rhamnose (47.2%), xylose (24.5%), and mannose (35.7%), but no glucose. Treatment of the product CWM1 with the two cellulases removed 83.1% of the polysaccharides, and 86.3, 74.8, and 83.5% of the glucose, xylose, and mannose, respectively. The cellulases solubilised 69.9% of the galacturonic acid of CWM1.

The combination of cellobiohydrolase and endoglucanase acted on CWM to remove 40.4% of the glucose, a notable proportion of the galacturonic acid,

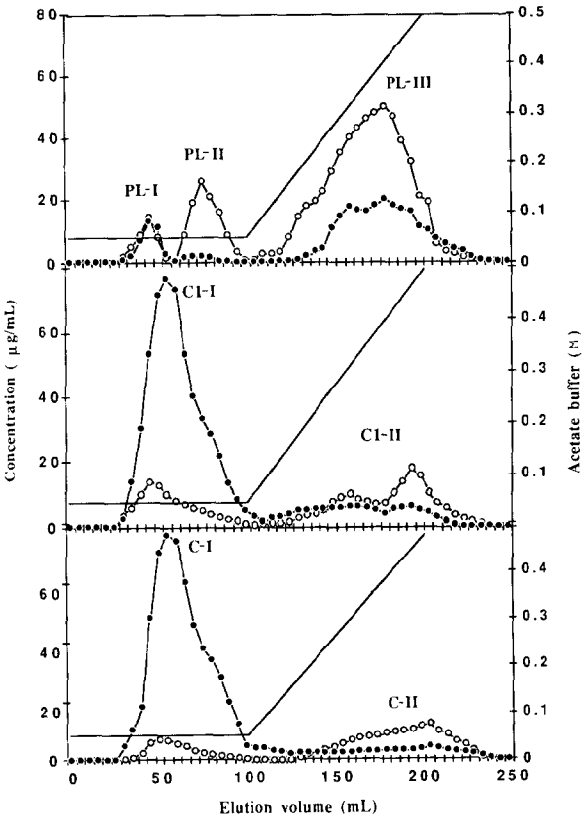

Fig. 2. Elution pattern of the endopectin lyase-soluble extract (PL) and the cellulases-soluble extract (C) from CWM, and the cellulases-soluble extract (C1) from CWM1 (see Fig. 1), from DEAE-Sephacel with sodium acetate buffer (pH 4.8) (see Experimental): ○, galacturonic acid; ●, neutral sugars determined by 3-hydroxybiphenyl and orcinol methods, respectively.

mannose, and xylose, and small proportions of rhamnose, arabinose, and galactose (Table II). Treatment of the residue (CWM') with endopectin lyase released 77.6% of the galacturonic acid, together with small proportions of glucose, mannose, and xylose. The final residues (CWM2 and CWM") had similar sugar compositions. However, the sequence endopectin lyase then cellobiohydrolase and endoglucanase degraded more CWM than the reverse sequence.

Fractionation of the enzyme-soluble extracts from CWM and CWM1. — The extracts from CWM and CWM1 (Fig. 1; ~5 mg) were fractionated by ion-exchange chromatography (Fig. 2) with recoveries of ~90%. The soluble polysaccharides (PL) in the endopectin lyase extract were separated into 3 fractions (PL-I/III). PL-I and PL-II, representing 3.3 and 5.6% of the CWM polysaccharides, respectively, were not bound to the gel in spite of the presence of galacturonic acid. The bound fraction (PL-III, 35.1% of the CWM polysaccharides) was eluted with ~0.4M acetate. The soluble polysaccharides (C and C1) in the cellulase extrats of CWM

