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Changes in the Structure of Apple Pectic Substances during Ripening and Storage

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SUMMARY

During ripening, the degree of polymerization, the degree of esterification, the neutral sugar content and the neutral sugar composition of extractable apple pectic substances did not change. Some xylose and glucose containing polysaccharides can be extracted from the ripe cell walls suggesting that changes in the hemicelluloses take place. In senescent apples, significant changes in the structure of apple pectic substances could be observed. The degree of polymerization of both the galacturonan chains and the arabinogalactan side chains decreased. The amount of water-extractable pectin molecules carrying 1,3/1,6-linked galactans increased. The degree of esterification and the distribution of the methoxyl groups in the apple pectic substances did not change very much.

INTRODUCTION

Many studies on cell wall changes during fruit ripening have been published. In spite of all these efforts, a clear understanding of the phenomena observed has not yet emerged. Undoubtedly, an important factor in this respect is the impact of the ripening and storage conditions. Differences between ripening on and off the tree have been observed (Esau *et al.*, 1962; Knee, 1973). Another problem is that no distinct stages can be defined: some changes can already be found

before ethylene production starts (Platt-Aloia *et al.*, 1979). Microscopic investigations show that during ripening the middle lamella changes. This has been observed for apples (Ulrich & Hartmann, 1967; Ben-Arie *et al.*, 1979; Mohr, 1979), strawberries (Neal, 1965) and avocados (Platt-Aloia *et al.*, 1979). In the early ripening stages of pears, however, changes in the primary cell wall have also been reported (Ben-Arie *et al.*, 1979). Dorofeeva *et al.* (1973) found differences in apple fruit collenchym and parenchym tissue.

In this study, structural differences among apple pectic substances of different stages were investigated. Pectins were extracted, purified by ion-exchange chromatography and gel filtration and degraded by pectolytic enzymes. The results are discussed in relation to a model of apple pectin molecules previously described (de Vries *et al.*, 1981, 1982, 1983*a*, *b*).

METHODS

Analytical methods

The anhydrouronic acid (AUA; $MW = 176$) content of pectin fractions was determined by an automated carbazole-sulphuric acid assay (van Deventer-Schriemer & Pilnik, 1976). The amount of AUA in the alcohol insoluble solids (AIS) preparations was determined according to Ahmed & Labavitch (1977) with *meta*-hydroxydiphenyl. The neutral sugars were analyzed by gas chromatography as their alditol-acetates (Albersheim *et al.*, 1967; Darvill *et al.*, 1975). The methoxy content was determined by gas chromatographic analysis of the methanol released on alkaline de-esterification (1 h at room temperature; 0·1 M KOH). Methanol was converted to methyl nitrite and determined according to Versteeg (1979).

Preparation of AIS

Golden Delicious apples were obtained from 'de Boutenburg', an experimental apple-orchard at Lienden, De Betuwe, The Netherlands. They were gathered in a pre-climacteric stage on 19 October 1980, and stored in the open until 23 October 1980 (*unripe apples*). Some of the apples were then allowed to ripen at 20°C, the first week in an

impermeable plastic bag in order to accelerate the ripening process (*ripe apples*). Some of the ripe apples were then stored at 20°C for two weeks and at 12°C for another two weeks (*senescent apples*). The temperature had to be lowered from 20°C to 12°C to avoid microbial spoilage. AISs were prepared from the unripe, ripe and senescent apples as described by de Vries *et al.* (1981).

Extractions

10 g of AISs were extracted on three occasions during 30 min (while stirred) with 300 ml 0·05 M sodium acetate buffer (pH = 5·2) at room temperature and the whole procedure was repeated at 70°C. Extraction with 0·05 M EDTA and 0·05 M ammonium oxalate in 0·05 M sodium acetate buffer was then applied (again three occasions, 30 min, 70°C). After washing with water this extraction was followed by an extraction with dilute hydrochloric acid (three occasions, 30 min, 70°C, pH = 2·5). The extracts were filtered and the pectins precipitated with ethanol at 70% concentration. In the text these four extracts are referred to as the cold buffer, hot buffer, oxalate and acid extracts.

The extraction scheme chosen was that previously used by the authors in studies on the structure of pectin substances (de Vries *et al.*, 1981, 1982, 1983*a*, *b*).

Enzymic degradation, ion exchange and gel permeation chromatography, pectin de-esterification and HPLC-analyses were performed as described by de Vries *et al.* (1983*b*).

