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Studies on Apple Protopectin. III: Characterization of the Material Extracted by Pure Polysaccharidases from Apple Cell Walls

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ABSTRACT

Plant cell walls contain polymers with complex and badly understood interactions that can be studied by enzymic extraction. Apple cell wall material, partially depectinated by a chelating agent, was treated with pure polysaccharidases (pectin-lyase, polygalacturonase, pectinesterase, endo-*β-(1,4)-glucanases,* exo-*β-(1,4)-glucanase,* endo-*α-(1,5)-arabinanase, arabinofuranosidase,* endo-*β-(1,4)-galactanase and* endo-*β-(1,4)-xylanase) and combinations of these enzymes. Glucanases, arabinanases and galactanase extracted neutral oligomers. Enzyme formulations active on highly methylated pectins freed galacturonate oligomers and high-molecular-weight material. Addition of* endo-*β-(1,4)-glucanase to these enzyme formulations increased the amounts of high-molecular-weight material in the extracts. One of the* endo-*β-(1,4)-glucanases had a more marked action. The high-molecular-weight material was rich in neutral sugars, notably arabinose and galactose, and was degraded to a large extent by arabinanases and galactanase. The results were analysed by principal component analysis.*

INTRODUCTION

Protopectin, the part of the pectic material which is strongly bound to the cell walls (Pilnik & Voragen, 1970), probably plays a major role in the

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connections between cell wall polymers. Though apple soluble pectins have been thoroughly investigated (Barrett & Northcote, 1965; Knee, 1973*a*, *b*, 1978*a*, *b*; Knee *et al.*, 1975; de Vries *et al.*, 1981, 1982, 1983*a*, *b*; de Vries, 1983; Aspinall & Fanous, 1984; Rouau & Thibault, 1984; Stevens & Selvendran, 1984), and their structure has been established by de Vries (1983), less is known about the structure of the less readily extractable pectic material.

Our aim is to study the protopectin and define the interactions that make pectin a protopectin. One promising possibility is the use of well-characterized polysaccharidases (Bauer *et al.*, 1973; Keegstra *et al.*, 1973; Talmadge *et al.*, 1973; Knee *et al.*, 1975; Voragen *et al.*, 1980; Ishii, 1981, 1982; Jarvis *et al.*, 1981; Konno & Yamasaki, 1982; Saulnier & Thibault, 1987; Thibault *et al.*, 1988; Massiot & Thibault, 1989). Pectolytic enzymes were the most often used, but cellulolytic enzymes (Bauer *et al.*, 1973; Knee *et al.*, 1975; Voragen *et al.*, 1980; Massiot & Thibault, 1989), xylanases (Massiot & Thibault, 1989), arabinanases (Knee *et al.*, 1975; Thibault *et al.*, 1988) and galactanases (Knee *et al.*, 1975; Jarvis *et al.*, 1981; Thibault *et al.*, 1988) have also been used.

In a preceding paper (Renard *et al.*, 1990*a*) we have investigated the effect of various polysaccharidases and their combinations on apple cell walls. Pectolytic enzymes were necessary for extraction of significant amounts of uronides. Non-pectolytic enzymes such as *exo-* and *endo*-β-(1,4)-glucanases, *endo*-β-(1,4)-xylanase, *endo*-(1,4)-β-galactanase, arabinofuranosidase and *endo*-α-(1,5)-arabinanase freed mostly neutral sugars. The combination of non-pectolytic and pectolytic enzymes led to increased degradation of the cell wall. Combinations of *endo*-β-(1,4)-glucanases, notably Endo Glu IV, with pectolytic enzymes extracted more uronides than combinations of arabinan-degrading enzymes, galactanase and pectolytic enzymes. Composition of the residues and principal component analysis of these results were also given.

The nature of the extracted material, especially its size and sugar composition, can give further insight into the nature of the interconnections between cell wall polymers. In this paper, we report results on the composition of the extracts and their size distribution.

MATERIAL AND METHODS

Partially depectinated cell wall material

Apple cell wall material free from soluble pectins was prepared as described by Renard *et al.* (1990*b*) by treatment of apple alcohol

insoluble solids by cyclohexane diamino tetraacetic acid (CDTA), giving the CDTA insoluble residue (CDTAIR).

Treatment of CDTAIR with enzymes

The enzymes and experimental conditions were the same as described previously (Renard *et al.*, 1990*a*).

Analytical methods

The galacturonic acid and neutral sugars determinations were performed as described previously (Renard *et al.*, 1990*a*). Derivatization of neutral sugars was carried out after hydrolysis by 1 M H_2SO_4 at 100° for 3 h. The molecular weight distribution of the extracted material was obtained by high-pressure gel-permeation chromatography (HPGPC) as described previously (Renard *et al.*, 1990*a*). The excluded volume of the column corresponded to an elution time of 20 min, and the included volume to an elution time of 38 min. Neutral monomers and small oligomers were identified and quantified by HPLC on an Aminex HPX-87P column (Bio-Rad Laboratories, Richmond, California, USA) as described by Voragen *et al.* (1986*a*). Saturated and unsaturated galacturonic acid monomers and oligomers were identified by HPLC on an Aminex 42H column (Bio-Rad Laboratories, Richmond, California, USA) eluted by 0·005 M H_2SO_4 at 30° at a flow rate of 0·6 ml/min. The eluent was monitored at 210 nm using a Varian 9060 polychrom UV detector.

