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Studies on Apple Protopectin. II: Apple Cell Wall Degradation by Pure Polysaccharidases and their Combinations

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ABSTRACT

Plant cell walls are complex associations of polymers, the interconnections of which can be studied by sequential enzyme degradation. Partially depectinated apple cell wall material was degraded by 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. Amounts of extracted material and composition of the residues were determined. Principal component analysis permitted an objective assessment of these results. Extraction of significant amounts of uronides was only possible with enzyme formulations active on highly methylated pectins. Addition of* endo-*β-(1,4)-glucanase enhanced cell wall degradation by enzyme formulations active on highly esterified pectins, but arabinanases and galactanase did not increase the extraction of uronides. Principal component analysis indicated that the effects of the* endo-*β-(1,4)-glucanases were related to degradation of a fucogalactoxyloglucan, and emphasized the specificity of the action of one of the* endo-*β-(1,4)-glucanases.*

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INTRODUCTION

In plant cell walls, cellulose microfibrils are embedded in a complex matrix of pectins and hemicelluloses. Pectins have a key role in the structure and properties of the cell wall. Pectic material in the plant cell wall can be divided roughly into two parts — 'soluble' pectin (extractable by non-degradative treatments such as water, buffers or chelating agents) and 'non-soluble' pectin or protopectin (Pilnik & Voragen, 1970). Most of the research on apple pectin focused on the soluble pectin, the structure of which has been studied in detail by de Vries (1983). Less is known about the residual pectic material, notably about what makes it insoluble.

The aim of our work is to characterize the pectic material remaining after extraction of the soluble pectins and study its connections with the other cell-wall polymers. Results of chemical extraction of protopectin using degradative procedures were described in an earlier paper (Renard *et al.*, 1990*a*). Diluted alkalis were the most efficient for extraction of protopectin. Though the neutral sugars/uronic acid ratio in the pectins varied widely with the different extractions, the relative proportions of the neutral sugars were fairly stable. The extracts obtained with the alkalis showed two fractions, a high-molecular-weight fraction rich in neutral sugars ('hairy' regions), and an almost pure pectate of lower molecular weight.

Another possibility for extracting the non-soluble pectin is to use pure enzymes to degrade specifically given types of linkages. The use of non-pectolytic enzymes might give access to an unmodified rhamno-galacturonic backbone, whereas pectolytic enzymes degrade it in a defined way. Enzymes have been used to study the structure of the cell walls from apples (Knee *et al.*, 1975; Voragen *et al.*, 1980; Thibault *et al.*, 1988) and other plants (Bauer *et al.*, 1973; Keegstra *et al.*, 1973; Talmadge *et al.*, 1973; Ishii, 1981, 1982; Jarvis *et al.*, 1981; Konno & Yamasaki, 1982; Saulnier & Thibault, 1987; Massiot & Thibault, 1989). Most of these authors used pectolytic enzymes, some of them glucanases (Bauer *et al.*, 1973; Knee *et al.*, 1975; Voragen *et al.*, 1980; Massiot & Thibault, 1989), arabinanases (Knee *et al.*, 1975; Thibault *et al.*, 1988), galactanases (Knee *et al.*, 1975; Jarvis *et al.*, 1981; Thibault *et al.*, 1988) or xylanases (Massiot & Thibault, 1989). The main problem in this approach is the difficulty of obtaining pure enzymes in order to get unambiguous results. This paper describes the degradation of apple cell walls free from soluble pectins by pure enzymes (pectin-lyase, polygalacturonase, pectinesterase, various *endo*-β-(1,4)-glucanases,

exo-β-(1,4)-glucanase, arabinofuranosidase, *endo*-α-(1,5)-arabinanase, *endo*-β-(1,4)-galactanase and *endo*-β-(1,4)-xylanase), alone and in combination, and the chemical characterization of the residual part of the cell wall.

EXPERIMENTAL

Plant material

Apples (*Malus malus* L., Rosaceae, var. Golden Delicious) were obtained from the Sprenger Institute (Wageningen, The Netherlands). They were harvested in 1986 and had been stored in a controlled atmosphere until mid-December 1986. They were then removed and stored at ambient conditions until mid-January 1987.

Partially depectinated cell wall material

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

Enzymes

Arabinofuranosidase (α-L-arabinofuranosidase B; E.C. 3.2.1.55; Arafase) and *endo*-arabinanase (1,5-α-L-arabinan arabinanohydrolase; E.C. 3.2.1.99; Endo Ara) were purified from a preparation of *Aspergillus niger* (Pectinase 29, Gist-Brocades, Delft, The Netherlands) as described by Rombouts *et al.* (1988).

Endo-galactanase (1,4-β-D-galactan galactanohydrolase; E.C. 3.2.1.89; Endo Gal) was isolated from the above preparation as described by van de Vis *et al.* (in press). The enzyme was ultimately purified by fast protein liquid chromatography (FPLC) on a MonoQ (Pharmacia, Uppsala, Sweden) column eluted at pH 5 by a NaCl gradient from 0 to 1 M in 0·02 M piperazine buffer.

