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ENZYMIC ANALYSIS OF CELL WALL STRUCTURE IN APPLE FRUIT CORTICAL TISSUE

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Abstract—Galactanase from *Phytophthora infestans* and an arabinosidase isoenzyme from *Sclerotinia fructigena* attacked the cortical cell walls of apple fruits liberating galactose and arabinose residues, respectively. Other arabinosidase isoenzymes from *S. fructigena* attacked cell walls very slowly. A *S. fructigena* polygalacturonase isoenzyme liberated half of the uronic acid residues with few associated neutral residues, while a second polygalacturonase isoenzyme released more uronic acid with a substantial proportion of arabinose and galactose and lesser amounts of xylose, rhamnose and glucose; reaction products of this enzyme could be further degraded by the first isoenzyme to give high MW fragments, rich in arabinose with most of the xylose, rhamnose and glucose, and low MW fragments rich in galactose and uronic acid. Endoglucanase from *Trichoderma viride* released a small proportion of the glucose residues from cell walls together with uronic acid, arabinose, xylose and galactose; more extensive degradation occurred if walls were pre-treated with the second polygalacturonase isoenzyme. Endoglucanase reaction products were separated into a high MW fraction, rich in arabinose, and lower MW fractions rich in galactose and glucose residues. The high MW polygalacturonase and endoglucanase products could be degraded with an arabinosidase isoenzyme to release about 75% of their arabinose. Cell walls from ripe fruit showed similar susceptibility to arabinosidase and galactanase to those from unripe apples. Cell walls from fruit, ripened detached from the tree were more susceptible to degradation by polygalacturonase than walls from unripe fruit or fruit ripened on the tree. Endoglucanase released less carbohydrate from ripe fruit cell walls than from unripe fruit cell walls.

INTRODUCTION

Chemical degradation of apple fruit cell walls to yield fragments, which were separated by ion exchange chromatography and gel filtration and analysed for their monomeric composition, has given a partial account of their structure [1]. Use of purified enzymes to degrade sycamore suspension culture wall polymers, and methylation analysis of fragments have contributed to a model of plant primary cell wall structure [2–4]. The model embodies a pectic framework of α-1,4 linked galacturonic acid residues, interrupted by rhamnose residues, and having side chains of β-1,4 galactosyl residues and branched arabinan [2], which is also supported by other evidence [5]. The suggestion that xyloglucan is covalently linked at some point to this structure [3,4]

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is new, and it could explain the presence of glucose and xylose residues in purified pectic polymers, observed in apple [1] and other plant tissues. If the xyloglucan is hydrogen bonded to cellulose microfibrils [3] this could partly explain the insolubility of pectic polymers [1], as well as suggesting ways in which wall extensibility could be controlled [4]. The model includes linkage of polygalacturonide to wall protein through arabino–galactan attached to seryl residues [4]. Protein–polysaccharide linkages in apple cell walls will be considered in a later paper [6].

This paper describes the use of purified glycanases in structural analysis of apple fruit cell walls. The enzymes were used firstly on walls from unripe fruit and, secondly, on walls from apples ripened on and off the tree. Previous work has shown that wall composition changes in different ways in these two kinds of fruit [7]. Most of the enzymes used were produced by plant pathogens grown in liquid culture; the enzymes used were pectinesterase (PE, pectin–pectyl hydrolase, E.C. 3.1.1.11), (endo-) galactanase, β-galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.23), β-glucosidase (β-D-glucoside glucohydrolase, E.C. 3.2.1.21), α-L-arabinofuranosidase (AF, α-L-arabinofuranoside arabinohydrolase, E.C. 3.2.1.55), polygalacturonase (PG, polygalacturonide glycanohydrolase, E.C. 3.2.1.15), pectin lyase [poly (methoxygalacturonide) lyase, E.C. 4.2.2.10] and (endo-) glucanase (β-1, 4-glucan 4-glucanhydrolase, E.C. 3.2.1.4). Where isoenzymes were purified by iso-electric focusing they are identified in the text by their iso-electric points (e.g. arabinosidase pI 6·5).

RESULTS

Composition of substrates

The residue from Me_2CO and buffer extraction of cortical tissue from Cox's Orange Pippin apples was used as a substrate for enzymes. A preparation from pre-climacteric fruit immediately after harvest (initial fruit cell wall material, ICW) was used most estensively. Further preparations were made from similar apples which had been allowed to ripen at 12° for 40 days (detached fruit cell wall material, DCW), or harvested 40 days later (attached fruit cell wall material, ACW). Analysis of the monosaccharide composition of these preparations gave results in agreement with previous findings [7], namely that galactose residues are lost during ripening and that insoluble polyuronide decreases in detached more than in attached fruit (Table 1). Estimation of neutral residues as their alditol acetates showed that rhamnose, fucose and mannose, whose TMSi ether derivatives were inseparable from major components on GLC, did not change during ripening. Analysis of monosaccharides in hydrolysates as alditol acetate and TMSi derivatives gave

