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Changes in the pectic substances of apples during development and postharvest ripening. Part 1: Analysis of the alcohol-insoluble residue

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Cell wall material in the form of an alcohol-insoluble residue (AIR) was prepared from apples during growth and maturation on the tree and at different stages of ripening during postharvest storage. The galacturonic acid, neutral sugar, starch content as well as the degree of acetylation of the AIR were determined for each stage as was the degree of methyl esterification of the pectic substances. During the first weeks of development, the AIR content of the fruit decreased; it then stayed constant until the end of storage. The galactose and arabinose content of the AIR decreased with time while the galacturonic acid content stayed almost constant. Hardly any changes were observed in the degrees of methyl esterification and acetylation. The unripe fruit contained appreciable amounts of starch whereas none could be determined shortly after harvest. The significance of the losses of arabinose and galactose residues will be examined in a further paper.

INTRODUCTION

Despite genetic results, which have downplayed the role of *endo*-polygalacturonase (*endo*-PG) in the softening of tomatoes, degradation of the pectic backbone of the middle lamella and primary cell wall remains a major feature of cell wall changes which accompany ripening in many fruit species (Giovannoni *et al.*, 1989). An increase in soluble pectin and a loss of non-cellulosic neutral sugars have been reported for most ripening fruit examined (Gross & Sams, 1984). The elucidation of the mechanism of cell wall changes which cause this softening is fundamental to the maintenance of fruit quality after harvest and is thus of economic importance. Work in this field also enables us to gain basic knowledge of plant cell walls and their functions, as well as of the mechanisms controlling plant growth and development. This has been a subject of research for many years, but the complexity of the cell wall polymers and their intermolecular links as well as their susceptibility to the methods of extraction have so far prevented a complete understanding of this process.

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The degradation of the pectic polymers has often been correlated with an increase in *endo*-PG and pectin methyl esterase (PME) activity (Huber, 1983). In some fruits, however, such as strawberries (Huber, 1984), muskmelon (McCollum *et al.*, 1989) or bananas (Wade *et al.*, 1992) no *endo*-PG activity has been detected, yet they also soften during the ripening process. The presence of *exo*-polygalacturonase (*exo*-PG) in apples has been known for some time (Bartley, 1978), while *endo*-PG has only been discovered recently (Wu *et al.*, 1993).

Knee and Bartley have carried out detailed studies on the cell wall changes in ripening apples (Knee, 1973, 1974, 1978*a*,*b*; Bartley 1976; Bartley & Knee, 1982). They observed a noticeable loss of galactose, a lesser one of arabinose and the appearance of an almost pure rhamnogalacturonan in the soluble fraction. Knee (1978*a*, *b*) postulated that the pectin solubilisation was caused by *de novo* synthesis of a highly methyl esterified polyuronide concurrently with the degradation by an *exo*-PG of the low esterified regions of the middle lamella pectin or else esterification of the existing pectin. This new pectin, because of its higher degree of methyl esterification (DM), would be less strongly bound in the middle lamella by ionic bonds and this could lead to cell separation. This dissolution is accompanied by the loss of galactan and arabinan side-chains, so in fact soft-

ening could result from changes in the interactions between the various cell walls polymers. This mechanism postulates a dynamic turnover of the cell wall components rather than a simple degradative process.

The present two-part study hopes to bring new clues to the changes in the structure of the pectic substances occurring during the development and ripening of apples. In this first part, the alcohol-insoluble residue (AIR) was isolated from apples in different stages of development on the tree and during postharvest storage. The galacturonic acid, neutral sugar content and the degree of acetylation of the AIR as well as the DM of the pectins were determined at each stage.

MATERIALS AND METHODS

Unless otherwise specified, all the chemicals were purchased from Fluka (Buchs, Switzerland) and were of analytical or puriss quality.

All the analyses were performed in duplicate and the coefficients of variation were usually less than 5%.

Sampling

The apples (cv. Golden Delicious) were provided by the Swiss Federal Research Station for Fruit Growing, Viticulture and Horticulture in Wädenswil.