Two *endo*-glucanases (1,4-β-D-glucan glucanohydrolases; E.C. 3.2.1.4; Endo Glu I and Endo Glu IV) and one *exo*-glucanase (1,4-β-D-glucan cellobiohydrolase; E.C. 3.2.1.91; Exo Glu III) were purified from a commercial preparation from *Trichoderma viride* (Maxazyme Cl, Gist-Brocades, Delft, The Netherlands), as described by Beldman *et al.* (1985).

Endo-xylanase (1,4-β-D-xylan xylanohydrolase; E.C. 3.2.1.8; Endo Xyl), was isolated from *Aspergillus awamori* as described by Kormelink *et al.* (1989) (Endo III).

Endo-pectin lyase type II (poly(methyl)-α-D-galacturonide lyase, E.C. 4.2.2.10; PL) was purified from a commercial preparation of *A. niger* (Ultrazym 100, Ciba-Geigy AG, Basel, Switzerland), as described by van Houdenhoven (1975).

Endo-polygalacturonase (poly(1,4-α-D-galacturonide)-glycanohydrolase, E.C. 3.2.1.15; PG) was isolated from a preparation of *Kluyveromyces fragilis* as described by Versteeg (1979).

Pectin methylesterase (pectin pectylhydrolase, E.C. 3.1.1.11) (PE) was purified from a culture broth of *Aspergillus niger* as described by Baron *et al.* (1980).

All enzyme activities were measured against their substrates at pH 5 and 30°C and are expressed in nkat. One nkat is the amount of enzyme that degrades 1 nmd of linkages per second.

Secondary activities

Secondary activities were detected by incubating the enzymes under the conditions used for the extraction of pectins (same enzyme concentrations, sodium succinate/succinic acid buffer 0·05 M pH 4·5, 40°C, 24 h) in presence of 0·1% of the appropriate substrates. The liberation of reducing end groups from polysaccharide substrates was detected by the Nelson-Somogyi test (Spiro, 1966). The substrates were: microcrystalline cellulose (Avicell type SF, Serva, Heidelberg, BRD); carboxymethylcellulose (CM cellulose type Akucell AF 0305, Akzo, Arnhem, The Netherlands); phosphoric acid-swollen cellulose prepared according to Wood (1971); potato β-(1,4)-galactan (type I galactan) and apple arabinan prepared as described by Rombouts *et al.* (1988); xylan from oat spelts (Koch-Light, Colnbrook, Bucks, UK) and polygalacturonic acid (I.C.N., Cleveland, Ohio, USA). Hydrolysis of para-nitrophenyl-glycosides (Koch-Light, Colnbrook, Bucks, UK) was measured spectrophotometrically at 400 nm in glycine buffer pH 9. The presence of PL activity was tested on pectin of degree of methylation 93% (van Deventer-Schriemer & Pilnik, 1976) followed by high performance gel-permeation chromatography (HPGPC) on combined Biogel TSK 40XL, 30XL and 20XL columns with a guard column (Bio-Rad Laboratories, Richmond, California, USA) eluted by acetate buffer 0·4 M pH 3 at 30°C, at a flow rate of 0·8 ml/min. The eluent was monitored by a Shodex SE61 refractive index detector.

Treatment of apple CDTAIR with enzymes

A suspension of CDTAIR (50 mg in 5 ml of previously sterilized sodium succinate buffer 0·05 M pH 4·5) was incubated with enzymes for 24 h at 40°C with end-over-end mixing. The amounts of enzymes added were calculated to degrade theoretically the corresponding polymer present in 24 h under standard conditions. However, Endo Ara and Endo Xyl, available in minute amounts, were used at half the amount so calculated. PG was used at the same concentration alone and with PE, regardless of the degree of methylation. After incubation, the suspension was centrifuged for 10 min at 3000 ***g***. The enzymes were inactivated by boiling the supernatants for 5 min. The pellets were washed three times with distilled water and freeze-dried. A blank was made under exactly the same conditions.

Analytical methods

The galacturonic acid (GalA) and total neutral sugars (NS) concentrations were measured by automated meta-hydroxy diphenyl (Thibault, 1979) and orcinol assays (Tollier & Robin, 1979), respectively. Corrections were made for the mutual interferences. Individual neutral sugars were analyzed as their alditol acetate derivatives by gas chromatography. Derivatization was carried out according to Englyst and Cummings (1984) after prehydrolysis by 13 M H_2SO_4 for 1 h followed by hydrolysis by 1 M H_2SO_4 at 100°C for 3 h.

Mathematical methods

Principal component analysis (PCA) was performed using software based on the programs of Foucart (1982) as described by Renard *et al.* (1987). Multidimensional analyses, such as PCA, are mathematical methods that produce simple representations of large data sets with minimum loss of information. They describe variation in multidimensional data by a few synthetic variables. These synthetic variables are linear combinations of the original variables and have the advantage of having no correlation with each other. In PCA, the synthetic variables are defined as the eigenvectors of XX', where X is the centered data table and X' is the transposed matrix. Variances along each axis are the eigenvalues of XX'. Percentages of total variance, which are the ratio (in percent) of the corresponding eigenvalues to the total variance, are commonly used to characterize the importance of the axes. PCA is a purely descriptive method, in which there is no transformation of the data and

no assumption is made on the relative importance of the measurements. This technique was used to draw maps of the compositions of the residues.