Table 1. Composition of insoluble residues used as enzyme substrates

Component	Analytical method	Anhydro-sugar content (mg/g fr wt) of insoluble residue from fruit ICW	DCW	ACW
Rha	Alditol acetates	0·11	0·10	0·10
Fuc		0·09	0·08	0·08
Ara		2·68	2·00	2·10
Xyl		0·79	0·73	0·70
Man		0·32	0·32	0·24
Gal		3·78	0·75	1·08
Glc		29·30	4·44	5·62
Ara	TMSi ether	2·76	2·27	2·41
Xyl		0·85	0·87	0·86
Gal	TMSi ether	3·80	0·80	1·45
Glc		30·90	4·43	6·32
Glc as starch	TMSi ether	27·00	1·77	2·36
Total Glc–starch Glc	TMSi ether	3·90	2·66	3·96
Uronic acid	Colorimetric	3·90	2·51	3·62

ICW—initial cell wall material from preclimacteric apples, DCW—detached cell wall material from preclimacteric apples ripened at 12° for 40 day, ACW—attached cell wall material from apples allowed to ripen for 40 days on the tree.

similar results for major components, arabinose, xylose, galactose and glucose. Products of enzymic digestion of cell walls were usually analysed as TMSi derivatives since this has proved a more rapid and reproducible method. No attempt was made to remove the large quantity of starch associated with ICW prior to enzyme treatments. Because of difficulties of hydrolysis and derivative formation, polyuronide was estimated colorimetrically in hot neutral EDTA extracts after chloral hydrate treatment of residues. Although this was expected to give total uronide values [8] it was found that enzymes, acting in sequence, could liberate more uronide than this chemical degradation.

Assay of enzymic wall degradation

All enzymes, except pectinesterase, were incubated with cell wall suspensions under continuous agitation at 20° for 72 hr. The digests were filtered, material in the filtrate was hydrolysed and neutral monomers estimated as their TMSi ether derivatives. Polyuronide was estimated colorimetrically and the results for all residues usually expressed as a percentage of the TMSi neutral sugar and uronide results (Table 1). Products of enzymic degradation of cell walls were routinely hydrolysed so that all sugar residues released would be detected whether they were released as monomers or in a polymeric state. Reducing sugar in enzymic products was estimated by Somogyi's [9] modification of Nelson's [10] method to give an indication of the time course and extent of the reaction. In this method molar *A* decreases as the degree of polymerisation of the saccharide increases, so the estimations are an indication of the presence of monomers and small oligomers. Negligible quantities of polysaccharide were released from cell wall preparations during incubation at pH 4 or pH 7 for 72 hr in the absence of enzyme.

Pectinesterase

Titrimetric assay [11] of citrus pectinesterase [12] on ICW showed 100-fold less activity than on citrus pectin. Because of this, long incubations and large amounts of enzyme were used to prepare de-esterified cell walls. There was no release of reducing sugar during this treatment, though 7·7% of total polyuronide present in ICW had passed into the filtrate. Traces (<2% of total in ICW) of arabinose, xylose, galactose and glucose were also present in hydrolysates.

Galactan degrading enzymes

A partially purified galactanase from *Phytophthora infestans* [13] released reducing sugar from de-esterified ICW, and after 72 hr, 30% of total galactose in ICW and 4·3% of total arabinose were found in the filtrates after hydrolysis together with 4·8% of total uronic acid. The enzyme did not liberate reducing sugar from walls unless they had been pre-treated with PE, and it was thought that PG contaminating the galactanase could be necessary for its activity. After a second purification by gel filtration, PG could not be detected, but the action of the galactanase on de-esterified walls was unimpaired.

Large amounts of β-galactosidase from *E. coli* are needed to release galactose residues from glycoproteins [14], and it was thought unlikely that this enzyme would liberate galactose from insoluble substrates. Preliminary work with barley seed β-galactosidase [15] suggested that unrealistically large amounts of this enzyme would be required for effective action on cell walls. When de-esterified ICW was incubated with a commercial β-galactosidase preparation from *S. fragilis* a slow, linear release of reducing sugar occurred, which, by 10 days, accounted for 2% of total galactose. No reducing sugar was released from cell walls pre-treated with galactanase. In later experiments it was found that a crude β-glucosidase from sweet almonds, which is known to have β-galactosidase activity [16] released a similar proportion of galactose, estimated with galactose oxidase, from a wall preparation.

Arabinosidase

Three arabinofuranosidase isoenzymes produced by *S. fructigena* [17,18] were used. In early experiments with AF pI 6·5, uronic acid was detected in filtrates from incubations with ICW in amounts corresponding to more than 80% of the polyuronide present in the wall material. This was caused by contamination with pectin lyase which was partially removed by ion exchange chromatography on carboxymethyl cellulose equilibrated with 0·01 M acetate buffer (pH 4·8). The AF, which eluted first, released reducing

sugar from de-esterified ICW at a similar rate to the enzyme contaminated with lyase. Analysis of material in the filtrate showed that 46·8% of arabinose, 3·2% of uronic acid and 0·6% of the galactose residues in the wall material had been released. When untreated ICW was incubated with the enzyme 39·8% of arabinose, 23·1% of uronic acid and 2·0% of galactose was released from the wall. This suggested that some lyase activity was still present.