Mathematical methods

Principal component analysis (PCA) was performed as described previously (Renard *et al.*, 1990*a*).

RESULTS

Composition of the extracts

The arabinanases and galactanase freed arabinose and galactose (Table 1). The combination Arafase + Endo Ara + Endo Gal solubilized 31% of the arabinose and 20% of the galactose. The *endo*-glucanases in the absence of Exo Glu released glucose but also high proportions of xylose,

TABLE 1
Yields and Sugar Composition of the Extracts from Enzyme Treatments: Non-pectolytic Enzymes

Sample	*Yield (% CDTAIR)*		*Composition of the extracts (mol%)*							
	NS[a]	*GalA*	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*
Blank	0·7	1·2	2·1	0·0	13·5	3·2	0·0	7·0	2·5	71·7
Arafase	3·2	2·1								
Endo Ara	2·4	1·5								
Endo Gal	1·3	1·8								
Endo Xyl	0·7	1·2								
Arafase + Endo Ara	5·8	2·1	0·7	0·0	57·2	1·1	0·0	3·4	1·4	36·2
Arafase + Endo Gal	4·0	1·6	0·7	0·0	24·8	1·1	0·0	35·4	1·3	36·7
Arafase + Endo Ara + Endo Gal	7·2	2·5	0·8	0·0	45·9	1·5	0·0	17·9	1·3	32·6
Exo Glu	1·0	1·8								
Endo Glu I	1·8	1·7	1·5	2·4	15·8	12·8	4·3	8·8	17·2	37·2
Endo Glu IV	4·2	2·0	1·1	4·4	8·1	20·6	0·0	9·1	28·1	28·7
Exo Glu + Endo Glu I	5·2	2·3	0·7	2·5	5·5	6·8	2·9	6·2	46·9	36·8
Exo Glu + Endo Glu IV	6·0	2·2	0·6	3·7	5·9	17·6	0·0	7·9	45·9	18·4
Exo Glu + Endo Glu I + Endo Glu IV	10·4	3·3	0·4	2·9	5·0	14·5	1·7	6·5	51·8	17·2
Arafase + Endo Ara + Endo Gal + Endo Glu I	9·7	3·0	0·5	1·2	41·5	3·9	0·8	22·1	6·7	23·3
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I	12·6	3·3	0·4	1·6	28·3	5·2	1·4	16·4	30·5	16·2
Arafase + Endo Ara + Endo Gal + Endo Glu IV	13·6	3·9	0·7	1·5	35·7	9·0	0·4	16·4	12·5	23·8
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu IV	18·8	5·2	0·4	1·8	28·7	8·8	0·1	15·9	25·8	18·5

[a]NS, neutral sugars.

fucose and some galactose. Endo Glu I liberated 4·5% of the fucose, 4·8% of the xylose, 2·5% of the galactose and 1·4% of the glucose initially present in the CDTAIR, and Endo Glu IV, liberated 16%, 15·3%, 5·6% and 4·5%, respectively. Endo Glu I was the only enzyme that released mannose. The addition of Exo Glu to the *endo*-glucanases led to an increase in the solubilization of glucose: up to 18·4% of the glucose was extracted with Exo Glu + Endo Glu I + Endo Glu IV.

The PL and PG + PE liberated essentially uronides (Table 2), but also arabinose and galactose in proportions almost as high as Arafase + Endo Ara + Endo Gal (PL: 23% of the arabinose, 15% of the galactose). The pectolytic enzymes liberated relatively little rhamnose: the PL solubilized 18·4% of the rhamnose with 44% of the uronides. With Arafase + Endo Ara + Endo Gal + PL or PG + PE, there was no synergism: 54·6% of the arabinose and 41·1% of the galactose were released by Arafase + Endo Ara + Endo Gal + PL, less than the sum of the amounts liberated separately. When the glucanases were combined to PL or PG + PE there was some synergism for the release of glucose (Exo Glu + Endo Glu I + Endo Glu IV + PL: extraction of 33·0% of the glucose), but higher increases were obtained for the other sugars. Endo Glu IV + PL extracted 55·8% of the arabinose, 58·3% of the galactose, 37·2% of the xylose, 30·0% of the fucose, 53·5% of the rhamnose and only 9·2% of the glucose. The amounts of arabinose and galactose released increased proportionally more than that of galacturonic acid, which increased from 44% to 65%. Endo Glu I gave the same type of effect as Endo Glu IV but less marked. The *endo*-xylanase used had no detectable effect alone, in combination with the PL or with Endo Glu I and the PL.

Molecular weight distribution

The blank showed a small peak at the excluded volume (Fig. 1). The non-pectolytic enzymes used separately produced extracts that contained low amounts of low-molecular-weight material (Fig. 1). Endo Glu IV was the only non-pectolytic enzyme that gave a rather high peak (at 33·3 min). PL and PG + PE released high-molecular-weight polymers in addition to oligomers. The PG extract differed from the blank only by the presence of a shoulder on the excluded peak.