RESULTS

Enzyme activities and experimental conditions

All the enzymes used in this study were shown previously to be electrophoretically pure. Secondary activities of the enzymes were determined because they were used at high concentrations for long treatments. Presence of trace activities, notably pectolytic activities, could modify the results drastically. As an example, in a preliminary experiment (results not shown) an Endo Ara preparation extracted almost all the uronides. However, this preparation contained traces of PL activity, which could not be detected by spectrophotometry at 325 nm but only by its effect on the HPGPC pattern of high methoxyl pectin.

Most of the enzymes showed no secondary activities. The Endo Ara had a slight activity on potato galactan, which was not due to galactanase activity but to the persistence of apparently linear arabinan in that substrate (Rombouts *et al.*, 1988). The Arafase was active both on *p*-nitro-phenyl-arabinose and arabinan: its main activity is to split terminal 1,3-α-L- or 1,2-α-L-linked arabinose substituents from the backbones of polysaccharides, but it is also active on *p*-nitro-phenyl-arabinose and small arabinose oligomers, and is even able to free arabinose from longer 1,5-α-L-linked chains (Voragen *et al.*, 1987). Endo Glu I and Endo Glu IV differed by their activity on xylans: Endo Glu I is only active on glucans, whereas Endo Glu IV will also hydrolyze xylans (Beldman *et al.*, 1985). The *exo*-glucanase Exo Glu III was chosen because it showed the highest synergistic action with the *endo*-glucanases (Beldman *et al.*, 1985). The *endo*-xylanase had no glucanase activity.

The extraction limits were always reached in less than 24 h for all the enzymes, and an incubation time of 24 h was chosen. Enzymes were added simultaneously rather than sequentially in order to take full advantage of eventual synergisms.

Yields of soluble products

The yields obtained using the various enzymes and enzyme combinations are given in Fig. 1. The reproducibility was good ($< 5\%$) and the

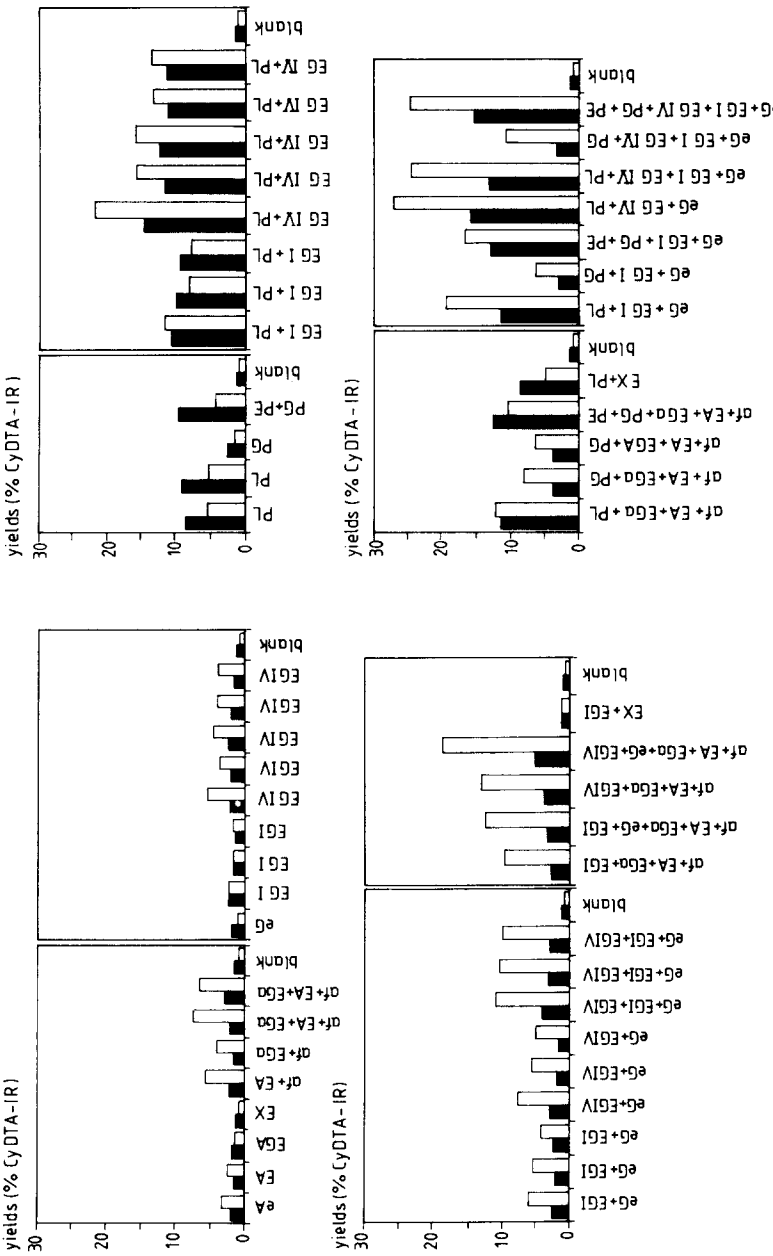

Fig. 1. Percent CDTAIR extracted by the enzymes and enzymes combinations. ■: Galacturonic acid; □: neutral sugars. af, arabinofuranosidase; EA, *endo*-arabinanase; EGa, *endo*-galactanase; EX, *endo*-xylanase; eG, *exo*-glucanase; EG I, *endo*-glucanase I; EG IV, *endo*-glucanase IV; PL, pectin-lyase; PG, polygalacturonase; PE, pectinesterase.