In later experiments advantage was taken of the affinity of pectin lyase for Sephadex gel filtration media. After passing a partially purified AF through Sephadex G-100 equilibrated with water the void fraction contained 0·0015 units of pectin lyase per unit of AF. This preparation released reducing sugar at similar rates from untreated and de-esterified walls, during a 6 hr incubation; there was no suggestion of an initial lag, which might be expected if lyase action were necessary to expose arabinosyl residues to attack by AF. Under similar conditions AF pI 4·5 released reducing sugar from de-esterified walls at 10% of the rate of AF pI 6·5.

An AF pI 3·0 preparation, contaminated with polygalacturonase, was incubated with de-esterified ICW. Analysis of the filtrate showed that only 2·9% of arabinose in the wall had been released though 16·4% of total polyuronide had been solubilized.

Polygalacturonate degrading enzymes

A polygalacturonase from *P. infestans* and two PG isoenzymes (pI 4·6 and 9·7) from *S. fructigena* [19] were tested for their wall degrading activity (Table 2). *Phytophthora* PG released no uronic acid from untreated walls and only a small proportion of that in de-esterified walls. *Sclerotinia* PG pI 4·6 released about 50% of total uronide and around 10% of arabinose and galactose. Pre-treatment with AF pI 6·5 decreased the neutral residues released by this PG. PG pI 9·7 solubilized a similar proportion of uronide to the chemical degradation used for "total" uronide estimation; it also released a large proportion of the neutral residues present in the wall. Pre-treatment of ICW with galactanase decreased the galactose and uronide released by this PG. Pre-treatment with PG pI 4·6 decreased the proportions of all residues released by pG pI 9·7. When it followed AF pI 6·5 and PG pI 4·6, the proportions of all residues released were smaller, although the proportion of arabinose was unexpectedly large. Neither PG pI 4·6 nor PG pI 9·7 seemed to be affected in their activity on walls by prior de-esterification. Other experiments showed that a PG present in a commercial "cellulase" preparation from *Trichoderma viride* released a similar proportion of total uronic acid to PG pI 4·6, and that pectin lyase purified from culture filtrates of *S. fructigena* would release a similar proportion of uronic acid to PG pI 9·7, from untreated walls but only 30% of the total from PE treated walls.

PG pI 9·7 (40 units) was used to treat de-esterified walls from 50 g pre-climacteric apple tissue. The concentrated filtrate was fractionated by gel filtration on Sephadex G-25 equilibrated with 0·1 M acetate (pH 4·8). Carbohydrate was eluted as a broad peak from the void to inclusion

Table 2. Sugar residues released from apple fruit cell walls (ICW) by polygalacturonase (PG) preparations

Enzyme	Pre-treatment of wall	Sugar residues released (% total in ICW) Uronic acid	Ara	Xyl	Gal	Glc
Phytophthora	None	0	—	—	—	—
	PE	4·6	0·25	0	3·1	0
Sclerotinia	None	50·2	13·8	0	11·3	1·8
pI 4·6	PE	53·1	10·9	0	7·9	1·3
	PE and AF pI 6·5	43·3	1·1	0	1·3	0
Sclerotinia	None	101	45·4	15·0	42·5	4·5
pI 9·7	PE	99	54·9	22·6	36·8	7·2
	PE + galactanase	35·8	38·1	14·1	4·1	4·5
	PE + PG pI 4·6	29·8	26·8	8·2	10·5	3·1
	PE, AF pI 6·5 + PG pI 4·6	16·4	41·3	11·8	17·4	3·8

PE, pectinesterase; AF, arabinofuranosidase.

Table 3. Gel filtration of polygalacturonase pI 4·6 degraded products of digestion of de-esterified apple fruit cell walls with polygalacturonase pI 9·7

Sugar residue	Percentage composition of fractions eluted between (ml/100 ml column volume) 43–60	70–80	83–93	93–106
Uronic acid	23·2	77·7	50·3	100
Rha	2·4	0·3	0·2	—
Ara	56·8	3·1	3·8	—
Xyl	5·5	0·8	0	—
Gal	7·2	14·7	45·5	—
Glc	4·9	3·4	0·2	—
	(10)*	(2)	(5)	(3)

* Relative amounts of fractions.
Neutral sugars in hydrolysates were analysed as their alditol acetates.

volumes, and colorimetric assays indicated that neutral monomers predominated in the high MW fractions while uronic acid predominated in the low MW fractions. The material which eluted from 43–60 ml/100 ml column volume was treated with PG pI 4·6 (8 units, 24 hr, 20°), concentrated and fractionated again by gel filtration. Carbohydrate was eluted in four distinct peaks whose monomeric composition is shown in Table 3. Most of the rhamnose, arabinose, xylose and glucose emerged in the highest MW peak, while the lowest MW peak gave colorimetric reactions similar to those of galacturonic acid. Most of the galactose residues were present in two peaks of intermediate MW which also contained uronic acid residues and small amounts of other neutral sugar residues.

