Pergamon

0031-9422(94)00927-9

*Phytochemistry*, Vol. 39, No. 3, pp. 491–494, 1995

0031 9422/95 $9.50 + 0.00

# PECTIN ESTERASE ACTIVITY AND PECTIN METHYL ESTERIFICATION IN HEATED GOLDEN DELICIOUS APPLES

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(*Received in revised form* 27 *October* 1994)

**Key Word Index**—*Malus domestica*; Rosaceae; apple; cell walls; heat treatment; pectin methyl esters.

**Abstract**—Pectin methylesterase activity and the degree of methyl esterification of cell wall pectins were measured in Golden Delicious apples after a 4 day heat treatment at 38° and after subsequent storage at 0° of heated and unheated apples. Enzyme activity increased similarly during storage in both heated and unheated apples, although heated fruit softened much less than unheated. During storage, the degree of esterification decreased in both heated and unheated apples in water- and CDTA-soluble pectin, but not in insoluble pectin. In a comparison of three methods of determining methyl groups, similar values were obtained for degree of bulk esterification of apple cell walls, regardless of treatment. Results presented do not confirm a role for pectin methylesterase or for de-esterification in apple fruit softening.

## INTRODUCTION

Apples exposed to a prestorage heat treatment of 4 days at 38° remained firmer during storage at 0° and softened more slowly during ripening at 20° after storage than unheated fruit [1]. The mechanism underlying this phenomenon has only partially been characterized. Pectin solubilization decreased [2] and calcium binding to cell walls increased [3] in heated apples, compared with unheated fruit. The purpose of this research was to investigate the effects of prestorage heating on pectin esterification in apple cell walls.

Since calcium binds to pectin polymers with a low degree of esterification, we hypothesized that pectin methylesterase. (EC 3.1.1.11) activity, with a temperature optimum *in vitro* above 40° [4, 5], might be enhanced by the heat treatment. This would lead to a reduction in the degree of pectin methylation, and in turn an increase in the binding of endogenous calcium to the cell wall, which would result in fruit remaining firmer.

We compared three methods of analysing the degree of methylation, each based on different physical characteristics of methylated cell walls. Cross polarization magic angle spinning-nuclear magnetic resonance (CPMAS-NMR) [6] can be used to quantitate O-Me, anomeric C, and carbonyl groups in cell walls, by measuring their resonance in blocks of critical point dried tissue which are otherwise unaltered and unextracted. The method of Klavons and Bennett [7] is based on hydrolysing pectin methyl esters to methanol and complexing the methanol to yield a colored product. A modification of the method of Maness *et al.* [8] is based on $NaBH_4$-mediated reduction of esterified uronosyl residues to galactose and measurement of the decrease in galacturonic acid compared with nonreduced samples.

## RESULTS

Pectin methylesterase activity was not affected by heating at 38°, either immediately after treatment or after 5 months of 0° storage (Table 1). Although activity of the enzyme more than doubled during storage, the firmness of heated fruit remained similar to that at harvest, while unheated fruit softened (Table 1).

The degree of pectin esterification in CDTA-soluble and insoluble pectin was about 60% at harvest, while water-soluble pectin was over 80% esterified (Fig. 1). The heat treatment caused a 30% drop in the amount of esterification of the water-soluble fraction, but no change in the other two fractions. However, the degree of esterification during storage decreased in both the water- and CDTA-soluble fractions of both heated and nonheated apples, while the insoluble fraction showed no change.

The overall level of esterification in the cell wall was about 60%, regardless of the method used to determine methylation. Degree of bulk esterification did not change as a result of either heat treatment or storage (Table 2). The changes recorded in the esterification of water- and CDTA-soluble fractions were obscured when total cell walls were assayed.

## DISCUSSION

Pectin methylesterase activity increased in apples during ripening immediately after harvest [9]. We found that the enzyme appears to be stimulated by low temperature

Table 1. Pectin methylesterase activity and fruit firmness in Golden Delicious apples at harvest, after 38° for 4 days, and after 5 months storage at 0°

| | Pectin methylesterase activity ($\mu$mol COOH $min^{-1}$ 100 $mg^{-1}$ cell wall) | Firmness (*N*) |
|---|---|---|
| At harvest | 29.6 | 66 |
| After 4 days at 38° | 29.0 | 65 |
| After 5 months at 0° | | |
| Unheated | 70.6 | 47 |
| Heated | 68.7 | 64 |