Combinations of non-pectolytic enzymes showed peaks at the same retention times as with the enzymes used separately (Fig. 2). Synergisms were visible, notably between Exo Glu and the *endo*-glucanases, where a typical peak at 35·5 min (cellobiose) was observed. Use of the PG

TABLE 2
Yields and Sugar Composition of the Extracts from Enzyme Treatments: Pectolytic Enzymes and Combinations of Pectolytic and Non-pectolytic Enzymes

Sample	*Yield (% CDTAIR)*		*Composition of the extracts (mol%)*							
	NS[a]	*GalA*	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*
PL	5·3	8·8	1·7	0·0	25·0	2·9	0·0	10·9	1·2	58·3
PG	1·3	2·3	1·5	0·0	18·2	2·3	0·0	8·1	1·5	68·4
PG + PE	4·2	9·5	2·9	0·0	35·6	13·1	0·0	12·4	tr.	46·1
Arafase + Endo Ara + Endo Gal + PL	12·1	11·3	1·7	0·0	35·7	2·5	0·0	18·1	1·0	41·0
Arafase + Endo Ara + Endo Gal + PG	7·1	3·6	0·8	0·0	45·1	1·0	0·0	15·7	1·0	36·4
Arafase + Endo Ara + Endo Gal + PG + PE	10·1	11·5	1·7	0·0	32·6	1·9	0·0	17·8	0·6	45·4
Endo Xyl + PL	4·8	8·5	2·4	0·0	23·0	0·7	0·0	10·3	0·9	62·7
Endo Glu I + PL	9·2	9·9	2·4	1·3	25·0	6·2	1·2	10·5	7·9	45·5
Endo Glu IV + PL	16·1	13	2·4	1·6	27·1	9·4	0·2	15·6	9·3	34·4
Exo Glu + Endo Glu I + PL	19·5	11·3	1·3	1·4	17·5	7·6	1·7	11·9	34·7	23·9
Exo Glu + Endo Glu I + PG	6·5	3	0·6	2·3	7·8	9·0	2·5	6·1	45·9	25·8
Exo Glu + Endo Glu I + PG + PE	16·5	13								
Exo Glu + Endo Glu IV + PL	27·1	15	1·5	1·5	22·5	9·1	0·3	13·5	25·5	26·2
Exo Glu + Endo Glu I + Endo Glu IV + PL	24·6	13·2	1·6	1·7	18·3	9·0	1·2	12·2	34·0	22·0
Exo Glu + Endo Glu I + Endo Glu IV + PG	10·6	3·3	0·4	2·8	6·5	14·4	1·7	7·1	49·4	17·7
Exo Glu + Endo Glu I + Endo Glu IV + PG + PE	24·7	15·3	1·1	1·6	15·9	8·8	1·4	10·2	35·1	25·9
Endo Glu I + Endo Glu IV + PG	6·5	2·9	1·0	4·1	9·9	17·1	1·2	9·3	26·3	31·1
Endo Glu I + Endo Glu IV + PG + PE	13	12·1	1·6	1·8	23·0	10·7	1·1	11·8	14·2	35·8
Arafase + Endo Ara + Endo Gal + Endo Glu I + PG	10·8	4·4	0·8	1·5	32·7	4·4	1·1	18·7	8·6	32·2
Arafase + Endo Ara + Endo Gal + Endo Glu I + PG + PE	12·5	10·9	1·5	1·3	26·7	4·6	0·9	16·2	7·3	41·5
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I + PL	23·9	12	1·1	1·3	19·5	7·5	1·5	12·7	33·2	23·2
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I + PG	14·8	5·1	0·5	1·5	25·2	5·5	1·5	15·4	32·3	18·1
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I + PG + PE	22·4	13·9	0·7	1·3	18·5	6·7	1·5	12·5	32·1	23·7

[a]NS, neutral sugars.

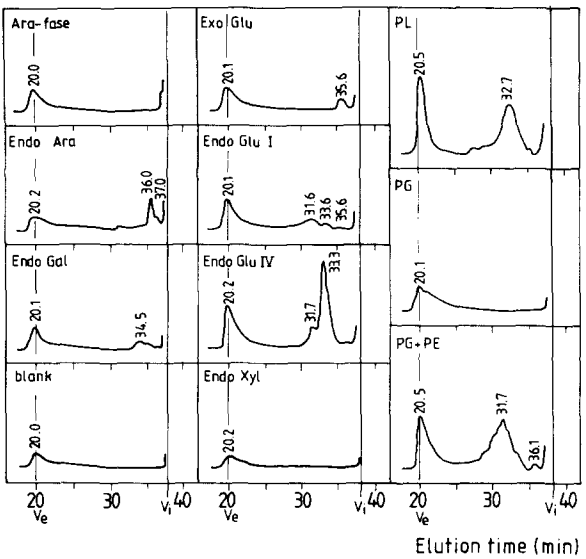

Fig. 1. HPGPC analysis of the size distribution of the extracts. Extracts obtained with the enzymes used separately.