Twenty apples, picked from five trees, were examined at each sampling time. Sampling (week 0) began immediately after the so-called 'June drop', which corresponds to the end of the cell division phase and was carried out at 3-week intervals during development on the tree and during storage. The apples were harvested in the autumn at optimum maturity (week 13) and stored in wooden crates, one per tree, at 4°C and 95% relative humidity until the end of February. At the end of the storage period a batch was left for 1 month at room temperature. These are the so-called 'senescent' fruit (week 38).

Sampling was carried out from July 1990 to March 1991. The average diameter (measured with callipers at widest part) and weight of the fruit as well as the water content of the fruit flesh were determined at each sampling date.

Preparation of the AIR

The unblemished fruit was peeled, cored and cut into small pieces which were plunged into boiling 96% ethanol (250 ml/100 g fruit flesh) for 10 min, then blended for 5 min with a Sorvall Omni-Mixer (DuPont Instruments, Newtown, CT, USA). The slurry was then homogenised with a Polytron PT 45-80 with rod PT-DA 6040/2M (Kinematica, Lucerne, Switzerland) and filtered through a G3 sintered glass filter funnel. The residue was washed with 96% ethanol followed by acetone and diethyl ether and dried overnight at 40°C under vacuum. The AIR was stored frozen until needed.

Determination of dry matter

The dry matter of the fresh fruit was gravimetrically determined by freeze-drying the grated fruit flesh. The dry matter of the AIR was determined either by drying at 105°C to constant weight or using a Karl-Fischer titrator DL 18 (Mettler Instruments, Greifensee, Switzerland).

Determination of galacturonic acid

The determination was carried out according to the method of Kintner & Van Buren (1982) with prehydrolysis of the samples as described by Ahmed & Labavitch (1977). The results are expressed as anhydrogalacturonic acid.

Determination of the sugars in the AIR

The sugars were derivatised to aldononitrile acetates (McGinnis, 1982) following Saeman hydrolysis according to the procedure described by Niederer (1993).

About 10–15 mg of AIR was weighed in a screw-cap test tube (Pyrex) and hydrolysed in 0·3 ml of 72% (w/w) (12 M) sulphuric acid at room temperature for 2 h followed by 3 h at 100°C after addition of 2·7 ml of distilled water while stirring. The cooled samples were then neutralised with 10 ml of 40% (v/v) *N*,*N*-dioctyldimethylamine in chloroform. After separation of the phases, the aqueous phase was washed twice with 5 ml of chloroform.

A 0·2 ml portion of the neutralised hydrolysate was added to a test tube followed by 0·4 ml of an *N*-methylimidazole solution containing 0·25 mg/ml of xylitol and 25 mg/ml of hydroxylamine hydrochloride. The reaction mixture was heated for 10 min at 80°C while stirring. After cooling, 1 ml of acetic acid anhydride was added. After cooling again, 1 ml of distilled water and 1·5 ml of methylene chloride were added and the contents of the test tube were thoroughly mixed with a Vortex mixer. After removal of the aqueous phase, the organic phase was washed twice with 1 ml of water and dried with sodium sulphate before being transferred to a GC vial.

The 0·15 μl samples were analysed by gas chromatography (Hewlett-Packard HP 5890 Gas Chromatograph, Hewlett-Packard, Palo Alto, CA, USA) on a Durabond DB 225 (J & W Scientific, Folson, CA, USA) capillary column (30 m × 0·32 mm, film thickness: 0·25 μm) at 215°C. The injector temperature was 240°C and the flame ionisation detector temperature 260°C.

Quantification was carried out using a standard mixture containing 1 mg/ml of each of the following

sugars: L-rhamnose, L-fucose, L-arabinose, D-xylose, D-mannose, D-glucose, D-galactose and xylitol. When the standard mixture was derivatised, an *N*-methylimidazole solution containing only 25 mg/ml of hydroxylamine hydrochloride was added. The sugars were quantified as anhydro-sugars, using xylitol as internal standard.

Determination of the degrees of methylation of the pectin and degrees of acetylation of the AIR

The degrees of methylation and acetylation were determined according to the method of Voragen *et al.* (1986). The values were calculated based on the galacturonic acid content of the AIR.

Determination of starch

The determination was carried out according to a slightly modified method from the 'Schweizerisches Lebensmittelbuch' (1991).