Endoglucanase

A commercial "cellulase" preparation from *T. viride* was fractionated by ion exchange and gel filtration chromatography [3]. A peak of PG activity eluted from the column between the two major endoglucanase peaks, overlapping with the second, so that the polygalacturonate degrading activity of the pooled endoglucanase peaks was one third of their carboxy-methyl cellulose degrading activity. As, mentioned previously the *T. viride* PG appears to be similar to that from *S. fructigena* PG pI 4·6, but the activity present in the endoglucanase incubations was not sufficient to cause maximum release of uronide from cell walls (Table 4).

The endoglucanase released a small proportion of the glucose residues from PE treated ICW, which was not increased by pre-treatment with PG pI 4·6. Pre-treatment with PG pI 9·7 led to a substantially higher proportion of glucose and other neutral monomers being solubilized by the endoglucanase. If, however, walls were pre-treated with AF pI 6·5 or galactanase and then PG pI 9·7, less glucose was liberated than from PE treated ICW (Table 4).

Endoglucanase (14 units) was incubated with PE and PG pI 9·7 treated cell walls from 50 g of pre-climacteric apple tissue. The filtrate was concentrated and fractionated by gel filtration on Sephadex G-25, from which carbohydrate was eluted as a distinct high MW peak, followed by three overlapping peaks. Arabinose, xylose, rhamnose and mannose were mainly in the high MW peak while fucose, galactose and glucose were mainly in the lower MW peaks (Table 5).

Arabinosidase and galactanase treatment of high MW products of PG and endoglucanase

Portions of the excluded fractions from gel filtration of PG and endoglucanase products were incubated for 24 hr at 20° with galactanase (2·5 units) and AF pI 6·5 (6 units), and subjected to further gel filtration. Material in the eluted peaks

Table 4. Sugar residues released from de-esterified apple fruit cell walls (ICW) by an endoglucanase preparation from *Trichoderma viride*

Pre-treatment of wall	Sugar residues released (% total in ICW) Uronic acid	Ara	Xyl	Gal	Glc
None	25·6	14·5	3·5	10·3	7·2
PG pI 4·6	15·6	14·8	7·1	11·8	8·2
PG pI 9·7	23·4	30·0	14·1	36·8	14·6
PG pI 4·6 + PG pI 9·7	21·6	21·7	10·6	27·1	11·5
AF pI 6·5, PG pI 4·6 + PG pI 9·7	24·9	1·1	2·3	6·8	1·5
Galactanase + PG pI 9·7	37·2	16·5	5·2	2·5	3·8

PG, polygalacturonase; AF, arabinofuranosidase.

Table 5. Gel filtration of products of endoglucanase attack on apple fruit cell walls pre-treated with pectinesterase and polygalacturonase pI 9·7

Sugar residue	Percentage composition of fractions eluted between (ml/100 ml column volume) 40–56	66–76	80–90	100–110
Uronic acid	33·6	8·4	25·7	0
Rha	2·3	1·5	0·7	0
Fuc	0	0·9	0	0
Ara	46·6	6·2	3·2	3·0
Xyl	6·1	3·1	3·8	3·5
Man	1·3	0	0	0
Gal	8·6	45·3	34·3	48·0
Glc	1·5	34·6	32·3	45·5
	(16)*	(5)	(7)	(0·2)

* Relative amounts of fractions.
Neutral sugars in hydrolysates were analysed as alditol acetates.

was analysed for its monomeric composition and results for the high MW fractions are shown in Table 6. After galactanase treatment galactose contents were slightly diminished, but after AF treatment the arabinose content of the high MW material had decreased by about 75%. Low MW fractions contained the galactose released by galactanase and most of the arabinose released by AF. Intermediate MW fractions from incubations with endoglucanase fragments were analysed and accounted for 15% of the arabinose released by AF.

Enzymic degradation of walls from ripe fruit

PE released less than 2% of uronide from ripe fruit cell walls and similarly small proportions of

Table 6. Composition of high MW reaction products of PG and endoglucanase with apple fruit cell walls after treatment with AF pI 6·5 and galactanase

Substrate	Enzyme	Anhydro-sugar content (mg) of 43–63 ml fraction from Sephadex G-25 column: Uronic acid	Rha	Ara	Xyl	Man	Gal	Glc
High MW fragments from PG action	None	0·41	0·04	1·06	0·10	0	0·13	0·09
	Galactanase	0·40	0·05	1·29	0·13	0	0·12	0·23
	AF pI 6·5	0·37	0·06	0·24	0·12	0	0·14	0·17
High MW fragments from Endoglucanase action	None	1·06	0·07	1·47	0·19	0·04	0·27	0·05
	Galactanase	0·79	0·07	1·38	0·20	0·05	0·25	0·07
	AF pI 6·5	0·82	0·10	0·41	0·19	0·05	0·34	0·07

Neutral sugars in hydrolysates were analysed as their alditol acetates.
PG, polygalacturonase.