RESULTS AND DISCUSSION

Table 1 shows the extractability of the pectic substances from the unripe, ripe and senescent apples used in our experiment. During ripening, a larger fraction of the pectic substances can be extracted due to loosening of the cell walls. This increase in pectin solubility has been found frequently, although not in every case (Esau *et al.*, 1962).

Many authors (e.g. Knee, 1978) have observed an increase in cold-water-soluble pectin during ripening, but in our case hardly any increase in cold-buffer-soluble pectin was found (Table 1).

Figure 1 and Table 2 give the results of the fractionation on DEAE-cellulose of the cold buffer extractable pectin from AIS from over ripe

TABLE 1

The Extractability and DE of Pectin Substances from Unripe, Ripe and Senescent Apples. Condition During the Extractions Described in the Text. Percentage of Total AUA: the Total Amount of Anhydrogalacturonate Material Extracted as Percentage of the Amount Present in the AIS

	AUA (mg/g AIS)			*DE (%)*		
	unripe	*ripe*	*senescent*	*unripe*	*ripe*	*senescent*
Cold buffer extract	28	30	30	74	79	82
Hot buffer extract	25	31	22	71	77	74
Oxalate extract	25	31	24	77	72	76
Acid extract	20	39	47	68	63	69
% of total AUA	38	48	42			

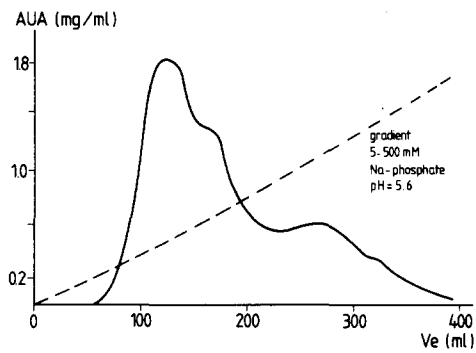

Fig. 1. Fractionation of the pectin from the cold buffer extract of senescent apple AIS on DEAE-cellulose. Ve = elution volume. The DEAE-cellulose column was eluted by a gradient of 5-400 mM sodium phosphate buffer pH = 5·6.

apples. In previous papers (de Vries *et al.*, 1983*a*, *b*), the occurrence of a pectin fraction with a high degree of esterification (DE) and containing only 1,3/1,6-linked galactan side chains has been described. Neutral sugar analysis of DEAE-cellulose purified pectins of various extracts (cold buffer, hot buffer and oxalate extracts of unripe, ripe and senescent apples) showed that this pectin fraction increases during ripening and storage from approximately 5% in the unripe stage to

TABLE 2
Neutral Sugar Composition of Pectin Fractions from the Cold Buffer Extract of AIS from Senescent Apples Fractionated as Shown in Fig. 1

	Elution volume (ml)			
	50–125	*125–200*	*200–300*	*300–400*
AUA, % (anhydrouronic acid)	13	44	30	13
Neutral sugars (mol/mol galacturonic acid residues)	0·20	0·15	0·28	0·36
Mol galactose/mol arabinose	0·79	0·74	0·68	0·96
Mol rhamnose/mol arabinose	0·08	0·18	0·10	0·15
Mol xylose/mol arabinose	0·02	0·06	0·09	0·14
Mol glucose/mol arabinose	0·03	0·03	0·10	0·19

about 12% in the senescent stage. Takeuchi & Komamine (1980) observed changes in cell wall 1,3/1,6-linked galactans during the growth of tobacco. Some authors concluded from the results of their experiments that during ripening a continuous synthesis and degradation of pectic substances occurs (Knegt *et al.*, 1974; Knee, 1978). According to Knee (1978), the newly synthesized material has a high DE. Due to the incompleteness of the extractions applied in these experiments, however, we cannot draw conclusions about the origin of the increased quantity of pectin extracted from senescent apple AIS. The molecular weight (*MW*) of the pectins does not change during ripening, but during storage after ripening a decrease can be observed (Fig. 2).

Gel filtration cannot be considered to be a reliable method of *MW* determination, but the conclusion that during storage after ripening a decrease in *MW* occurs is hardly questionable. Because the neutral sugar content of the extracted pectins is low (about 10%) and does not change very much, it is likely that the galacturonan chains are degraded during senescence.