About 30 mg of cell wall material (CWM) was weighed in a test tube, and 25 ml of distilled water was added. The test tubes were then placed in a boiling-water bath. Once the mixture had reached a temperature of 85°C, 200 μl of Termamyl (Termamyl 120 L, Novo Nordisk Ferment, Dittingen, Switzerland) was added and the mixture was left to react for 15 min while stirring occasionally. After cooling to room temperature, 5 ml of sodium acetate buffer at pH 4·6 (70 ml of 0·5 M NaOH + 950 ml of distilled water and adjusted to the pH with glacial acetic acid) was added, then 1 ml of an amyloglucosidase (No. 208469, Boehringer, Mannheim, Germany) solution (10 mg/ml in sodium acetate buffer, pH 4·6) was added and the mixture was stirred for 30 min in a water bath at 60°C. After cooling, the solution was transferred to a 50 ml volumetric flask and filled up to the graduation mark with distilled water. The solution was filtered through a folded filter and the first few millilitres were discarded; 0·1–0·4 ml of solution were used for the determination of glucose, depending on the starch content. Glucose was determined enzymatically, using a test-kit (No. 716 251, Boehringer).

Determination of amino acid content as a measure of protein

The samples (20–30 mg) were hydrolysed in 1 ml of 6 M hydrochloric acid at 110°C for 24 h after degassing with nitrogen for 10 min (Lebet *et al.*, 1994). The samples were then dried by evaporation at 50°C under vacuum with a rotatory evaporator and dissolved in 4 ml of loading buffer (sodium citrate/hydrochloric acid, 0·2 M, pH 2·2, No. 4130-131, Pharmacia LKB, Uppsala, Sweden). The samples were filtered (syringe filter, 0.45 μm, Gelman Sciences, Ann Arbor, MI, USA) before analysis.

The amino acid analysis of the samples was carried out by automated analysis on an Alpha Plus Amino Acid Analyser, 4151 (Pharmacia LKB) with a four-buffer system, using an internal method.

Determination of ash

Ash was determined gravimetrically by heating about 1 g of material in a platinum crucible at 550°C for 16 h in a muffle furnace (Solo, Käsemann and Spérisen, Biel, Switzerland).

RESULTS AND DISCUSSION

The changes in the pectic polymers from the AIR were studied for two apple varieties, Golden Delicious and Glockenapfel, for two seasons, and the same trends were observed in all cases. Only the values for the Golden Delicious apples during one season are presented here for the sake of simplicity. The data from another harvest will only be mentioned if it differs markedly. The complete data were collected in a PhD thesis (Fischer, 1993).

Development of the fruit

The weight and diameter measurements were made to monitor the overall development of the fruit during the sampling period (Fig. 1). The size and weight of the apples increased steadily during the first 10 weeks after which growth ceased and there was no more visible change. The pattern of growth followed the single sigmoidal curve characteristic of the apple (Rhodes, 1980). There was a very slight decrease in weight and size during storage probably due to loss of water by evaporation, but also to loss of dry matter because of metabolic activity. The evolution of the dry matter during storage depends on these processes, which are influenced by the storage conditions such as temperature and humidity (Perring, 1989). The water content has an effect on the texture of the fruit since it determines turgor pressure, a decrease in which can lead to cell separation (Bartley & Knee, 1982). The water and AIR content of the fruit flesh can be seen in Table 1. The AIR content of the fruit flesh declined during the first 18 weeks to reach a relatively constant value of about 1·9%. This decrease is mainly due to the disappearance of starch during development, but it also points to the fact that during growth, synthesis of cell wall polysaccharides is slower than water uptake and cell expansion (Proctor & Peng, 1989; Smock & Neubert, 1950).