Table 7. Sugar residues released by various enzymes from cell walls of apple fruits ripened on or off the tree

Enzyme	Substrate	Pre-treatment	Sugar residues released (% total in cell wall): Uronic acid	Ara	Xyl	Gal	Glc
Galactanase	ACW	PE	3·9	0·9	0	24·1	0
	DCW	PE	12·3	1·8	0	23·7	0
AF pI 6·5	ACW	None	28·2	54·9	0	14·5	0
	DCW	None	39·0	71·5	0	21·2	0
	ACW	PE	2·2	27·8	0	7·6	0
	DCW	PE	5·2	38·8	0	15·0	0
PG pI 4·6	ACW	PE	50·8	2·9	0	2·8	0
	DCW	PE	68·8	10·6	0	6·3	0
PG pI 9·7	ACW	PE + PG pI 4·6	23·5	31·6	7·0	17·9	2·3
	DCW	PE + PG pI 4·6	21·5	32·2	6·9	16·3	3·0
Glucanase	ACW	PE	13·8	4·6	2·3	9·6	2·8
	DCW	PE	25·5	7·1	1·1	7·5	1·1
	ACW	PE, PG pI 4·6 + PG pI 9·7	14·1	6·2	2·3	6·2	0·5
	DCW	PE, PG pI 4·6 + PG pI 9·7	13·9	3·1	1·1	3·7	0·8

For substrate abbreviations see Table 1.
PE, pectinesterase; PG, polygalacturonase; AF, arabinofuranosidase.

neutral residues relative to those from ICW. The proportions of residues released by other enzymes from cell walls of apples, ripened on (ACW) or off (DCW) the tree are shown in Table 7. Although the galactose contents of these walls were low, galactanase released a similar proportion of this sugar to that released from ICW. No reducing sugar was released from ripe fruit cell walls on incubation with β-galactosidase. The AF pI 6·5 preparation released more uronic acid and arabinose from untreated walls from ripe fruit, especially DCW, than was liberated from ICW. However, the proportions released from de-esterified ICW, DCW and ACW were more consistent.

PG pI 4·6 released a similar proportion of total polyuronide from ACW and ICW, but a much higher proportion from DCW. PG pI 9·7 preceded by treatment with PG pI 4·6 solubilized similar proportions of uronic acid and neutral monomers from ICW, ACW and DCW. Endoglucanase liberated less glucose from ACW than from ICW, and less from DCW than from ACW, and after pre-treatment with PG pI 9·7, did not release greater amounts of glucose, or other neutral monomers from ACW and DCW.

DISCUSSION

An enzyme will release carbohydrate from a plant cell wall only if it can permeate the gel structure to reach a linkage which it can cleave, and if one of the products of cleavage is a monomer or soluble oligomer. The experimental data gives no direct information about glycosidic linkages in products of enzymic digestion of cell walls. Terminal glycosidases such as arabinosidase and galactosidase would release essentially monosaccharides, although their action might increase the porosity of the gel structure of the wall sufficiently to allow dissolution of entangled polysaccharides. It is known that random cleavage enzymes, such as galactanase, polygalacturonase and endoglucanase degrade their homopolymeric substrates incompletely to liberate the monomer and a series of oligomers. Action of these enzymes in the wall would be expected to release other polymers entangled with or covalently bonded with their substrates. Incomplete release of monosaccharide residues may mean some residues are in linkages which the enzyme cannot cleave or that some are in relatively inaccessible positions in the wall.

Heating apple fruit cell walls at pH 6·9 and 75° solubilized two polyuronide fragments with respectively high and low proportions of neutral sugar residues linked to them [1], and subsequent (unpublished) work has shown that these fragments are excluded from Sephadex G-200. This could mean that neutral glycan branches are not attached to every polyuronide chain, or that they are unevenly distributed along the chains so that random cleavage yields large fragments with varying proportions of neutral glycan attached. At least half of the uronic acid residues released by PG pI 4·6, are in chains without neutral glycan branches or in unbranched regions of longer chains. PG pI 4·6 apparently degrades the middle lamella leading to cell separation in plant tissues [19], and it is probable that the material which it releases from cell walls derives from a distinct, unbranched polyuronide in the middle lamella. The present work shows that PG pI 9·7 is more effective in releasing polyuronide from the cell wall, and it is surprising that it does not macerate plant tissue [19]. When PG pI 9·7 is incubated with wall preparations the surface of the primary wall is exposed and the solubilization of pectic fragments would be aided by attached neutral residues. PG pI 4·6 may be unable to attack this branched polymer in the primary wall, although it is able to degrade further the fragments released by PG pI 9·7. This phenomenon could be due to an inability of PG pI 4·6, which has a MW of 75000, to permeate the primary wall, while PG pI 9·7 (MW 37000) and pectin lyase (MW 26000) [19] can permeate it readily. *P. infestans* PG (MW *ca* 200000) is, presumably, completely excluded from the wall.