repeatability acceptable. Some uronides and neutral sugars (6% of the uronides and 1% of the neutral sugars) were extracted by the buffer alone. When used individually, the non-pectolytic enzymes mostly had very limited effects. Endo Xyl had no significant effect. Endo Glu IV, Arafase, Endo Ara, Endo Glu I, Endo Gal and Exo Glu freed small amounts of neutral sugars. The maximum degradation was obtained with Endo Glu IV with about 6% of the neutral sugars. The synergisms between *exo*- and *endo*-enzymes were clearly visible for the arabinan-degrading enzymes and for the glucanases. The synergism between Arafase and Endo Gal was lower than between Arafase and Endo Ara. The addition of Exo Glu to either Endo Glu I or Endo Glu IV induced a marked rise in the amount of neutral sugars liberated (respectively, 8·0 and 9·5% of the neutral sugars). This effect was higher with Endo Glu I, as the degree of synergism (defined as the ratio of the neutral sugar concentration obtained with the combined enzymes to the sum of the neutral sugar concentrations obtained by each enzyme) was 2·8 with Endo Glu I versus 1·3 with Endo Glu IV. All these enzymes and their combinations extracted only a small proportion of the uronides. Arafase + Endo Ara + Endo Gal extracted 12·5% of the uronides and Exo Glu + Endo Glu I + Endo Glu IV 16·5%, versus 6% in the blank. More uronides were liberated with glucanases than with arabinanases and galactanase. The PL and the combination PG + PE (Table 1) freed large proportions of the uronides (44% and 47·5%), while the PG had a limited effect (extraction of 11·5% of the GalA). The pectolytic enzymes also extracted neutral sugars. The combination PG + PE liberated less neutral sugars (6·5%) than PL (8·5%). When non-pectolytic enzymes were combined with the PL or PG + PE more uronides and more neutral sugars could be extracted. This was particularly striking with the cellulases. The highest yields of uronides (65%) were obtained with the combination Endo Glu IV + PL; 25·5% of the neutral sugars were also extracted by this combination. The amounts extracted showed the existence of a synergism between pectolytic enzymes and *endo*-glucanases for degradation of the CDTAIR, especially with Endo Glu IV. The increase was more marked for the neutral sugars. The addition of Exo Glu led to a further increase of the yields of both uronides (75%) and neutral sugars (43%). With the arabinanases and galactanase all these effects were lower: Arafase + Endo Ara + Endo Gal + PL led to the solubilization of 56% of the uronides and 19% of the neutral sugars, Arafase + Endo Ara + Endo Gal + PG + PE solubilized 62% of the uronides and 16% of the neutral sugars. With the xylanase no difference was detected. When a mixture of pectolytic enzymes, cellulases, arabin-

anases and galactanases was used, the supernatants contained little more neutral sugars and no more galacturonic acid than without the arabinanases and galactanases. The PG when used without PE produced very limited effects in the combinations.

Composition of the residues

Yields of the residues have not been measured systematically due to difficulties in recovering quantitatively the insoluble material. The sugar compositions of the residues (Tables 1 and 2) combined with the data for the yields of soluble material allow a number of conclusions to be drawn.

The arabinanases and galactanase gave residues where the proportion of all the sugars except arabinose and galactose were slightly increased. This effect was more marked with the combinations Arafase + Endo Ara, Arafase + Endo Gal and Arafase + Endo Ara + Endo Gal. The combination Arafase + Endo Ara + Endo Gal extracted a higher proportion of arabinose than of galactose. The residues from Exo Glu and Endo Xyl did not show any significant difference from the blanks. Endo Glu IV gave residues where the proportions of glucose surprisingly were not diminished. Higher proportions of fucose and xylose had been extracted, as well as a little galactose. The proportions of uronides, arabinose, rhamnose and mannose were increased. Endo Glu I led to a decrease of the mannose content; the composition of the residue was otherwise very similar to that of the blank. Addition of Exo Glu increased the degradation of the cellulose as can be seen from the decrease of the proportion of glucose in the residues (Exo Glu + Endo Glu I 37·7 mol%, Exo Glu + Endo Glu IV 39·5 mol%). The residues from PL and PG + PE action contained less uronides but also less arabinose, galactose and rhamnose. The neutral sugars that had been extracted were thus mostly arabinose and galactose. The residues from PL action contained less uronides than those from PG + PE action (respectively, 6·6 and 10·3 mol%). The residue from PG was little different from the blank, showing only a slight decrease of the uronide content. The Rha/GalA ratio was increased in the residues (blank 1/15, PL 1/6 and PG + PE 1/9). The residues obtained after the action of Arafase + Endo Ara + Endo Gal + PL or PG + PE contained a still lower proportion of arabinose and galactose. However, more marked effects were obtained with Endo Glu IV + PL, which gave a residue with an important enrichment in mannose (3·7 mol%) and glucose (73 mol%). The uronides as well as arabinose, galactose, rhamnose, fucose and xylose had been solubilized for the most part. The addition of Exo Glu led to