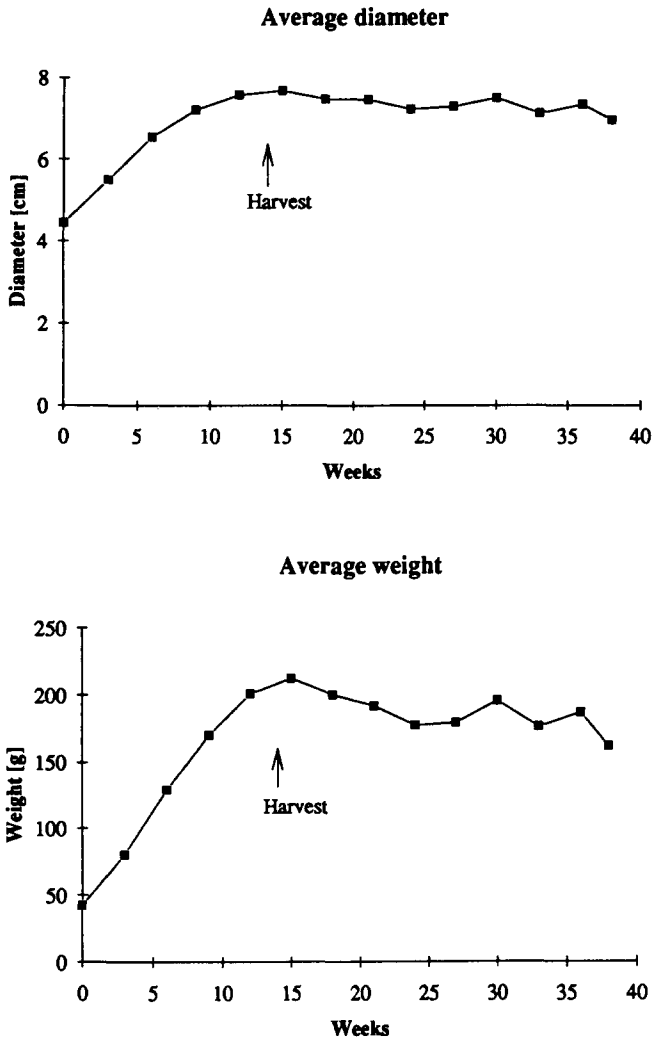

Fig. 1. Average diameter and weight of Golden Delicious apples ($n = 20$) during growth and ripening.

Table 1. AIR and water content of the fruit flesh

Weeks	AIR (g/100 g)	Water (g/100 g)
0	5·48	85·84
3	5·21	85·95
6	4·90	81·83
9	4·54	85·03
12	2·84	77·83
15	2·14	84·41
18	1·94	82·91
21	1·84	82·99
24	1·96	83·36
27	1·85	83·36
30	1·84	82·72
33	1·82	82·66
36	1·82	84·43
38	1·94	83·14

Composition of the AIR and changes during development

The molar ratios of galacturonic acid and neutral sugars in the AIR are shown in Table 2, the sum of both being taken as 100. The values for glucose have been corrected for the starch content. The last column presents the weight of these components in the starch-free fractions. The balance is made up of proteins, water and ash, and the mass balance of the AIR from a ripe sample (week 21) is presented in Table 3. No significant loss in galacturonic acid residues could be observed during growth and ripening whereas the neutral sugar content increased overall despite the general loss in non-cellulosic neutral sugars.

The galactose and arabinose content of the AIR declined with time (Table 2). In the senescent fruit (week 38), 71% of the initial galactose was lost. The decrease in arabinose content was less marked, being 34%. In contrast, the xylose and glucose contents rose markedly. Xylose, one of the main hemicellulosic monosaccharides, almost doubled in value. In the case of glucose this change was due to an increase in the relative amount of cellulose coupled to the loss in pectic polysaccharides. This was also the reason for the overall increase in total neutral sugars noted above.

In the unripe fruit, starch accounted for about 2% (w/w) of the fruit flesh or up to 40% of the AIR (Table 4). Just before harvest (week 12), the amount of starch had declined by about half, and a few weeks after harvest (week 21) no starch could be detected. It is known that the starch content in apples reaches a maximum during growth and begins to decrease before harvest with decline being very rapid during ripening (Smock & Neubert, 1950). Eggenberger (1949), in his investigation of apples during ripening and storage, found values around 1·7% (w/w) in the flesh of unripe fruit and 0·2% (w/w) at harvest for Boskoop apples. The amount of starch contained in the fruit depends on the variety, but the results found here are consistent with Eggenberger's.