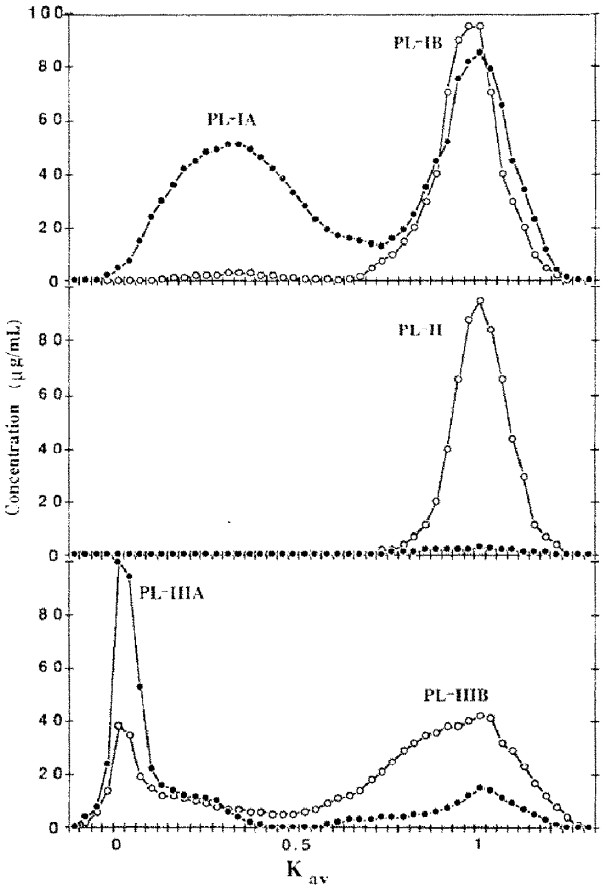

Fig. 3. Elution pattern of PL-I, PL-II, and PL-III (see Fig. 2) from Sephacryl S-200 (see Experimental): ○, galacturonic acid; ●, neutral sugars determined as in Fig. 2.

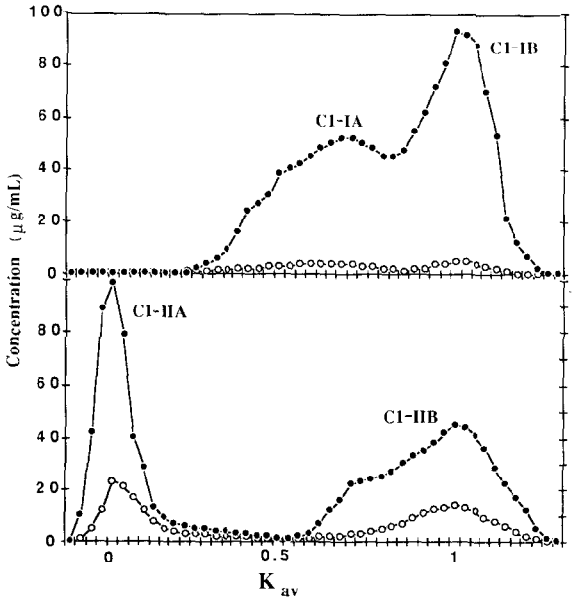

Fig. 4. Elution pattern of C1-I and C1-II (see Fig. 2) from Sephacryl S-200 (see Experimental): ○, galacturonic acid; ●, neutral sugars determined as in Fig. 2.

and CWM1 had similar elution patterns with two main fractions, CI and C1-I, which were not bound to the gel and amounted to 20.2 and 40.0%, respectively, of the initial weight of CWM polysaccharides. Two minor fractions, CII and C1-II, were eluted with the acetate gradient.

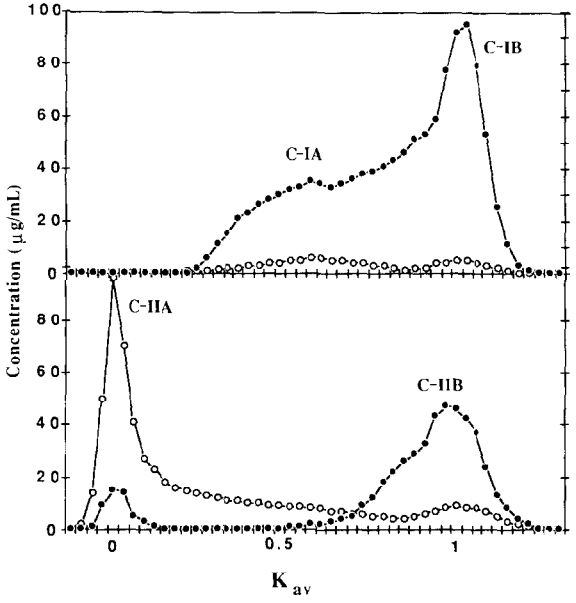

Fig. 5. Elution pattern of CI and CII (see Fig. 2) from Sephacryl S-200 (see Experimental): ○, galacturonic acid; ●, neutral sugars determined as in Fig. 2.