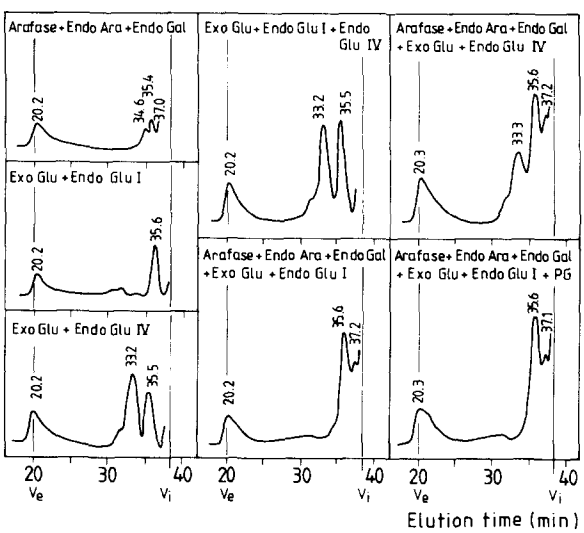

Fig. 2. HPGPC analysis of the size distribution of the extracts. Extracts obtained with enzymes combinations that did not include enzyme formulations active on highly methylated pectins.

without PE in combination with non-pectolytic enzymes led to the presence of a shoulder on the excluded peak (Fig. 2), as could be noticed for PG alone.

Two different situations occurred for combinations including PL or PG + PE and non-pectolytic enzymes (Fig. 3). With *endo*-glucanases the area of the excluded peak increased, indicating that an increased amount of high-molecular-weight polymers had been extracted. The highest excluded peak was obtained with Endo Glu IV plus PL. In contrast, when Arafase + Endo Ara + Endo Gal were added to PL or PG + PE, the area of the excluded peak diminished. In both cases the amount of low-molecular-weight products increased.

Composition of the high-molecular-weight soluble fragments

Dialysis was used to remove the oligomeric products; composition of the high-molecular-weight fractions of the extracts was determined. Dialysis retentates from extracts of arabinanases and galactanase (Table 3) had compositions drastically different from the whole extracts. Galactose and, to an even higher degree, arabinose were lost during dialysis. Loss

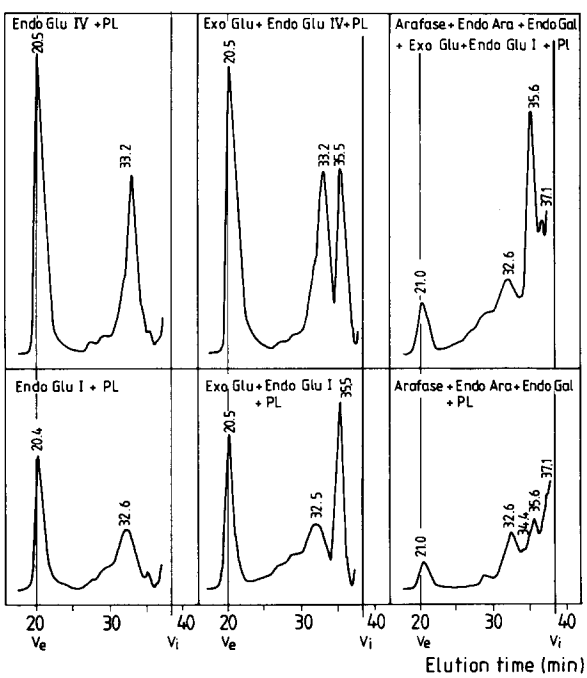

Fig. 3. HPGPC analysis of the size distribution of the extracts. Extracts obtained with combinations of pectin-lyase and non-pectolytic enzymes.

TABLE 3
Sugar Composition of the Dialysis Retentates of the Extracts from Enzymes Treatments: Non-pectolytic Enzymes

Sample	*Composition of the dialysis retentates (mol%)*							
	Rha	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*
Blank	1·9	0·0	14·7	4·3	0·0	7·1	5·8	66·2
Arafase + Endo Ara	1·8	0·0	6·0	4·7	0·0	6·9	7·9	72·7
Arafase + Endo Ara + Endo Gal	1·9	0·0	6·1	4·7	0·0	5·0	4·1	78·2
Endo Glu I	1·4	2·5	13·0	8·5	tr.	10·3	14·2	50·1
Endo Glu IV	2·1	2·8	14·3	10·7	0·0	8·7	12·4	49·0
Exo Glu + Endo Glu I	1·1	2·7	10·4	13·6	tr.	7·7	17·4	47·1
Exo Glu + Endo Glu IV	1·1	1·2	13·1	7·0	0·0	7·6	9·7	60·3
Exo Glu + Endo Glu I + Endo Glu IV	1·4	3·3	12·7	11·9	0·8	8·9	17·0	44·0
Arafase + Endo Ara + Endo Gal + Endo Glu I	1·6	2·6	4·9	8·1	3·3	5·7	12·8	61·0
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I	1·5	2·8	3·4	11·3	tr.	6·1	15·1	59·8
Arafase + Endo Ara + Endo Gal + Endo Glu IV	2·3	1·5	5·8	9·6	0·8	6·6	8·8	64·6
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu IV	1·9	2·8	5·4	10·2	0·0	6·8	12·1	60·8

of uronides was visible for the PL extract (Table 4). The retentate of the combination Arafase + Endo Ara + Endo Gal + PL contained very low proportions of arabinose and galactose. Retentates from combinations of glucanases and PL (or PG + PE) had the highest arabinose and galactose contents. GalA content of these retentates was lower than in the retentate of the PL extract. Some fucose and xylose remained after dialysis. At this point sugars covalently linked to the pectin and oligomers too large to dialyse could not be distinguished. The proportion of glucose remaining in the retentates, always lower than in the corresponding extracts, was lower than in the retentate of the PL with Endo Glu IV, but higher with Endo Glu I.