Figure 2 shows the differences in the gel filtration pattern of the cold buffer extracts, but similar changes can be observed in the pectins of the other extracts. In literature on ripening, much attention is paid to changes in the activities of pectolytic enzymes. In tomatoes, polygalacturonase (PG) activity increases during ripening, but the increase

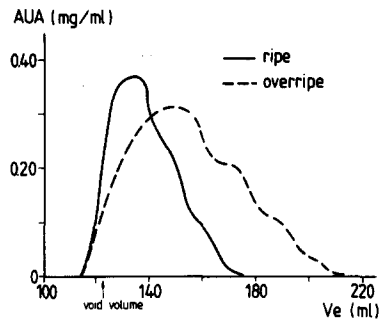

Fig. 2. Gel permeation chromatography on Sephacryl S-300 of the pectin from the cold buffer extract of ripe and senescent apple AIS. Ve = elution volume. The eluent was 0·1 M sodium phosphate buffer pH = 5·1.

in the water-extractable pectin fraction has already started before the rise in PG activity begins (Sawamura *et al.*, 1978). In tomatoes, PG is certainly not the only factor involved in fruit softening (Wallner & Bloom, 1977). Apples seem to have only exo-PG activity (Ben-Arie *et al.*, 1979). The interpretation of the results of experiments, in which enzyme activities are monitored during ripening, is difficult since a higher enzyme activity can result from better extractability, *de novo* synthesis or activation by changes in inhibitor-concentrations. Interestingly, Ben-Arie *et al.* (1979) observed that senescent apple tissue looks like the tissue of unripe apples treated with tomato endo-PG. It is possible (and in agreement with the data of Fig. 2) that in apples the pectic substances are affected by PG in senescence. However, not only the galacturonan chains are degraded during senescence but also the arabinogalactan side chains. The 'hairy' regions (the pectin main chain segments carrying the neutral sugar side chains; see de Vries *et al.*, 1983*a*) appear in higher elution volumes (Fig. 3, Table 3) than those of unripe or ripe apple pectin (de Vries *et al.*, 1983*b*). Pectin molecules that can be characterized as degraded hairy regions (molecules of 'type E', de Vries *et al.*, 1982) can also be found in unripe and ripe apples. But especially in the period of storage after ripening, the amount of 'type E' pectin increases (as can be concluded from the increased neutral sugar content and the decreased molecular weight of the acid-extractable pectin) and it is striking that this pectin mainly occurs in the acid extracts (de Vries *et al.*, 1982). This suggests that pectin molecules contain acid-labile bonds.

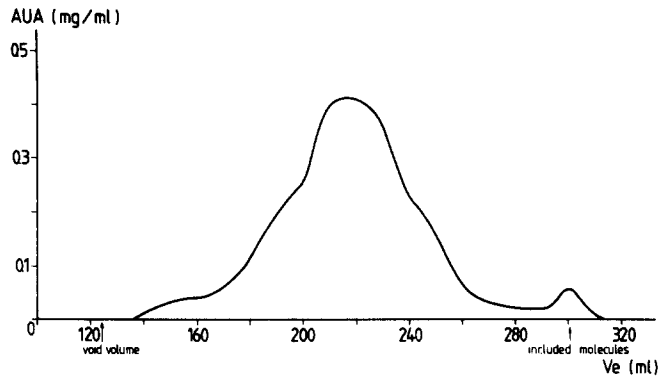

Fig. 3. Gel permeation chromatography on Sephacryl S-300 of a pectate lyase degraded pectin. Ve = elution volume. The substrate was a DEAE-cellulose purified pectin from the oxalate extract of AIS from senescent apples. Degradation with pectate lyase as described by de Vries *et al.* (1983*a*). Extent of degradation was 4%. The eluant was water.

TABLE 3
Neutral Sugar Composition of Pectate-Lyase-Degraded Pectin Fractions Fractionated as Shown in Fig. 3

	Elution volume (ml)			
	130–180	*180–210*	*210–250*	*250–320*
Anhydrouronic acid, %	8	28	58	6
Neutral sugars (mol/mol galacturonate residues)	1·01	0·30	0·08	0·05
Mol rhamnose/mol arabinose	0·13	0·06	0·11	0·04
Mol galactose/mol arabinose	1·20	0·80	0·70	0·90
Mol xylose/mol arabinose	0·04	0·06	0·13	0·08
Mol glucose/mol arabinose	0·06	0·07	0·16	0·08

In the theory of elongation growth of Cleland (1971), a role for acid-labile bonds has been postulated. The neutral sugar composition of the acid extractable pectin does not differ from that of the buffer- and oxalate-extractable pectin, suggesting that the acid-labile bonds broken during acid extraction are not arabinose glycosidic linkages.