TABLE III

SUGAR COMPOSITION OF SOLUBLE FRACTIONS ISOLATED FROM ENDOPECTIN LYASE-SOLUBLE EXTRACT (PL) FROM CWM, CELLOBIOHYDROLASE AND ENDOGLUCANASE-SOLUBLE EXTRACT (C1) FROM CWM1, ALKALI-SOLUBLE EXTRACT FROM CWM1, AND CELLOBIOHYDROLASE AND ENDOGLUCANASE-SOLUBLE EXTRACT (C) FROM CWM (SEE FIG. 1)

Fraction[a]	*Yield*[b]	*Sugar composition (mol/100 mol of monosaccharide)*								
		GalA	*Rha*	*Fuc*	*Rib*	*Ara*	*Gal*	*Xyl*	*Man*	*Glc*
PL-IA	1.3	7.3	3.9	1.2	2.0	12.7	64.8	3.0		5.1
PL-IB	2.0	42.8	2.2	1.2		25.7	2.3	4.1	14.0	7.7
PL-II	5.6	97.0	1.0		0.2	0.3	0.4	0.3	0.6	0.2
PL-IIIA[c]	13.7 (100)	33.3	2.6			19.3	33.7	10.1		1.0
excluded	(55.5)	5.9	5.1			29.2	53.1	5.1		1.6
included	(35.1)	80.3				15.0	2.8	1.9		
PL-IIIB	21.4	89.7	1.2			4.5	1.3	1.4	0.9	1.0
C1-IA	19.7	11.5	1.1		1.5	4.0	2.4	0.9	5.1	73.5
C1-IB	20.3	4.3	1.0	0.5		0.6	0.5	3.5	1.3	88.3
C1-IIA	1.7	37.2	1.6			31.6	25.1			4.5
C1-IIB	3.3	26.8				21.1	4.0	5.3	11.7	31.1
Alkali-soluble fraction from CWM1	5.8	35.2	8.7			31.6	24.5			
CIA	6.4	20.4	3.6			34.9	7.3			33.8
CIB	13.8	16.1	0.4	0.3	0.4	1.7	0.7	5.2	1.3	73.9
CIIA	1.9	76.6	1.6	0.1		4.0	3.9		3.1	10.7
CIIB	2.2	18.9				10.2	7.8	7.8	5.0	50.3

[a]Material of Figs. 3–5. [b]% of total sugars in CWM. [c]PL-IIIA was treated with endopolygalacturonase and chromatographed on Sephadex G 50 (see Fig. 1 and text).

TABLE IV

PARTIALLY METHYLATED ALDITOL ACETATES FROM POLYSACCHARIDES PRESENT IN FRACTIONS OBTAINED FROM THE ENDOPECTIN LYASE-SOLUBLE EXTRACT (PL) FROM CWM, AND FROM THE CELLOBIOHYDROLASE AND ENDOGLUCANASE-SOLUBLE EXTRACT (C1) FROM CWM1, AND IN CWM2 (SEE FIG. 1)