TABLE 1

Enzymatic Treatment of Apple Cell Walls with Pectolytic Enzymes and Combinations of Pectolytic and Non-pectolytic[a] Enzymes: Yields and Sugar Composition of the Residues

Samples	*Yields residue (% CDTAIR)*	*Composition of the residues (mol%)*							
		Rha	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*
PL	73	1·2	2·0	13·6	10·7	2·6	10·4	52·9	6·6
PG	nd	1·5	2·1	15·5	9·2	2·0	10·6	44·5	15·0
PG + PE	nd	1·2	2·0	14·1	10·2	2·2	10·9	49·1	10·3
Arafase + Endo Ara + Endo Gal + PL	nd	1·3	2·3	6·7	12·3	2·9	7·0	60·7	6·8
Arafase + Endo Ara + Endo Gal + PG	68	1·4	1·9	9·4	10·5	2·3	8·0	49·0	17·5
Arafase + Endo Ara + Endo Gal + PG + PE	nd	1·4	2·2	8·7	11·9	2·8	7·5	56·9	8·6
Endo Xyl + PL	74	0·9	1·9	13·7	10·3	2·5	10·3	52·4	8·0
Endo Glu I + PL	67	0·9	1·9	11·9	9·8	2·1	8·8	57·9	6·6
Endo Glu IV + PL	48	0·4	1·4	5·3	7·8	3·7	5·8	73·2	2·4
Exo Glu + Endo Glu I + PL	nd	1·6	2·0	16·8	12·0	2·3	10·6	48·5	6·2
Exo Glu + Endo Glu I + PG	nd	1·7	1·7	18·5	9·2	1·8	12·1	37·6	17·4
Exo Glu + Endo Glu I + PG + PE	nd	1·5	1·8	17·1	11·0	2·0	12·8	42·8	11·0
Exo Glu + Endo Glu IV + PL	nd	0·7	1·7	6·9	8·7	5·0	6·2	68·1	2·7
Exo Glu + Endo Glu I + Endo Glu IV + PL	nd	1·8 #		8·0	7·7	4·5	7·0	65·6	5·4
Exo Glu + Endo Glu I + Endo Glu IV + PG	nd	1·8	1·2	20·1	7·1	2·1	12·2	34·6	20·9
Exo Glu + Endo Glu I + Endo Glu IV + PG + PE	nd	1·4	1·5	15·5	9·3	3·5	11·4	53·0	4·4
Endo Glu I + Endo Glu IV + PG	nd	1·4	1·5	17·1	7·2	1·8	10·2	44·0	16·8
Endo Glu I + Endo Glu IV + PG + PE	nd	1·0	1·4	12·4	7·6	2·4	9·8	56·7	8·7
Arafase + Endo Ara + Endo Gal + Endo Glu I + PG	nd	1·6	1·9	9·7	10·2	2·0	7·7	49·6	17·3
Arafase + Endo Ara + Endo Gal + Endo Glu I + PG + PE	nd	1·4	2·3	8·9	10·6	2·1	7·0	57·7	10·0
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I + PL	nd	1·7	2·1	9·2	13·1	3·0	7·3	54·9	8·7
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I + PG	nd	2·0	1·9	10·9	11·1	2·4	8·2	44·1	19·4
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I + PG + PE	nd	1·7	2·0	12·4	12·7	2·4	8·1	49·2	11·5

[a]nd, not determine; #, rhamnose and fucose not separated.

TABLE 2
Enzymatic Treatment of Apple Cell Walls with Non-pectolytic Enzymes: Yields and Sugar Composition of the Residues[a]