TABLE 4

Free Glycan Sugars Present in the Oxalate Extracts of AIS from Apples of Different Stages of Ripeness. Free Glycan Sugars: Separable from Galacturonan by DEAE-Cellulose Ion Exchange Chromatography (mg/g AIS)

Sugar residue	*Unripe*	*Unripe cellulase*[a]	*Ripe*	*Senescent*
Rhamnose/fucose	0	0	0	0
Arabinose	0	0·02	0·04	0·10
Xylose	0·07	0·09	0·15	0·10
Galactose	0·03	0·03	0·08	0·13
Glucose	0·09	0·11	0·15	0·21
Total	0·19	0·25	0·42	0·55

[a] AIS from unripe apples treated with cellulase as described by Voragen *et al.* (1979).

What happens during acid-extraction remains unclear. The events occurring may have a physical rather than a chemical nature. The amounts of neutral polysaccharide residues, which are not covalently linked to galacturonans, present in the oxalate extract, are given in Table 4. During ripening, polysaccharides containing xylose and glucose residues can be extracted, suggesting that during ripening changes in the hemicelluloses take place. Furthermore the other extracts contain some free xylose and glucose containing polysaccharides. The larger part (about 75%) of the glycan residues in the extracts, however, is covalently bound to galacturonan residues. After ripening, the amounts of free galactose and arabinose residues also increase. This enhanced extractability probably results from wall loosening. Release of xyloglucans and glucans during growth has been reported (Johnson, 1979; Yamaota *et al.*, 1980). Ripening and growth are made possible through the same processes: in both cases, cell wall loosening occurs.

As reported in a previous paper (de Vries *et al.*, 1981), ripening of apples can to some extent be simulated by cellulase action. However, cellulase activity is probably absent in apples (Ben-Arie *et al.*, 1979). During growth, the activity of 1,3-glucanases increases (Goldberg, 1980). This may also happen during the ripening of apples.

TABLE 5

Distribution of the DP of Oligogalacturonides Present in the Pectate Lyase Digests of AIS from Unripe, Ripe and Senescent Apples Represented as a Percentage of the Total Amount of Anhydrouronic Acid Present in the AIS. The Pectate Lyase Digests were Alkali-Saponified and the DP of Oligomers Present was Determined by HPLC (de Vries *et al.*, 1983*a*)

DP	*Unripe*	*Ripe*	*Senescent*
2	0·05	0·02	0·31
3	–	–	0·09
4	–	–	–
5	–	–	–
6	1·2	0·9	1·2
7	5·6	5·1	6·0
8	7·0	8·0	8·1
9	8·5	13·1	12·6

As reported in a previous paper, the impact of pectin esterase (PE) on the distribution of the methoxyl groups in pectic substances can be investigated by HPLC-analysis of the pectins after degradation with pectate lyase and subsequent de-esterification (de Vries *et al.*, 1983*b*). After PE-action, an increased amount of di- and tri-galacturonic acid can be found in the chromatograms, due to the preferential attack of pectate lyase on blocks of de-esterified residues. Table 5 shows the results of pectate lyase degradation of apple AIS of different stages. In the senescent stage, some PE activity appears to be present. It cannot be excluded, however, that this PE activity can only be observed in senescence because of a better accessability of the pectate lyase to the substrate. It has been shown that it is unlikely that the biosynthesis of apple pectic substances passes through a stage of 100% esterification after which partial de-esterification by pectin esterase occurs. The overall DE is fairly constant during ripening (Table 1); the same has been reported in literature for apples (Knee, 1978), pears (Esau *et al.*, 1962) and strawberries (Neal, 1965). In avocado ripening, the DE decreases (Dolendo *et al.*, 1966; Eaks & Sinclair, 1978). No general

relation between PE level and ripening can be deduced from literature data. In apples, PE activity increases during ripening (Lee, 1969). Our results suggest that PE activity is not an important factor in the ripening process. However, when a combined PE/polygalacturonase action occurred the resulting pectin fragments were probably not detected.