Derivatives	*PL-IA*	*PL-IB*	*PL-IIIA*	*PL-IIIB*	*C1-IA*	*C1-IB*	*C1-IIA*	*C1-IIB*	*CWM2*
2,3,4,6-Me_4-Glc[a]					8.8	36.6	3.3	19.6	1.1
2,3,6-Me_3-Glc	3.0[b]	8.4	2.6	7.0	63.1	46.3	3.5	25.7	72.8
$^{2,3\text{-}Me_2}$-Glc		4.8		4.5	7.9	5.8		5.9	1.4
2,6-Me_2-Glc					1.2	1.6			
3,6-Me_2-Glc					1.1	2.1		3.1	
Total	3.0 (5.7)[c]	13.2 (15.2)	2.6 (6.6)	11.5 (10.5)	82.1 (85.0)	92.4 (93.5)	6.8 (7.1)	54.3 (45.6)	75.3 (74.8)
2,3,4,6-Me_4-Gal		2.0	4.9			0.6			
2,3,4-Me_3-Gal	2.2		4.3				3.2	2.9	0.6
2,3,6-Me_3-Gal	44.5	5.0	35.3	11.6	1.4		26.0		5.4
2,4,6-Me_3-Gal	4.1		0.8				2.6		
2,3-Me_2-Gal			5.4	3.6					
2,4-Me_2-Gal							6.2		
2,6-Me_2-Gal	15.4		3.4						0.4
Total	66.2 (72.8)	7.0 (4.4)	54.1 (50.3)	15.2 (14.7)	1.4 (2.6)	0.6 (0.5)	38.0 (39.0)	2.9 (5.9)	6.4 (6.8)
2,3,5-Me_3-Ara	8.2	17.1	5.3	19.4	1.8	0.5	11.9	3.5	3.0
2,3-Me_2-Ara	6.3	9.0	22.0		2.9		29.1	13.4	0.6
2,5-Me_2-Ara		7.7	3.0	17.4			4.6	2.1	0.5
2-Me-Ara		4.1					2.4	2.6	0.9
Ara	1.1	2.7					2.2		
Total	15.6 (11.3)	40.6 (40.7)	30.3 (25.9)	36.8 (40.4)	4.7 (3.8)	0.5 (0.5)	50.2 (50.9)	21.6 (25.2)	5.0 (6.8)
3,4-Me_2-Rha			1.0			0.5			
3-Me-Rha	11.4	4.2	4.4	12.5	0.3	0.6	3.0		1.8
Total	11.4 (4.0)	4.2 (3.9)	5.4 (3.9)	12.5 (12.1)	0.3 (1.2)	1.1 (1.0)	3.0 (2.7)		1.8 (1.8)
2,3,4-Me_3-Xyl	3.8	4.9	4.2	4.1		1.8		6.8	1.0
2,3-Me_2-Xyl			1.0	11.4	1.7	3.6			6.6
2-Me-Xyl			2.4			0.5			0.8
Total	3.8 (2.8)	4.9 (6.5)	7.6 (13.3)	15.5 (12.3)	1.7 (0.9)	3.9 (3.1)		6.8 (6.3)	8.4 (6.5)
2,3,6-Me_3-Man		30.1 (27.2)		8.5 (10.0)	9.8 (6.5)	1.5 (1.4)		14.4 (17.0)	3.1 (3.3)

[a]2,3,4,6-Me_4-Glc denotes 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, *etc.* [b]% of total area of peaks identified by g.l.c. [c]Values in parentheses are based on analysis of alditol acetates.

Gel-filtration chromatograms of PL-I/III are shown in Fig. 3. PL-I gave two fractions, PL-IA, which was composed essentially of neutral polysaccharides, and PL-IB, which contained neutral and acidic sugars of low molecular weight. The chromatogram of PL-II shows a peak rich in galacturonic acid eluted in the total volume of the column, suggesting the presence of monomers or small oligomers. PL-III was separated in two fractions, PL-IIIA, eluted in the void volume of the column, and PL-IIIB, which was heterogeneous in molecular size and was eluted at K_{av} 0.5–1.

On gel filtration (Fig. 4), C1-I and C1-II were shown to be heterogeneous in molecular size, and four fractions, C1-IA, C1-IB, C1-IIA, and C1-IIB, were collected.

Gel-filtration chromatograms of CI and CII are given in Fig. 5. The elution patterns of these fractions were similar to those of C1-I and C1-II (*cf.* Fig. 4), respectively, except that the ratio neutral sugar/galacturonic acid for the sub-fraction CIIA was reversed compared to that of C1-IIA.

Analysis of the isolated polysaccharides. — Table III gives the composition of the polysaccharides isolated by chromatography, and Table IV shows the results of methylation analysis of the fractions from PL and C1 extracts, and of the final insoluble residue CWM2. There was good agreement between the results of the analysis of alditol acetates and partially methylated alditol acetates.

PL-IA was composed mainly of galactose and arabinose, whereas PL-IB contained galacturonic acid, arabinose, and mannose. Small proportions of glucose and xylose were present in these two fractions. The methylated derivatives indicate that PL-IA contained a high proportion of (1→4)- and (1→3,4)-linked and terminal galactose, (1→5)-linked arabinose, and (1→2,4)-linked rhamnose. PL-IB was characterised by the presence of a branched arabinan with a large proportion of terminal arabinosyl groups and (1→4)-linked mannosyl residues. These results suggest that the PL-I polysaccharides were type I arabinogalactans with branched arabinan chains.

PL-II was composed nearly exclusively of galacturonic acid and h.p.l.c. showed that only 1% was monomeric. These results indicate that PL-II was formed from an esterified homogalacturonan.