Samples	*Yields residue (% CDTAIR)*	*Composition of the residues (mol%)*							
		Rha	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*
CDTAIR	100	1·3	1·7	15·3	8·5	1·9	10·3	38·9	22·1
Blank	91	1·2	1·7	16·0	8·8	2·0	10·6	42·0	17·7
Arafase	nd	1·2	1·7	14·7	9·5	1·9	10·8	42·6	17·6
Endo Ara	nd	2·8 #		12·3	9·3	2·4	11·1	42·9	19·2
Endo Gal	nd	2·7 #		15·9	9·5	2·2	9·2	42·0	18·5
Endo Xyl	90	1·2	1·6	16·3	8·8	1·9	10·6	41·6	18·0
Arafase + Endo Ara	nd	1·7	2·1	9·8	9·7	1·9	11·7	46·2	16·9
Arafase + Endo Gal	nd	1·5	1·9	14·7	9·5	1·9	8·1	44·6	17·8
Arafase + Endo Ara + Endo Gal	82	1·2	1·8	9·6	10·0	2·2	8·9	47·3	19·0
Exo Glu	nd	2·8 #		15·7	9·2	2·3	22·2	41·0	17·9
Endo Glu I	87	1·1	1·4	16·7	9·0	1·9	11·2	40·9	17·8
Endo Glu IV	82	1·1	1·1	16·9	6·9	2·2	10·4	41·6	19·8
Exo Glu + Endo Glu I	81	1·2	1·4	17·7	8·4	1·8	11·5	37·7	20·3
Exo Glu + Endo Glu IV	79	1·4	1·0	17·9	6·9	2·3	11·3	39·5	19·7
Exo Glu + Endo Glu I + Endo Glu IV	nd	1·8	1·0	20·2	6·7	2·1	12·1	34·9	21·2
Arafase + Endo Ara + Endo Gal + Endo Glu I	nd	1·9	1·8	9·9	10·5	2·4	7·7	47·9	17·9
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu I	nd	2·0	1·8	11·0	10·9	2·3	8·0	43·0	21·0
Arafase + Endo Ara + Endo Gal + Endo Glu IV	nd	1·3	1·3	6·9	8·7	2·7	7·6	51·4	20·1
Arafase + Endo Ara + Endo Gal + Exo Glu + Endo Glu IV	nd	2·3	1·5	8·5	9·6	3·5	7·2	47·8	19·6

[a]nd, not determined; #, rhamnose and fucose not separated.

some decrease of the glucose content as this enzyme increased the degradation of the cellulose. With Endo Glu I+PL or PG+PE, the effects were much lower.

Mathematical treatments

Principal component analysis was used in order to have a global view of the data. An intuitive approach to PCA is to see a sample characterized by N-measurements as a point in an N-dimensional space. Similarly, a set of samples forms a multidimensional volume in that space. Each dimension then corresponds to a measurement. Some of these measurements, however, may be correlated or may be constant. The resulting redundancy can be eliminated by creating a more suitable system of axes. The first improvement is to place the origin of the axes at the center of the multidimensional volume, eliminating the data that do not vary significantly. A rotation of axes is then performed to put the first axis (first principal component) in the largest dimension of the volume (largest variation). The second component is chosen to be orthogonal to (i.e. uncorrelated with) the first one, taking the greatest width into account. Subsequent components are calculated in the same way, each at right angles to (non-correlated with) all the preceding components and adjusted to the largest remaining dimension. The coordinates from the samples in that new system of axes are obtained by projection on the new axes (principal components); similarity maps can be drawn by selecting two of the new axes and associating to the samples their coordinates on these axes, i.e. projecting the samples on that plane. PCA was performed using the composition of the residues and the yields of uronides and neutral sugars. One of the first steps of PCA is the calculation of the correlation coefficients (r) between the original variables (Table 3). Some of those correlations could be attributed to the structure of the set of samples, for example the strong negative correlation ($r=-0{\cdot}91$) between the uronides concentration in the extracts and the proportion of uronides in the residues. Some correlations, however, showed relationships between the data which could not be seen on the raw results, notably the high correlation between fucose and xylose proportions ($r=0{\cdot}85$), which indicated that these sugars were extracted together. The correlations between these sugars and glucose were low ($r=0{\cdot}20$ and $0{\cdot}17$), emphasizing the facts that the *exo*-glucanase was necessary for extraction of important amounts of glucose, while those by the *endo*-glucanases alone were low. Arabinose, galactose and uronides proportions had strong negative correlations with the glucose (respectively $r=-0{\cdot}85$, $-0{\cdot}83$ and $-0{\cdot}89$), showing the existence of an opposition

TABLE 3
Principal Component Analysis: Correlation Coefficients between the Yields and the Sugar Composition of the Residues of Enzymatic Extraction

	Yields		*Sugars in the residues (mol%)*							
	NS[a]	*GalA*	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*
Yields										
Neutral sugars	1									
GalA	0·77	1								
Sugars in the residues										
Rha	0·01	−0·32	1							
Fuc	0·02	0·24	0·25	1						
Ara	−0·57	−0·59	0·28	−0·35	1					
Xyl	0·14	0·32	0·32	0·85	−0·37	1				
Man	0·76	0·72	−0·36	−0·08	−0·69	0·00	1			
Gal	−0·59	−0·57	0·26	−0·25	0·89	−0·25	−0·7	1		
Glc	0·61	0·82	−0·56	0·20	−0·85	0·17	0·79	−0·83	1	
GalA	−0·53	−0·91	0·51	−0·30	0·60	−0·30	−0·68	0·57	−0·89	1

[a]NS, neutral sugars.

between extractable and resistant sugars (glucose and mannose) in the system of enzymes used. The first principal component (55·8% of the variation of the data) was strongly correlated to the glucose and mannose contents of the residues ($r = 0{\cdot}92$ and $0{\cdot}72$) and to the uronide concentration in the extracts ($r = 0{\cdot}79$), and negatively correlated to uronides, arabinose and galactose contents ($r = -0{\cdot}77$, $-0{\cdot}75$ and $-0{\cdot}71$). The second principal component (20·8% of the variation) was defined essentially by the xylose and fucose contents ($r = 0{\cdot}80$ and $0{\cdot}78$). The plane defined by these two principal components is shown in Fig. 2. It opposed along axis 1 the samples treated by *endo*-glucanases and *endo*- plus *exo*-glucanases (where the glucose content and in the case of Endo Glu I the mannose content were lower) to the samples that had been extensively degraded (samples treated by pectolytic enzymes combined to Endo Glu IV or to Arafase + Endo Ara + Endo Gal, eventually plus