The galactose rich fragments among wall degradation products were of low MW and contained uronide residues. It seems likely that short chains of galactose residues are attached to galacturonic acid, rather than rhamnose as has been suggested [3] and that at least some of these chains have free non-reducing ends which can be attacked by β-galactosidase. The limited release of galactose from cell walls and soluble fragments by galactanase may be due to this enzyme's limited ability to hydrolyse short oligomers. The absence of xylose and glucose from products of

action of galactanase on cell walls contradicts the suggestion [2,3] that xyloglucan is attached to galactan branches of the rhamnogalacturonan.

The arabinose rich fragments among wall degradation products were of high MW and associated with uronide and rhamnose residues. Possibly, rhamnose residues in rhamnogalacturonan are branch points for arabinan chains. A purified α-L-arabinosidase from *Aspergillus niger* hydrolysed lateral 1,3 linked residues of sugar beet arabinan to leave a core of 1,5 linked residues [20]. If the AF isoenzymes produced by *S. fructigena* act similarly, arabinose remaining in the high MW fraction after AF treatment of pectic fragments, or in the wall after AF treatment would presumably contain a predominance of 1,5 linkages. AF pI 3·0 (MW 220000) and AF pI 4·5 (MW 350000) hydrolyse arabinan at similar rates to AF pI 6·5 (MW 40000) [17,18], but attack cell walls much more slowly than the latter, presumably because their molecular size prevents their entry into the wall. Pre-treatment of walls with AF pI 6·5 resulted in a diminution of the arabinose residues subsequently released by PG pI 4·6, but surprisingly not of those released by PG pI 9·7.

The presence of xylose and glucose residues in the reaction products of PG pI 9·7 on cell walls suggests that they are linked to pectic polymers. The predominant hemicellulose isolated from ripe apples was a xyloglucan [1], and a similar polymer in sycamore suspension cultures is thought to be linked through galactan to rhamnogalacturonan [2,3]. However, endoglucanase treatment of walls from unripe fruit solubilized high MW fragments containing most of the xylose residues released, and low MW fragments containing most of the glucose. This suggests that there are separate xylan and glucan polymers which could hydrogen bond to cellulose microfibrils [21], and, through their terminal linkages to pectic polysaccharides, could retain pectic fragments in the wall after PG treatment.

After heating apple cell walls at pH 6·9 and 75° a proportion of the polyuronide remains insoluble and can be extracted in 0·5 M NaOH; these pectic fragments, purified by DEAE cellulose chromatography were found to contain xylose and glucose residues [1], and similar binding by attached hemicelluloses could explain their insolubility. It is also possible that attachment to wall protein renders them insoluble [1]. So far there is no direct evidence on the mode of attachment of hemicelluloses to pectic polysaccharides.

Because of its different source the endoglucanase preparation may not be equivalent to that previously described [3] the PG present might have acted in collaboration with the endoglucanase, although like PG pI 4·6 it has a limited ability to attack the wall. PG pI 9·7, through its ability to permeate the primary wall, aided more extensive endoglucanase attack. The solubility of attached pectic fragments would be expected to aid the release of glucose oligomers from their attachment to microfibrils. The 1,5 linked cores left after action of AF on arabinan are much less soluble than the parent molecule [20] and this could explain the failure of endoglucanase to attack walls after AF pre-treatment.

The most obvious compositional changes in the cell walls of ripening apple fruits are the disappearance of galactose residues, presumably from the primary wall, and the increase of soluble polyuronide [7]. The absence of neutral residues from purified soluble polyuronide probably reflects its origin in the middle lamella, rather than the action of glycosidases to remove side chains, as has been suggested [1,7].

The susceptibility of PE treated walls from ripe and unripe fruit to galactanase and AF pI 6·5 was similar; the greater effect of AF pI 6·5 on untreated walls from ripe fruit was probably a result of more extensive attack by the pectin lyase impurity than on unripe fruit cell walls. Degradation of middle lamella structure, particularly during the ripening of detached fruit, could account for this effect, as well as the greater susceptibility of walls from this fruit to attack by PG pI 4·6. Endogenous β-galactosidase is probably responsible for the loss of galactose residues during ripening [22], but, if there is a linkage between glucan and galactan, it would have to be broken before this enzyme could act. The absence of a linkage to solubilizing pectic fragments could explain the lower release of glucan from ripe fruit walls by endoglucanase. Glucose residues lost from the cell walls of detached fruit during ripening, probably came from hemicellulose rather than cellulose; this would, of course, preclude endoglucanase attack on these walls.