Table 5 lists the DM of the pectin and the degrees of acetylation (DA) of the AIR during growth and ripening. The DM of the pectin was higher than that reported by de Vries *et al.* (1981) (65–70%) or Renard (1990) (72%) since it lay around 80% in fruit of a comparable degree of ripeness and even almost reached values close to 100% several weeks after harvest. The value at week 21 was particularly high. The reason for this is unknown, but could be attributed to formation of an artefact preparation of the AIR. Also, the galacturonic acid content at week 21 is significantly lower than the other values, which could account for an overestimation of the DM. The method for DM determination was tested with commercial pectins of known DM so a systematic error during analysis can probably be excluded. In addition, the values during the first year trials were noticeably lower (50–79%), but followed a similar trend. Probably environmental factors influence the DM, but no reference to this has been found in the literature. An increase was observed at the begin-

Table 2. Galacturonic acid and neutral sugar distribution (mol%) of the AIR of Golden Delicious apples during growth and ripening

Week	GAlUA	Rha	Fuc	Ara	Xyl	Man	Glu	Gal	Total (μg/mg)
0	33·1	1·4	3·6	23·4	4·8	4·7	Trace	29·0	512
3	24·0	2·6	3·9	18·2	4·2	4·2	20·1	22·8	808
6	32·3	1·0	4·7	18·4	4·9	3·4	9·8	25·5	573
12	24·0	1·7	1·2	18·2	6·2	2·2	26·8	19·7	822
18	28·3	1·4	1·2	19·0	7·2	2·1	26·9	14·0	693
21	17·2	1·5	1·1	20·3	7·9	2·4	37·6	12·0	866
24	22·0	1·8	1·9	17·2	6·8	2·1	36·3	11·8	798
27	24·5	1·3	1·1	17·2	7·1	2·3	34·9	11·6	750
30	25·1	1·8	2·3	15·5	7·2	2·1	35·7	10·4	801
33	23·2	1·1	1·0	17·9	8·5	3·4	34·8	10·0	951
36	28·0	2·2	2·8	15·0	6·9	2·2	33·6	9·4	780
38	21·2	1·3	1·0	15·4	10·1	6·6	36·0	8·4	983

Table 3. Mass balance of the AIR from ripe (week 21) Golden Delicious apples

Galacturonic acid	16·8
Neutral sugars	69·8
Ash	2·3
Protein (sum of amino acids)	4·1
Water	11·0
Total (g/100 g AIR)	104.0

Table 4. Starch content (g/100 g) of the AIR and fruit flesh of Golden Delicious apples

Weeks	Starch in AIR	Starch in fruit flesh
0	34·1	1·9
3	36·8	1·9
6	41·6	2·0
12	24·1	0·7
18	8·6	0·2

Table 5. DM of the pectin and the DA of the AIR of Golden Delicious apples during growth and ripening (%)

Weeks	DM	DA
0	79·1	33·9
3	81·8	29·0
6	88·7	30·4
9	93·6	30·0
12	80·5	29·4
15	89·8	26·9
18	85·3	23·2
21	120·3	35·6
24	92·2	31·1
27	100·1	30·1
30	73·7	26·5
33	83·1	25·7
36	75·8	27·2
38	72·8	26·3

ning of the development and a very slight downward tendency in the senescent fruit, but it is difficult to make out a consistent trend. De Vries *et al.* (1981) report no change in the DM of AIR from apples during ripening, but the apples they examined did not present the same extremes in their development as those from this study. Knee (1978*a*,*b*) also observed no change in the overall DM of apple cell walls although that of the water-soluble pectin increased. A high DM would theoretically disfavour calcium bonding, thus decreasing cell wall cohesion although the exact role of calcium in complexation of the galacturonic acid residues has recently been questioned (McFeeters & Fleming, 1989).

The DA was high, almost 30%, and it declined slightly in the senescent fruit. The values for the first year trials were also slightly lower (25%); once again this difference could be attributed to environmental factors. Renard *et al.* (1990) also report a high DA (26%) for the AIR of ripe apples. This high value can be explained by the fact that the DA is calculated in relation to the galacturonic acid content yet other cell wall polymers, such as xyloglucans, can also carry acetylated groups, which will be detected at the same time (Carpita & Gibeaut, 1993).

CONCLUSIONS

During growth and ripening of Golden Delicious apples, the galactose content of the pectic polymers of the AIR decreased. The galacturonic acid content of the AIR changed little. The neutral sugars associated with the hemicellulosic polymers, xylose and glucose, showed an increasing trend during ripening. These facts point to changes in the structure of the pectic material of the cell wall and these will be examined in more detail in a further paper where purified pectic fractions will be investigated (Fischer *et al.*, 1994).

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