Low-molecular-weight products

HPLC techniques were used to investigate the nature and composition of the low-molecular-weight material. Low-molecular-weight products of the non-pectolytic enzymes were identified by HPLC on an Aminex HPX-87P column either by comparison with authentic standards or by their relative retention times (Voragen *et al.*, 1986*a*) (Fig. 4, Table 5). The arabinofuranosidase produced exclusively arabinose; when Endo Ara was used in combination with the arabinofuranosidase, arabinose was again the main product with low concentrations of arabino-biose, and -triose and higher oligomers (not separated). The main product of the galactanase was galactobiose. The *endo*-glucanases produced mostly oligomers that had degrees of polymerization too high to be separated on this column. Cellobiose and some glucose and cellotriose were produced in the presence of Exo Glu. In the presence of PL or PG + PE, the amounts of monomers and oligomers increased.

PL and PG + PE produced a wide range of oligomers (Fig. 5). Comparison with standards from PG- or PAL-treated polygalacturonic acid showed the presence of more peaks than only those of the non-esterified oligomers. The unsaturated oligomers produced by PL had slightly different retention times than the oligomers produced by PG + PE. PG + PE extracted more oligomers of higher degrees of polymerization than the PL.

Mathematical treatments

PCA was carried out on the concentrations in the extracts. Two groups of strongly positively correlated sugars could be distinguished (Table 6) — the uronide/rhamnose/arabinose/galactose group ('pectin-related sugars') and the fucose/xylose/glucose group ('xyloglucan-related sugars'). Correlation coefficients (r) above 0·9 were obtained between

TABLE 4

Sugar Composition of the Dialysis Retentates of the Extracts from Enzymes Treatments: Pectolytic Enzymes and Combinations of Pectolytic and Non-pectolytic Enzymes

Sample	*Composition of the dialysis retentates (mol%)*							
	Rha	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*
PL	3·5	0·0	29·5	5·2	0·0	14·8	4·0	43·0
Arafase + Endo Ara + Endo Gal + PL	4·7	0·0	8·9	8·0	0·7	6·3	4·2	67·2
Arafase + Endo Ara + Endo Gal + PG	2·2	0·0	5·3	3·7	0·0	4·9	3·4	80·5
Arafase + Endo Ara + Endo Gal + PG + PE	4·1	0·0	6·5	5·9	0·9	5·0	3·3	74·3
Endo Xyl + PL	2·9	0·0	30·7	3·9	0·0	13·7	1·8	47·0
Endo Glu I + PL	3·2	0·9	30·0	6·5	0·9	16·3	5·1	37·1
Endo Glu IV + PL	3·0	0·5	35·7	5·9	0·0	19·4	2·4	33·1
Exo Glu + Endo Glu I + PL	2·6	2·3	26·1	10·5	0·5	18·0	11·9	28·1
Exo Glu + Endo Glu I + PG	1·4	3·4	13·7	11·1	0·7	8·8	15·8	45·1
Exo Glu + Endo Glu I + PG + PE	2·0	1·9	27·2	9·8	0·8	14·2	10·8	33·3
Exo Glu + Endo Glu IV + PL	2·8	1·0	35·2	7·7	0·3	19·5	4·4	29·1
Exo Glu + Endo Glu I + Endo Glu IV + PG	1·5	3·4	13·9	12·6	0·8	9·4	16·4	42·0
Exo Glu + Endo Glu I + Endo Glu IV + PG + PE	2·5	1·3	27·3	8·0	0·6	16·1	7·0	42·0
Endo Glu I + Endo Glu IV + PG	1·8	2·5	17·2	11·2	1·2	9·8	14·0	42·3
Endo Glu I + Endo Glu IV + PG + PE	2·8	1·2	27·2	6·6	0·5	12·4	6·4	42·9
Arafase + Endo Ara + Endo Gal + Endo Glu I + PG + PE	3·9	1·0	5·9	8·0	0·4	6·4	7·3	67·1
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I + PL	4·2	3·5	6·0	16·1	0·6	10·2	17·1	42·1
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I + PG	2·2	3·1	4·1	11·1	0·7	6·3	14·0	58·5

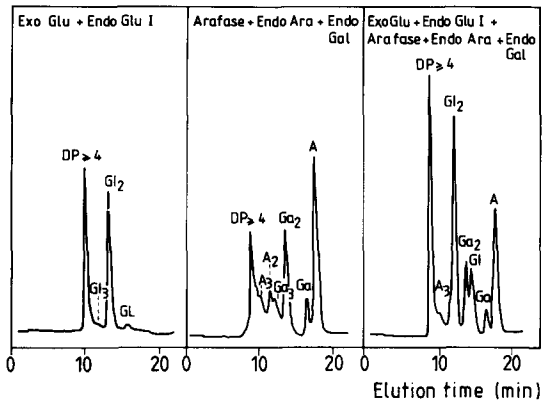

Fig. 4. HPLC analysis of the monomeric and oligomeric reaction products of the non-pectolytic enzymes. A, arabinose; Ga, galactose; Gl, glucose; A2, arabinobiose, etc.