It has been suggested that the ability to initiate attack on plant cell walls is restricted to polygalacturonases from certain sources [2,23]; the present results show that pectin lyase, AF pI 6·5 and probably galactanase can attack cell walls independently, but that enzymes with MW above 10^5 are excluded from the wall and cannot degrade it, irrespective of their activity on soluble substrates. Enzymes of this kind, secreted by pathogens, would be active around the surface of invading cells or hyphae, and may actively degrade the middle lamella causing cell separation, whereas low MW enzymes would diffuse through the cell walls causing more extensive damage. Polygalacturonase, pectin lyase, endoglucanase and probably galactanase would be expected to cause structural damage and aid pathogen penetration, but arabinosidase would be more likely to contribute to its carbohydrate requirement [18].

EXPERIMENTAL

Preparation and analysis of cell walls. Two samples of Cox's Orange Pippin apples were harvested from 12-yr-old trees on M26 rootstocks at East Malling in September 1972. Cell walls were prepared from one sample immediately (ICW), and from the other after 34 days storage at 12° (DCW). Cell walls were prepared from a further sample, harvested from the same trees on 18 October (ACW). Cortical tissue was disintegrated in Me_2CO (4 ml/g), filtered and washed with Me_2CO–H_2O (4:1). The residue was extracted by resuspensions and filtration with successively, 5 mM Na–Pi (pH 7), 0·1 M Tris–HCl, containing M KCl and 0·1% Triton X-100 (pH 7·5), and H_2O. After resuspension in H_2O (2 ml/g tissue) aliquots were stored at −20°. For analysis starch was solubilized with chloral hydrate [7], recovered by precipitation with 4 vol. Me_2CO, hydrolysed with 0·4 M H_2SO_4 at 121°, neutralized with dioctylmethylamine [8], and estimated as glucose TMSi ether. Polyuronide was degradatively extracted after starch removal by heating in 0·05 M EDTA, 0·1 M Na_2HPO_4 (pH 6·9) for 4 hr at 95°, filtering and estimated in the filtrate by an automated carbazole–H_2SO_4 procedure [1]. Samples of suspension were filtered, washed with Me_2CO and allowed to dry. The dry material was dissolved in 72% (w/w) H_2SO_4, dild to 0·4 M H_2SO_4 and autoclaved at 121° [24]. The hydrolysed samples were neutralized with an excess of 10% dioctylmethylamine in $CHCl_3$ [8] and evaporated to dryness at 40° for analysis of neutral monosaccharides as TMSi ether or alditol acetate derivatives.

Sources and purification of enzymes. "Lactase Grade II" (β-galactosidase) from *Saccharomyces fragilis* was purchased from Sigma, crude β-glucosidase from sweet almonds from BDH, and crude cellulase from *Trichoderma viride* from Aldrich. Pectinesterase was prepared from orange peel [12] and stored in soln at −20°. Galactanase and polygalacturonase were separated from the 80% $(NH_4)_2SO_4$ fraction of culture filtrates of *Phytophthora infestans* [25], by gel filtration on a column (40 × 2·5 cm) of Sephadex G-200 equilibrated with 0·1 M citrate–Pi buffer (pH 4) at 2° [13]. Fractions (5 ml) containing the appropriate activities were pooled and stored at −20°. AF pI 3, AF pI 6·5, PG pI 4·6, PG pI 9·7 and pectin lyase were purified from culture filtrates of *Sclerotinia fructigena* by iso-electric focusing [26] over a wide pH range (3–10) [17,19], followed by gel filtration on Sephadex G-100 (0·05 M Tris–HCl, pH 7·6, 0·1 M KCl) for AF pI 6·5, PG pI 4·6, PG pI 9·7 and pectin lyase and on Biogel p-300 (same buffer) for AF pI 3. AF pI 4·5 was obtained by a similar procedure from a mycelial homogenate of the same organism [17]. AF pI 6·5 was further purified by narrow range (pH 5–8) iso-electric focusing and column chromatography as described in Results. Crude cellulase from *Trichoderma viride* (1·5 g) was dissolved in 15 ml 0·02 M Na–Pi buffer (pH 7) and centrifuged at 1000 *g* for 5 min. The supernatant was applied to a column (40 × 2·0 cm) of Sephadex G-25 and eluted with 0·02 M Pi buffer (pH 7). After the void vol. 20 ml was collected and re-applied to the column. This procedure was repeated and the eluate applied to a column (15 × 2·5 cm) of DEAE Sephadex A-50. After elution with 100 ml 0·02 M Pi buffer a linear gradient formed from 100 ml each of 0·02 M Pi and 0·5 M NaCl in 0·02 M Pi buffer (pH 7) was applied [3]. Fractions (5 ml) containing the appropriate activities were pooled and stored at −20°.