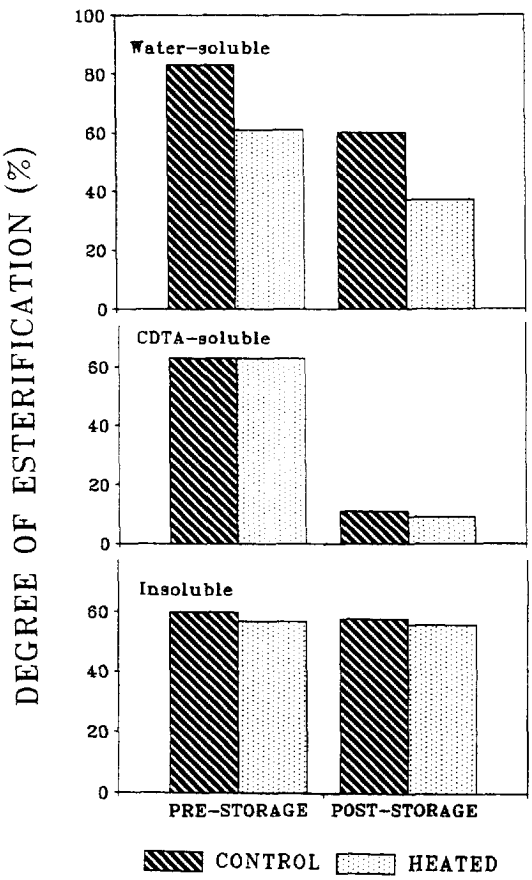


Fig. 1. Degree of methyl esterification in three cell wall fractions (water-soluble, CDTA-soluble, insoluble) from heated and unheated apple fruit before storage and after 5 months of 0° storage.

storage, as well, as already shown for nectarines [10]. The lack of change of esterification in the insoluble pectin may mean that portions of this fraction which become demethylated move into the water- or CDTA-soluble fractions. Plant pectin esterases cause a blockwise de-esterification [11], and continued activity during storage could result in the observed progressive decrease in methyl groups in the two soluble fractions.

Although there have been previous reports on the degree of apple pectin esterification during ripening directly after harvest, to the best of our knowledge there have been no studies of changes after extended storage. Knee [12] found that total methylation remained at 78% during 25 days of ripening, although the esterification in water-soluble pectin increased from 60% to 80% during that time. Yoshioka *et al.* [13] also found an increase in esterification of water-soluble pectin during apple ripening and a decrease in the EDTA-soluble fraction (which is structurally similar to the CDTA-soluble fraction reported here). These results support our findings regarding the degree of esterification in bulk tissue and in the CDTA-soluble fraction, although our results with water-soluble pectin differ.

Determination of the degree of esterification has often been judged problematic, due to the possibility of artifactual results. The non-invasive NMR method and colorimetric techniques based on cell wall digestions all gave almost identical values for degree of bulk esterification. Since each method used is based on different biochemical or biophysical principles of analysis, the uniformity of the results is indicative of the reliability of the value obtained.

Neither pectin methylesterase activity, which increased in apples of both treatments during storage, nor degree of esterification were correlated with fruit softening. Ripening-impaired tomato mutants *nor* (nonripening) and *Nr* (never ripe) also do not soften, despite having pectin esterase activity similar to that of normally-ripening tomatoes [14]. Loss of cell-wall galactose residues, a feature of both apple [15] and tomato [16] ripening, was also not affected in either heated apples [17] or ripening-impaired *rin* tomatoes [16]. The mode of action of heat treatments in maintaining apple fruit firmness remains unresolved.

Solubilization of polyuronides is the only degradative activity in the cell walls of ripening apples shown thus far to correlate with softening (or lack thereof) in either heated or nonheated fruit [15, 17]. However, there is insufficient polygalacturonase in apples to account for the extent of polyuronide solubilization [15]. Yoshioka *et al.* [13] have previously pointed out that pectin methylesterase activity in apples does not always correlate with loss of firmness, which is confirmed by the results presented here. Nonetheless, both Knee [12] and Yoshioka *et al.* [13] suggested that the de-esterification process itself, regardless of its catalysing agent, is closely linked with apple fruit softening. Our data do not confirm

Table 2. Comparison of CPMAS-NMR, hydrolytic and reductive methods of determining pectin methyl esterification in Golden Delicious apples

| | CPMAS-NMR | Hydrolytic* | Reductive† |
|---|---|---|---|
| | Degree of methyl esterification (%) | | |
| At harvest | — | 60 | 59 |
| After 4 days at 38° | — | 59 | 58 |
| After 5 months at 0° | | | |
| Unheated | 59 | 58 | 61 |
| Heated | 56 | 57 | 59 |

*Method of Klavons and Bennet [7].
†Modified method of Maness *et al.* [8].

a role for de-esterification of pectins in apple fruit softening, since results were similar in soft unheated fruit and in firm heated fruit.