arabinose and galactose ($r=0{\cdot}97$), fucose and xylose ($r=0{\cdot}96$), and rhamnose and galacturonic acid ($r=0{\cdot}95$). These pairs of sugars were extracted together and in almost constant proportions. The intensity of the correlations decreased for arabinose/galacturonic acid ($r=0{\cdot}89$), galactose/galacturonic acid ($r=0{\cdot}88$), arabinose/rhamnose ($r=0{\cdot}85$), galactose/rhamnose ($r=0{\cdot}85$), fucose/glucose ($r=0{\cdot}79$) and xylose/glucose ($r=0{\cdot}75$), as the intensity of the links between the sugars decreased. These pairs were still extracted together, but in increasingly variable proportions. The fucose and xylose had lower correlations with the glucose than with one another. The presence of Exo Glu was the main factor for liberation of high amounts of glucose, though some glucose was extracted by the *endo*-glucanases together with xylose and fucose. Arabinose and galactose were extracted together, either with arabinanases and galactanases (used in combination) or with pectolytic enzymes. However, arabinanases and galactanases extracted little pectin, so that the correlations between arabinose or galactose on the one hand and galacturonic acid or rhamnose on the other hand were slightly lower.

The first principal component, which took into account 67·9% of the total variance of the data, was strongly correlated to all the sugars (with the same sign) (correlation coefficients from 0·77 to 0·42). This kind of correlation pattern corresponds to an 'intensity axis', i.e. an axis that takes into account the global intensity of each sample (here the total concentration) (Robert & Bertrand, 1985). Axis 2 (18·5% of the variance) was correlated to arabinose, galacturonic acid and rhamnose, and negatively correlated to glucose, fucose and mannose. Axis 3 (9·1% of the variance) was correlated to the fucose and xylose contents of the

TABLE 5
Concentrations of the Neutral Monomers and Small Oligomers Produced by the Non-pectolytic Enzymes[a]

Relative retention time:	*1*	*0·93*	*0·89*	*0·81*	*0·78*	*0·69*	*0·65*	*0·58*	*0·54*	*0·5*
Identification:[b]	*Ara*	*Gal*	*Xyl*	*Glc*	*Gal2*	*Glc2 Gal3*	*Ara2*	*Glc3 Gal4*	*Ara3*	*DP ≥ 4*
Arafase	130									
Endo Ara	tr.						60		60	130
Endo Gal					60	50				tr.
Endo Xyl										
Exo Glu						80				tr.
Endo Glu I						tr.				170
Endo Glu IV						tr.				650
Arafase + Endo Ara + Endo Gal	430	60			160	100	70	30	50	70
Arafase + Endo Ara + Endo Gal + PL	590	130			350	160	150			560[c]
Exo Glu + Endo Glu I + Endo Glu IV				80		800				1100[d]
Exo Glu + Endo Glu I + Endo Glu IV + PL				130		1140				1850[d]

[a]Concentrations are given in μg/ml.
[b]Ara, arabinose; Ara2, arabinobiose, etc. DP ≥ 4, oligomers with polymerization degrees equal or superior to 4.
[c]Gal4 and Ara3 are included as shoulders on this peak.
[d]Glu3 is included as a shoulder on this peak.

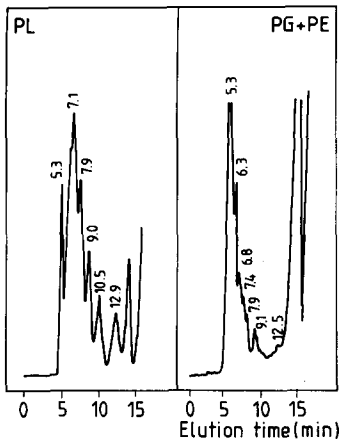

Fig. 5. HPLC analysis of the oligomeric reaction products of the pectolytic enzymes. Elution times for non-esterified standards: galacturonic acid, 12·5 min; digalacturonic acid, 9·1 min; trigalacturonic acid, 7·4 min; tetragalacturonic acid, 6·3 min; unsaturated digalacturonic acid, 10·1 min; and unsaturated trigalacturonic acid, 8·0 min.

TABLE 6
Principal Component Analysis: Correlations between the Sugar Concentrations in the Extracts

	Correlation coefficients							
	Rha	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*
Rha	1							
Fuc	0·52	1						
Ara	0·85	0·41	1					
Xyl	0·68	0·96	0·53	1				
Man	0·36	0·51	0·42	0·46	1			
Gal	0·82	0·55	0·97	0·65	0·51	1		
Glc	0·39	0·79	0·39	0·75	0·81	0·54	1	
GalA	0·95	0·49	0·89	0·63	0·42	0·88	0·41	1

extracts, and negatively correlated to the mannose content. These three axes together took into account 95·5% of the total variance.

On the plane defined by axes 1 and 2 (86·4% of the total variance) (Fig. 6), extracts were classified along axis 1 according to their total concentration. The blanks, i.e. the samples where the concentrations were the lowest, were at one extremity of axis 1. The extracts obtained by combinations of *exo*-glucanase, *endo*-glucanases and pectolytic enzymes, for which the yields were the highest, were at the other extremity. Samples were distributed along axis 2 according to the relative intensities of pectin extraction on one side and cellulose degradation, which was significant only in the presence of Exo Glu, on the other side.