Enzyme assays. Enzyme activities of the fractions from column chromatography were assayed by an automated procedure incorporating a ferricyanide–ferric sulphate system for reducing sugars [27]. Fractions were sampled, 40/hr at 0·2 ml/min, segmented with air at 1 ml/min and mixed with substrate at 0·8 ml/min. After passing through a delay coil (15 min) at 30° the enzyme reaction was stopped by mixing with the alkaline ferricyanide reagent (1 ml/min). The subsequent procedure was similar to that described in Ref. [27] except that sensitivity was reduced by measuring *A* at 550 nm. Fractions from *P. infestans* were assayed against H_2O to check for endogenous reducing sugars, polygalacturonic acid (0·1 mg/ml) adjusted to pH 4 (NaOH) for PG, and potato pectin [28] (0·1 mg/ml) for galactanase. Fractions from *T. viride* were assayed with 0·1 M NaOAc (pH 4·8), for reducing sugars, and, in the same buffer, $NaBH_4$ reduced cellobiose (0·1 mg/ml) for β-glucosidase, polygalacturonic acid (0·1 mg/ml) for PG, and sodium carboxymethylcellulose (0·1 mg/ml) for endoglucanase. Pectinesterase activity was assayed against citrus pectin at 20° [11]. Endoglycanase activities for cell wall digests were assayed at 25° as reducing sugar [9,10] released in reaction mixtures as follows. Galactanase was incubated with potato pectin (1 mg) and 1 ml citrate (0·1 M) Pi (pH 4) in a total vol. of 2·5 ml; *P. infestans* PG was incubated in a similar mixture but with polygalacturonic acid (1 mg) as substrate. *S. fructigena* and *T. viride* PGs were incubated with polygalacturonic acid (1 mg), 1 ml 0·1 M NaOAc (pH 4·8) in a total vol. of 2·5 ml; *T. viride* endoglucanase was assayed in a similar mixture but with Na carboxymethylcellulose (1 mg) as substrate. Pectin lyase was assayed at 20° in a mixture of enzyme, citrus pectin (1 mg), 1 ml 0·1 M acetate (pH 4·8) in a total vol of 2 ml, by increase of *A* at 235 nm. Arabinosidase was assayed with *p*-nitrophenyl α-L-arabinofuranoside at pH 4·8 and 30° [15]. Activity of β-galactosidase in emulsin was assayed similarly, but with *p*-nitrophenyl β-D-galactopyranoside as substrate, and in the *S. fragilis* preparation with this substrate and 0·05 M Pi (pH 7) instead of OAc. For all enzymes the unit of activity was the amount which released 1 μmol of product per hr under the assay conditions.

Enzyme treatment of cell walls. For pectinesterase treatment 100 ml wall suspension was incubated with 100 units of enzyme and 100 ml 0·1 M Tris–HCl (pH 7·5) under toluene at 20° for 5 days. For most glycanase treatments, duplicate

samples of 5 ml wall suspension (*ca* 30 mg dry wt of wall), enzyme, and 2 ml buffer in a total vol of 10 ml were incubated under toluene at 20°, with continuous agitation on a bottle roller for 72 hr. Amounts of enzyme with buffers used were, 0·8 units galactanase with citrate (0·1 M) Pi (pH 4), 500 units *S. fragilis* β-galactosidase with 0·05 M Na–Pi (pH 7), 500 units almond β-galactosidase with 0·1 M NaOAc (pH 4·8), 14 units AF with 0·1 M acetate (pH 4·8), 2 units *P. infestans* PG with citrate (0·1 M) Pi (pH 4·0), 2 units *S. fructigena* PG with 0·1 M NaOAc (pH 4·8), 0·08 units pectin lyase with the same buffer, and 0·7 units endoglucanase with 0·15 M OAc (pH 4·8). Incubations on a larger scale used the same proportions of cell wall, buffer and enzyme but agitation was by magnetic stirrer. At the end of incubation reaction mixtures (10 ml) were filtered through sintered glass (porosity 3), and larger scale digestions through glass fibre paper. Reducing sugar [9,10], and uronic acid [1] in the filtrate were estimated colorimetrically. Portions of filtrates were passed through columns (2 × 1 cm) of Zerolit 225 (H^+ form) resin, concentrated and H_2SO_4 added to 0·4 M. After hydrolysis at 121° for 60 min samples were neutralized with $BaCO_3$, evaporated at 40° and dried over P_2O_5 *in vacuo*. Unless otherwise stated neutral sugars were estimated as TMSi derivatives.

GLC of monosaccharide derivatives. Sugars in most samples were estimated as their TMSi ethers [1]. Sugars in selected samples were reduced with $NaBH_4$ and acetylated with Ac_2O in C_5H_5N [29]; inositol was added as internal standard for quantitative analysis. An equal vol of H_2O was added to the products which were then dried *in vacuo* over P_2O_5 and NaOH. Samples were dissolved in CH_2Cl_2 and 1 μl of the solns injected onto a column (2 m × 2 mm i.d.) of 3% ECNSS–M on Gas Chrom Q using an injection temp. of 250°, column temp. 180°, and N_2 carrier at 25 ml/min.

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