## EXPERIMENTAL

Golden Delicious apple fruits were stored directly after commercial harvest at 0° or held for 4 days at 38° before storage. Fruits were removed from storage for examination after 5 months. Fruit firmness was measured at each examination on opposite pared sides of each of 10 apples, using a Hunter-Spring penetrometer equipped with an 11 mm diam. probe.

Pectin methylesterase activity was measured in triplicate samples of water-insoluble solids (WIS). This fr. was prepd from cortical tissue from 5 apples (200 g) by homogenization for one min with 200 ml of 25 mM ascorbate buffer (ph 4.5). The homogenate was centrifuged at 23 000 g for 15 min, after which the pellet was re-extracted ( × 2) in ascorbate buffer. The final pellet was lyophilized and stored in an air-tight container. Activity was measured by suspending 50 mg of WIS in 0.2M NaCl and 0.2% pectin (Sigma) having 81% esterification. The reaction was performed at 38° in a Radiometer pH stat by holding the pH at 6.5 by titrating with 0.1N NaOH [18]. After 15 min, the amount of NaOH added was determined and the results were expressed as μmol COOH-$min^{-1}$ 100 $mg^{-1}$ WIS.

Degree of esterification was determined by 3 methods. In all cases, analyses were performed using a minimum of 3 replicate samples. For the NMR measurements, 5 × 5 × 3 mm blocks of tissue were prepd from 5 apples and critical-point dried. About 400 mg of tissue blocks were used for each analysis. NMR spectra (*ca* 18 kHz spectral width) were obtained at a $^{13}C$ frequency of *ca* 75 HMz (*ca* 4.7 Tesla); 512 points were sampled. All chemical shifts were assigned relative to the anomeric C resonance ($\delta$105). Samples were spun at *ca* 4 kHz at the magic angle (54.7°). Spectra used for quantitative measurements were acquired using $^{1}H$–$^{13}C$ polarization transfer times of 25, 50, 100, 200, 400, 800, 1000, 2000, 4000 and 6000 μs. Individual CPMAS NMR resonances ($R_n$; n = carbonyl including spinning sidebands, anomeric C and O–Me) were linear baseline corrected and integrated by standard numeric methods. The degree of methyl esterification was calcd as in ref. [6] with the modification that $I_{n,max}$ was used rather than $I_n$. $I_{n,max}$ was calcd using a modified Gauss–Newton curve-fitting procedure [19, 20] and was used in these calcns because of the heterogeneous nature of the rate constant describing $^{1}H$–$^{13}C$ polarization transfer.

For the hydrolytic and reductive esterification assays, cell walls were prepd by homogenizing 300 g of cortical tissue from 10 apples with 700 ml of 70% EtOH for 2 min. The residue was filtered onto glass fibre paper and homogenized ( × 2) with 70% EtOH, once with 100% EtOH, and left to dry at room temp. overnight. Samples (100 mg) of the resulting alcohol insoluble substances (AIS) were serially extracted with $H_2O$ and with 0.02% CDTA, pH 6.5.

Both unextracted AIS and the 3 frs (water-, CDTA-soluble and insoluble residue) were measured by the method of ref. [7], which is based on the hydrolysis of pectin methyl esters to MeOH, oxidation of the MeOH to HCHO, and condensation with 2,4-pentanedione to yield a coloured product.

The reductive method used was a modification of the method of ref. [8]. Duplicate 2 mg samples of AIS were weighed into screw cap test tubes, and one sample was incubated overnight in 1 ml of 10 mg $ml^{-1}$ $NaBH_4$ in 50% EtOH. This sample was then neutralized with HOAc, dried, and washed a number of times with HOAc: MeOH (1:9) and then MeOH. Both duplicates were then dissolved in 67% $H_2SO_4$ and the uronic acid content determined. Incubation with $NaBH_4$ converted esterified uronosyl residues to galactose, so the difference in uronic acid content between the duplicate samples was the amount of uronic acid which contained Me esters.

*Acknowledgements*—This research was funded by a grant from the United States–Israel Binational Agricultural Research and Development Fund. This is publication 1205-E, 1993 series from the Agricultural Research Organization-Volcani Center, Bet Dagan, Israel.

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