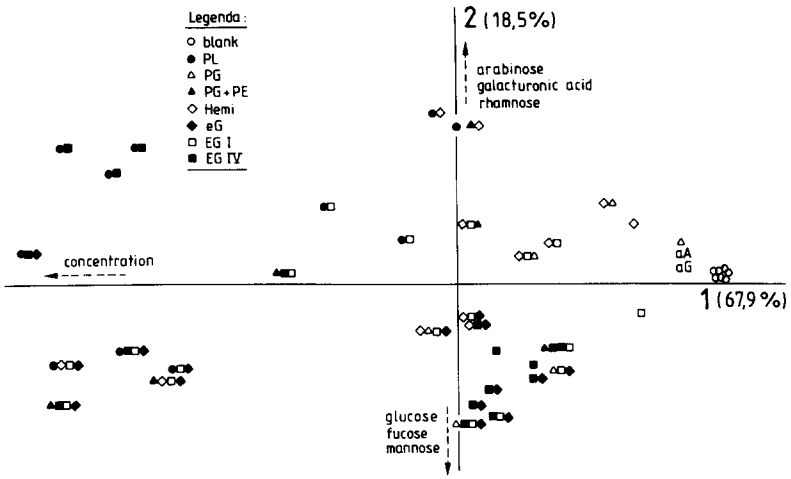

Fig. 6. Principal component analysis: similarity map defined by principal components 1 (67·9% of the variance) and 2 (18·5% of the variance). aA, arabinofuranosidase + *endo*-arabinanase; aG, arabinofuranosidase + *endo*-galactanase; Hemi, arabinofuranosidase + *endo*-arabinanase + *endo*-galactanase; eG, *exo*-glucanase; EG I, *endo*-glucanase I; EG IV, *endo*-glucanase IV; PL, pectine-lyase; PG, polygalacturonase; PE, pectinesterase.

Extracts from combinations containing the *exo*-glucanase were opposed to all other extracts.

On plane 2 × 3 (Fig. 7), the blanks were close to the origin. As on plane 1 × 2, the samples were classified along axis 2 according to the proportion of glucose in the extracts. Axis 3 opposed the extracts obtained with Endo Glu IV, which released xylose and fucose, to the extracts obtained with Endo Glu I, which was able to solubilize mannose. Samples extracted without *endo*-glucanase or with both *endo*-glucanases were not differentiated along axis 3.

DISCUSSION

Non-pectolytic enzymes

Arabinanases and galactanase freed arabinose and galactose oligomers similar to the reaction products obtained with model substrates. The *endo*-glucanases liberated fucose, xylose, galactose and glucose. The molar ratios of these sugars correspond to the fucogalactoxyloglucan which is the main hemicellulose of apple (Aspinall & Fanous, 1984; Stevens & Selvendran, 1984; Voragen *et al.*, 1986*b*). Though xylo-

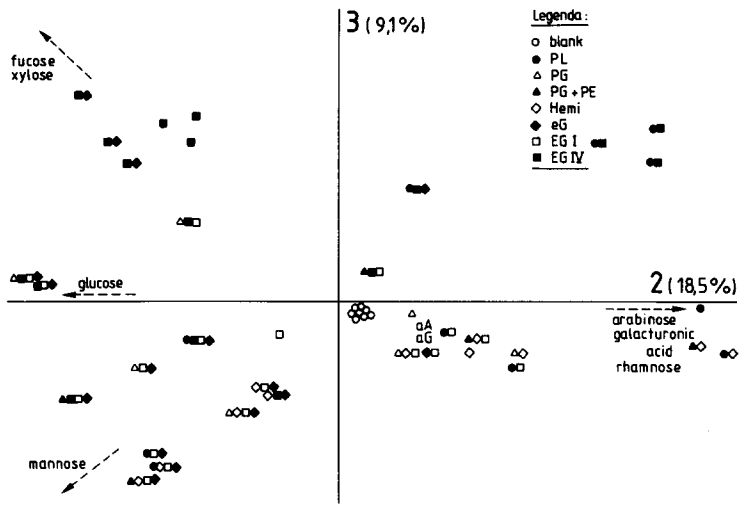

Fig. 7. Principal component analysis: similarity map defined by principal components 2 (18·5% of the variance) and 3 (9·1% of the variance). aA, arabinofuranosidase + *endo*-arabinanase; aG, arabinofuranosidase + *endo*-galactanase; Hemi, arabinofuranosidase + *endo*-arabinanase + *endo*-galactanase; eG, *exo*-glucanase; EG I, *endo*-glucanase I; EG IV, *endo*-glucanase IV; PL, pectine-lyase; PG, polygalacturonase; PE, pectinesterase.

glucans are not the preferential substrate of *endo*-glucanases, they are more accessible than the cellulose and are hydrolysed first (Hayashi *et al.*, 1984). The cellulase preparation used by Voragen *et al.* (1980) also solubilized a high proportion of fucose, xylose and galactose from apple cell walls. Endo Glu IV has a more intense effect. It is highly active on xylans (Beldman *et al.*, 1985, 1988) and xylans are known to be present, albeit in low amounts, in apple cell walls (Stevens & Selvendran, 1984; Voragen *et al.*, 1986*b*). However, none of the reactions products of Endo Glu IV on xylans (Beldman *et al.*, 1988) could be detected. Endo Glu I partially degraded the (gluco-)mannan detected by Voragen *et al.* (1986*b*) in the hemicelluloses of apple. Addition of the *exo*-glucanase greatly increased liberation of glucose. Up to one-fifth of the glucose could be solubilized in the absence of pectolytic enzymes i.e. in spite of the pectic wrap. This glucose was mostly of cellulosic origin, as 95% of the glucose present in the original apple alcohol-insoluble solids (AIS) could be identified as cellulose (Renard *et al.*, 1990*b*). Voragen *et al.* (1980) liberated 19·8% of the glucose from apple AIS and 50·1% from water-insoluble solids (WIS) with a cellulase preparation devoid of pectolytic activities. Massiot and Thibault (1989) freed significant proportions of glucose from carrot cell walls with glucanases in the absence of pectolytic enzymes. According to Ben-Shalom (1986), the ability

of the pectic wrap to protect cellulose from enzymic degradation is diminished by preparation of cell wall material. The *endo*-xylanase had no detectable effect. The arabinanases, galactanase and glucanases were unable to extract significant amounts of uronides.