PL-IIIA contained mainly galacturonic acid, galactose, arabinose, and xylose. The galactose was principally (1→4)-linked, but also (1→3)-, (1→6)- and (1→3,6)-linked. The arabinose was mainly (1→5)-linked and the xylose was terminal, (1→3,4)- and (1→4)-linked. The rhamnose was (1→2)- and (1→2,4)-linked. Polysaccharides of this fraction were chemically de-esterified and hydrolysed by an endopolygalacturonase. The hydrolysate was fractionated on Sephadex G-50 to give two peaks (Table III). The first peak, eluted in the void volume of the column, contained mainly galactose, arabinose, and some (~5%) galacturonic acid, rhamnose, xylose, and glucose. The second peak, eluted in the total volume of the column, was composed of galacturonic acid, arabinose (76% of the neutral sugars), and small proportions of galactose and xylose, but no rhamnose. These results

suggest that PL-IIIA was composed of rhamnogalacturonans with side chains of arabinans and types I and II arabinogalactans. These side chains contained xylose or were associated with fragments of xylan. The branched points involved position 4 of rhamnose, but the last result indicates that neutral sugars (especially arabinose) could be directly linked to galacturonic acid. PL-IIIB contained >50% of the galacturonic acid of the cell wall, some arabinose, and minute proportions of other sugars. Of the arabinose, ~50% was terminal and the remainder was (1→3)-linked. All the rhamnose present was (1→2,4)-linked, suggesting that the side chains were at position 4. The fractions derived from fragments released by the endopectin lyase contained low proportions of glucose, mainly (1→4)-linked. Contamination with glucose from DEAE-Sephacel or Sephacryl S-200 gels was not likely because all the fractions contained different amounts of glucose.

Glucose was the main sugar in C1-IA and C1-IB, and represented >80% of the initial glucose. These fractions also contained small proportions of galacturonic acid. C1-IA was characterised by a high proportion of (1→4)-linked glucose with ~10% in terminal positions. This fraction was hydrolysed by a β-D-glucosidase, and chromatography on Sephacryl S-200 (Fig. 6) showed that it was extensively degraded, suggesting the presence of (1→4)-linked oligomers of β-D-glucose. C1-IB was composed mainly of (1→4)-linked and terminal glucosyl residues and h.p.l.c. confirmed the presence of cellobiose. Galacturonic acid, arabinose, and galactose in similar proportions, and small proportions of glucose were present in C1-IIA. Methylation analysis (Table IV) of this fraction revealed a branched arabinan, a linear galactan, and a type II arabinogalactan. C1-IIB contained mainly glucose, galacturonic acid, and arabinose, but no rhamnose. The nature of the partially methylated alditol acetates suggested the presence of cellobiose (confirmed by h.p.l.c.), arabinose-containing branched oligomers, and terminal xylosyl groups.

In order to detect the ester linkages, CWM1 was treated with 0.05M NaOH at 4°. The polysaccharides released represented 5.8% of the initial CWM polysaccharides and were composed (Table III) of galacturonic acid, arabinose, and galactose in proportions similar to those of C1-IIA. This fraction was rich in rhamnose but contained no glucose and xylose.

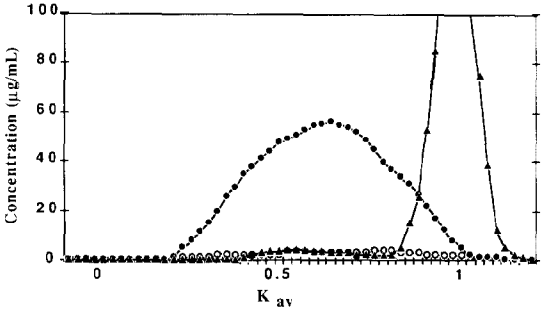

Fig. 6. Elution pattern of C1-IA (see Fig. 4) from Sephacryl S-200 before and after hydrolysis with β-D-glucosidase (see Experimental): galacturonic acid (○), neutral sugars before (●) and after (▲) hydrolysis, determined as in Fig. 2.

The final insoluble residue CWM2 was characterised (Table IV) by a high proportion of (1→4)-linked glucose with a small proportion of non-reducing terminal glucose, suggesting that microfibrils of cellulose were not extensively degraded. CWM2 contained terminal, (1→4)- and (1→3,4)-linked xylose and terminal arabinosyl groups, suggesting the presence of arabinoxylans.

CIA, CIB, CIIA, and CIIB (Fig. 5) were the fractions released by the cellulases. CIA contained mainly arabinose, glucose, and galacturonic acid, whereas CIB contained ~30% of the glucose of CWM together with a small proportion of galacturonic acid. In contrast, CIIA contained essentially galacturonic acid and a low proportion of glucose. CIIB comprised ~50% of glucose and a low proportion of galacturonic acid, but no rhamnose.