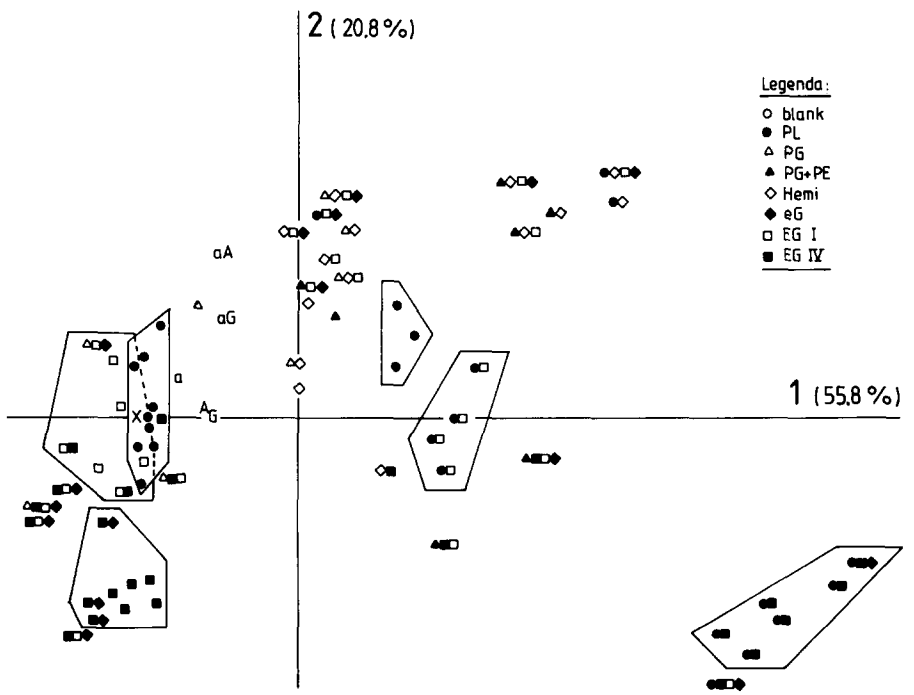

Fig. 2. Principal component analysis: similarity map defined by principal components 1 (55·8% of the variance) and 2 (20·8% of the variance). a, Arabinofuranosidase; A, *endo*-arabinanase; G, *endo*-galactanase; aA, arabinofuranosidase + *endo*-arabinanase; aG, arabinofuranosidase + *endo*-galactanase; Hemi, arabinofuranosidase + *endo*-arabinanase + *endo*-galactanase; X, *endo*-xylanase; eG, *exo*-glucanase; EG I, *endo*-glucanase I; EG IV, *endo*-glucanase IV; PL, pectin-lyase; PG, polygalacturonase; PE, pectinesterase.

glucanases). Along axis 2 there was a separation between the samples treated with Endo Glu IV, which extracted most efficiently fucose and xylose, and all the other samples. Some clear groupings were visible. One striking feature was the extreme position of the samples treated by Endo Glu IV + PL. The samples treated by Endo Glu I + PL were in an intermediate position but closer to those treated by PL alone, showing the existence of the same type of effects but less pronounced. The samples treated by combinations of Arafase + Endo Ara + Endo Gal and pectolytic enzymes were not as far along axis 1 as those treated by Endo Glu IV + PL: the highest synergism in degradation of the cell wall and more specifically extraction of the uronides was obtained not with the arabinanases and galactanase but with *endo*-glucanases, more specifically, Endo Glu IV. Samples treated by isolated enzymes (except PL and Endo Glu IV) were close to the blanks, showing again that isolated enzymes had had limited effects.

DISCUSSION

All the enzymic extractions were carried out at pH 4·5. At this pH the solubilization in the blanks was minimal and the polysaccharidases were active. Pectate-lyase was not used in this study, in spite of its efficiency in extracting uronides from cell walls (Konno & Yamasaki, 1982), because of its high optimum pH. The optimal pH range of this enzyme is such that there is spontaneous degradation of the pectins by β-elimination, and the other polysaccharidases are not active.

The amounts released without enzymes were lower than those reported by Voragen *et al.* (1980), who extracted in the controls 14·6% of the galacturonides and 6·8% of the neutral sugars from apple water insoluble solids (WIS), and 27% of the galacturonides and 3·8% of the neutral sugars from apple alcohol insoluble solids (AIS). One reason could be that these authors used substrates that were not free from 'soluble' pectins. Knee *et al.* (1975), using buffer-extracted (at pH 7·5) cell wall material, found that only negligible amounts of polysaccharides were released after incubation at pH 4 or 7 (at 20°C) for 72 h in the absence of enzymes. In our case the extraction limit was reached in the blank in the initial stages of the incubation, suggesting that some molecules were liberated by the mechanical effects of the grinding of the CDTAIR. The maximum degradations were of a lesser extent than reported by Voragen *et al.* (1980) or Knee *et al.* (1975) but these authors used partially purified enzymes on cell wall material that had not been freed of soluble pectins prior to enzyme action. The amounts of enzymes we used were

low, due to the difficulties in obtaining sufficient amounts of pure enzymes, although the degradation limits were reached in 24 h.