In conclusion, our results suggest that the pectin molecules are hardly affected by ripening. In senescence degradation occurs. O'Beirne *et al.* (1981) observed a substantial decrease in DE during storage of apples at 0°C for 11 months. It is possible, that our conclusions do not apply to ripening and storage under other conditions and to other apple varieties.

REFERENCES

Ahmed El Rayah Ahmed & Labavitch, J. K. (1977). *J. Food Biochem.* **1**, 361.

Albersheim, P., Nevens, D. J., English, P. D. & Karr, A. (1967). *Carbohyd. Res.* **5**, 340.

Ben-Arie, R., Kislev, N. & Frenkel, C. (1979). *Plant Physiol.* **64**, 197.

Cleland, R. (1971). *Ann. Rev. Plant Physiol.* **22**, 197.

Darvill, A. G., Roberts, D. P. & Hall, M. A. (1975). *J. Chromatog.* **115**, 319.

van Deventer-Schriemer, W. H. & Pilnik, W. (1976). *Lebensm.-Wiss. u.-Technol.* **9**, 42.

Dolendo, A. L., Luh, B. S. & Pratt, H. K. (1966). *J. Food Sci.* **31**, 332.

Dorofeeva, L. S., Sapozhnikova, E. V. & Kiseleva, R. E. (1973). *Applied Biochem. Microbiol.* July, 54. (Translated from Russian, original, 7 (1), 1971.)

Eaks, I. L. & Sinclair, W. B. (1978). *J. Am Hortic. Sci.* **103**, 846.

Esau, P., Joslyn, M. A. & Claypool, L. L. (1962). *J. Food Sci.* **27**, 509.

Goldberg, R. (1980). *Physiol. Plant.* **50**, 261.

Johnson, K. G. (1979). *Plant Physiol.* **63**, 5 suppl., 142.

Knee, M. (1973). *Phytochemistry* **12**, 1543.

Knee, M. (1978). *Phytochemistry* **17**, 1261.

Knegt, E., Kramer, S. J. & Bruinsma, J. (1974). *Colloq. Int. CNRS* **238**, 355.

Lee, Y. S. (1969). *Measurement, characterization and evaluation of pectinesterase in apple fruits.* PhD Thesis, University of Maryland.

Mohr, W. P. (1979). *J. Food Technol.* **14**, 521.

Neal, G. E. (1965). *J. Sci. Food Agric.* **16**, 604.

O'Beirne, D., van Buren, J. P. & Mattick, L. R. (1981). *J. Food Sci.* **47**, 173.

Platt-Aloia, K., Thomson, W. W. & Young, R. E. (1979). *Plant. Physiol.* **63**, 5 Suppl., 111.

Sawamura, M., Knegt, E. & Bruinsma, J. (1978). *Plant Cell Physiol.* **19**, 1061.

Takeuchi, Y. & Komamine, A. (1980). *Physiol. Plant* **50**, 113.

Ulrich, R. & Hartman, C. (1967). *Ann. Nutrition Aliment.* **21**, B168.

Versteeg, C. (1979). *Pectinesterases from the orange fruit – their purification, general characteristics and juice cloud destabilizing properties.* PhD Thesis, Agricultural University, Wageningen, The Netherlands.

Voragen, A. G. J., Krist, R., Heutink, R. & Pilnik, W. (1979). Apple cell wall digestion by polysaccharide degrading enzymes. In: *Food Process Engineering*, Vol. 2: Enzyme Engineering in Food Processing, eds P. Linko & J. Larinkari, Applied Science Publishers, London, pp. 264–75.

de Vries, J. A., Voragen, A. G. J., Rombouts, F. M. & Pilnik, W. (1981). *Carbohyd. Polymers* **1**, 117.

de Vries, J. A., Rombouts, F. M., Voragen, A. G. J. & Pilnik, W. (1982). *Carbohyd. Polymers* **2**, 25.

de Vries, J. A., den Uijl, C. H., Voragen, A. G. J., Rombouts, F. M. & Pilnik, W. (1983*a*). *Carbohyd. Polymers* **3**, 161.

de Vries, J. A., Rombouts, F. M., Voragen, A. G. J. & Pilnik, W. (1983*b*). *Carbohyd. Polymers* **3**, 245.

Wallner, S. J. & Bloom, H. L. (1977). *Plant Physiol.* **60**, 207.

Yamaota, R., Sakurai, N., Shibata, K. & Masuda, Y. (1980). *Plant Cell Physiol.* **21**, 373.