DISCUSSION

The foregoing results show that pure enzymes can partially degrade and solubilise carrot cell-wall polysaccharides. The solubilised polymers or oligomers may be the degradation products or molecules released after their association with other polysaccharides has been broken. For instance, the cellulases released 14% of the galacturonic acid of CWM, a part of which was a pectic polymer (fraction CIIA). At the same time, ~30% of the cellulose was degraded to oligomers (fraction CIB).

The sequence of the enzymes has an effect on the degradation limit of the cell wall. The action of endopectin lyase followed by cellulases solubilised more material than the opposite sequence, in agreement with the literature[6,7]. This finding suggests that, by removing the bulk of the pectic polysaccharides (~80% of the galacturonic acid content), the pectinase had increased the accessibility of cellulose to the cellulases. Moreover, the direct action of cellulases is hindered by the presence of pectic matrix[27]. The endoglucanase alone is inactive, and the cellobiohydrolase alone degrades only ~10% of the glucans. However, the combination of the two cellulases has a synergistic action on the cellulose in spite of the pectic wrap. Some zones of glucans (~10% of the cell-wall glucose) are probably accessible to the cellobiohydrolase which has endo activity[28]. Thus, the endoglucanase could act and release cellulose fragments (~30% of the glucose). The cellobiohydrolase removes cellobiose (fraction C1-IB) from cellulose, whereas the endoglucanase releases larger fragments that will be hydrolysed by the cellobiohydrolase (fraction C1-IA), but the association of cellulose to xyloglucans, arabinoxylans, or residual pectic substances, to lignin, or to proteins can hinder the cellulase action. Some fragments of glucans could therefore remain intact (fraction C1-IA).

On the other hand, since the cellobiohydrolase, endoglucanase, and xylanase released 6–10% of the galacturonic acid and since the combination of cellobiohydrolase and endoglucanase removed pectic polysaccharides (*e.g.* CIIA), it is likely that pectins are associated to cellulose. Covalent linkages have been detected[29] between cellulose and homogalacturonan in the primary cell wall of *Rosa*.

It has been suggested[30] also that some galacturonic acid residues could be ester-linked to glucans. These ester linkages could be present in C1-IIA and would be hydrolysed by sodium hydroxide which released the same pectic fragment but without glucose (alkali-soluble fraction, Table III). The fact that the addition of xylanase to cellulases increases the solubilisation of galacturonic acid by 4.2% suggests an association between xylans and pectic substances[5]. The xylose involved in these linkages would be part of the side chains of pectic fractions, as suggested by the composition of the excluded peak of PL-IIIA on Sephadex G50 (Table III) which contains ~20% of the cell-wall xylose. Therefore, pectic polysaccharides could be bound directly to xyloglucans or xylans, as has been suggested for pectic fragments of apple cell-wall[6]. Nevertheless, because endopectin lyase alone released "hairy" regions of pectins (PL-I, PL-III), numerous side-chains amounting to 64 and 80% of the arabinose and galactose, respectively, are probably not linked to other polysaccharides.

The pectic polysaccharides removed by endopectin lyase are highly branched[9] and are divided into two fractions. In the first one (8.9% of the CWM polysaccharides), the galacturonans are methyl-esterified, allowing the enzyme action (fractions PL-I and PL-II). The short fragments released are associated either with branched side-chains as galactan or arabinogalactan (fraction PL-IA) or to short chains of arabinose (fraction PL-IB) or are methyl-esterified oligogalacturonic acids (fraction PL-II). The second fraction (35.1% of the CWM polysaccharides) is partially methyl-esterified because the released fragments are bound to the ion-exchange gel (fraction PL-III). The degradation of fraction PL-IIIA by the endo-polygalacturonase led to fragments carrying arabinogalactans on the rhamnose (excluded peak of PL-IIIA on Sephadex G50) and homogalacturonans carrying short side-chains of arabinose (included peak of PL-IIIA on Sephadex G50). Moreover, some rhamnose may carry short side-chains of arabinose as in potato pectic polymers[8] (*cf.*, fraction PL-IIIB).

The structure of carrot cell-wall is a complex association of all these fragments. The present results show that some pectic polysaccharides may be associated to the molecules of cellulose either with direct ester linkages[30] and/or *via* side chains of xylose[6] and/or *via* xyloglucans[5].

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