Non-pectolytic enzymes had limited actions on the apple cell wall. The cellulases extracted less neutral sugars than mentioned by Voragen *et al.* (1980) from apple AIS (19·8%) and WIS (42·6%). No comparison could be made with the data reported by Knee *et al.* (1975), as they applied to deesterified apple cell walls a cellulase preparation that they reported to be contaminated with PG. The action pattern of the cellulases was similar to that obtained by Massiot and Thibault (1989) on carrot cell walls. The main difference was the absence of action of Exo Glu alone, but its specificity was slightly different from that of the cellobiohydrolase fom *Trichoderma reseei* used by these authors, which had some *endo*-activity (Henrissat *et al.*, 1985). Composition of the residue of Endo Glu IV and results from PCA pointed to degradation of a fucogalactoxyloglucan, such as reported in apple cell walls by Aspinall and Fanous (1984), Stevens and Selvendran (1984) and Voragen *et al.* (1986), rather than of cellulose itself. Voragen *et al.* (1980) have reported extraction of fucose, xylose, mannose and galactose from apple cell walls by a partially purified glucanase preparation devoid of pectolytic activities. It is known that, in the absence of *exo*-glucanase, *endo*-glucanases will degrade preferentially the xyloglucans (Hayashi *et al.*, 1984).

The combination Arafase + Endo Ara + Endo Gal freed less neutral sugars (11%) than the cellulases (Exo Glu + Endo Glu I + Endo Glu IV: 16%). This could be due (i) to the very composition of the CDTAIR, especially its high glucose content and relatively low arabinose and galactose content, (ii) to the fact that these enzymes constitute *per se* a less efficient synergistic system than the *endo*- and *exo*-glucanases, and (iii) to the structure of apple arabinans and arabinogalactans. These arabinans and arabinogalactans are highly branched, include some (1,3)/(1,6)-galactans (type II galactans) and may carry such substituents as arabinopyranose that hinder action of the enzymes (de Vries *et al.*, 1983).

The very limited pectin-solubilizing effect of the arabinanases and galactanase does not agree with the idea that pectin is linked to other cell-wall polymers via arabinose and galactose side-chains. Thibault *et al.* (1988) also reported failure to extract pectin from apple marks using a *Bacillus subtilis* preparation containing *endo*-arabinanase and *endo*-galactanase activities.

An enzyme formulation active on highly esterified pectins was necessary to extract uronides from CDTAIR. The low action of PG was due to the high degree of methylation (70%) (Renard *et al.*, 1990*a*) of the pectin present in the CDTAIR and not to acetylation of the pectin or an acces-

sibility problem, such as mentioned by Knee *et al.* (1975) and Jarvis *et al.* (1981) with PG from *Phytophtora infestans*, as the combination PG + PE was highly active. PG extracts high proportions of pectins only from deesterified apple cell walls (Knee *et al.*, 1975; Voragen *et al.*, 1980).

The neutral sugars that were extracted by pectolytic enzymes were probably present as side chains of the pectins. Composition of the residues and the strong positive correlations between arabinose, galactose and uronides contents of the residues show that these sugars were mostly arabinose and galactose. To be extractable by pectolytic enzymes alone, these side-chains had to be linked in the cell wall only to the rhamnogalacturonic backbone. This supports the idea that arabinans and arabinogalactans side chains are for a great part unconnected to any other cell wall polymer. Addition of the arabinanases and galactanase led to little increase in the amounts of uronides extracted, which again suggests a limited role for type I arabinogalactans in the connection between pectins and other cell wall polysaccharides.

Degradation of the cell wall increased when glucanases were combined with the pectolytic enzymes. This effect is partly due to the existence of mutual protection of the cellulose and the pectin toward enzymic attack (Voragen *et al.*, 1980; Ben-Shalom, 1986; Massiot & Thibault, 1989). Composition of the residue of Endo Glu IV + PL, however, indicated that other sugars were more solubilized than glucose. The extreme position of the residues from the action of Endo Glu IV + PL on the similarity map emphasizes the existence of a special effect of that *endo*-glucanase. When used alone Endo Glu IV extracted more specifically high proportions of xylose and fucose. It thus seems that this strong effect on uronides extraction is related to an action on the (fuco)xyloglucan, and not on xylans as (i) the *endo*-xylanase has little or no effect and (ii) if Endo Glu IV acted on xylans, fucose would not be extracted at the same time and in similar proportions. The xyloglucans thus seem to play an important role in the connection between pectins and other cell wall polymers, notably cellulose. The next step to confirm these results is the study of the extracts. This will be described in the accompanying paper (Renard *et al.*, 1990*b*).

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