Pectolytic enzymes

The enzyme formulations active on highly methylated pectins, i.e. PL and PG + PE, freed high proportion of uronides, rhamnose, arabinose and galactose. Analysis of the residues (Renard *et al.*, 1990*a*) had shown a loss of arabinose and galactose together with uronides after treatment with these enzymes. The uronides were mostly present as oligomers. The neutral sugars (arabinose, galactose and rhamnose) were present in the high-molecular-weight fraction. By PG treatment, Talmadge *et al.* (1973) extracted from sycamore cell walls 39% and 33%, respectively, of the arabinose and galactose present in the cell wall. Knee *et al.* (1975) extracted up to 45% and 43%, respectively, of the arabinose and galactose in apple cell wall material using PG from *Sclerotinia infestans*, and 55% and 37% when this enzyme was combined with a PE. Voragen *et al.* (1980) extracted 48% and 22% of the arabinose and galactose of apple WIS by PG treatment, and with PG + PE 59% and 43% of the arabinose and galactose of apple AIS. Massiot and Thibault (1989) liberated 67% and 69% of the arabinose and galactose of carrot cell walls using a PL. Previous results (Renard *et al.*, 1990*a*) already suggested that a high proportion of the arabinans and arabinogalactans side chains had thus 'loose', 'free' ends, i.e. were connected in the cell wall only to the rhamnogalacturonic backbone. The PL extracted 23% of the arabinose and 15% of the galactose: these sugars were included not only in 'loose', 'free' side chains but actually in whole 'loose', 'hairy regions'. Enzyme formulations active on highly methylated pectins degraded preferentially material originating from homogalacturonan regions, thus solubilizing some 'loose', 'hairy' fragments. The PG had a limited action, as could be expected from the high degree of methylation of the pectin in the CDTAIR (Renard *et al.*, 1990*b*).

Combinations of pectolytic and non-pectolytic enzymes

Combinations of the arabinanases and the galactanase with PL showed no synergism in the amounts of material extracted. This is in disagreement with the idea that the rhamnogalacturonan is connected to the other cell wall polymers via the arabinans and arabinogalactan side chains. However, such a covalent connection cannot be ruled out in the absence

of an enzyme able to degrade the (1,3)/(1,6)-galactans present in apple cell walls (de Vries *et al.*, 1983*b*). The amounts of arabinose and galactose in the extract were no higher than the sum of the amounts extracted separately by the PL and the combination Arafase + Endo Ara + Endo Gal. However, the diminution of the excluded peak in HPGPC, the composition of the dialysed extract and the increased concentrations of arabinose and galactose oligomers showed that conversion of the arabinans and galactans to oligomers had been more intense. This could be because of higher activity of the enzymes on the side chains solubilized by the pectolytic enzymes or because of easier access to the arabinans and galactans in the cell wall after removal of the pectins.

On the contrary, *endo*-glucanases and enzyme combinations active on highly methylated pectins showed synergism for extraction of uronides and even more of arabinose and galactose. HPGPC patterns of these extracts showed a larger increase of the excluded peak than of the oligogalacturonides peak. The high-molecular-weight polymers were, as with the enzyme combinations active on highly methylated pectins, rich in arabinose and galactose. The *endo*-glucanases apparently increased extraction of the 'hairy regions' rather than degradation of the homogalacturonic zones. Another possibility is that degradation of the homogalacturonic zones was already nearly complete with the PL or PG + PE. Endo Glu IV was more efficient than Endo Glu I. The specificity and high efficiency of the combination Endo Glu IV + PL was emphasized by the PCA results. Addition of Exo Glu resulted in increased cellulose degradation. Removal of the 'pectic wrap' increased the amounts of oligomers produced by these enzymes.

A polymer that is degraded or liberated by the *endo*-glucanases, especially Endo Glu IV, plays a major role in the binding of the rhamnogalacturonans to the rest of the cell wall. As the addition of Exo Glu has little additional effect in extraction of pectins, and no evidence was found for the intervention of xylans, xyloglucans (fucogalactoxyloglucans) appear to be one of the main links between rhamnogalacturonans and cellulose. Though the *endo*-glucanases and notably Endo Glu IV enhance the effect of the pectolytic enzymes, they are not able themselves to extract uronides. Possible explanations are that (i) the intact rhamnogalacturonans have other connections to the cellulose framework or (ii) though having no other connection, the physical enmeshment is such that they cannot pass in the buffer. Arabinans and type I arabinogalactans could be involved in that enmeshment. Among other possible connections, the most likely appears to be intervention of glycoproteins, notably arabinogalactan proteins. Intervention of phenolic acids such as

ferulates does not seem very likely in this material (Renard *et al.*, 1990*b*).

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