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USE OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY TO DETERMINE CELL WALL CHANGES IN APPLE FRUIT

J.D. Klein, S. Lurie, P. Irwin

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USE OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY TO DETERMINE CELL WALL CHANGES
IN APPLE FRUIT

Investigators' Names
(Principal listed first)

J D KLEIN

S LURIE

P L IRWIN

Investigators' Institutions

VOLCANI CENTER -ARO

VOLCANI CENTER- ARO

USDA-ERRC

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Principal Investigator

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C. Abstract

Prestorage heating (38°C for 4 days) of apples leads to enhanced retention of fruit firmness upon removal from storage, compared to unheated fruit, but the mechanism of action is unknown. Postharvest treatment with calcium is also known to extend storage life of apples. Chemical fractionation of cell walls and determination of the uronide portion and degree of methyl esterification of pectic components was performed in order to compare standard methods with 'less invasive' nuclear magnetic resonance (NMR) techniques. Cortical tissues of heated fruit had more insoluble and less water soluble pectin than unheated fruit, although the total pectin content was similar in both treatments. The average degree of methyl esterification was 59%. No effect of calcium or heat treatment on degree of methyl esterification was observed in pectic fractions or in critical-dried whole tissues, using colorimetric and NMR techniques, respectively. Perturbations of the polyuronide motions in apple cell walls, as measured by relaxation behavior, followed the order: local < internal < supramolecular. Taken together, these data reinforce the notion that, despite the increased solubility of pectin in softening fruit, the disruption of cohesion in apple cortical tissues seems to result from processes other than the cleavage of the polyuronide backbone.

D. Objectives of the original research proposal

The major objective of this research was to examine cell wall changes in apple fruits that had undergone prestorage heating (38°C for 4 days), compared with fruit that had been placed in storage directly after harvest. In addition, prior to storage, separate lots of heated and unheated fruit were dipped in CaCl_2 , which is known to help maintain postharvest quality. Changes were to be identified using nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopy of intact cell walls. Chemical fractionation of cell walls and determination of the uronide portion and degree of methyl esterification of pectic components was also performed in order to compare standard methods with less 'invasive' NMR techniques. For the sake of completeness, ripeness characteristics, ethylene and CO_2 production, and Ca concentration in the flesh were also examined, although we had previously published these data from similar experiments (Klein and Lurie, 1990; Lurie and Klein, 1992).

E. Research report

I. Ripening indices, uronide content, and degree of methyl esterification of heated and calcium-treated apples

Materials and methods

'Golden Delicious' apple fruits were stored directly after harvest (controls), or dipped for 5 minutes in 3% CaCl_2 prior to storage. A third group was held for 4 days at 38°C and was then divided into two groups. One subgroup was dipped in CaCl_2 as described, while the other was stored directly at 0°C. Fruit firmness, skin color, titratable acidity, and soluble solids content were examined upon removal from 4 months of storage and after a further 7 days shelf life at 20°C, as described previously (Klein et al, 1990).

Alcohol insoluble solids (AIS) were prepared 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 fiber paper and homogenized twice more with 70% EtOH, once with 100% EtOH, and left to dry at room temperature overnight. Samples (100 mg) of AIS were acid digested and assayed for Ca content using atomic absorption spectrometry.

Both degree of methylation and galacturonic acid residues were determined using NMR and colorimetric (heated and unheated fruit only) techniques. For the NMR measurements, critical-point dried tissues were treated as described earlier (Irwin, et al., 1985). For

the colorimetric assays, serial extraction of AIS was performed with water and 0.02M CDTA pH 6.5. Uronic acid content of each filtrate was measured by the method of Blumenkranz and Asboe-Hansen (1973). Degree of methylation was measured by the method of Klavons and Bennet (1986), which is based on the hydrolysis of the pectin methyl esters to methanol, oxidation of the methanol to formaldehyde, and condensation with 2,4 pentanedione to yield a colored product.

In a separate experiment, we used unheated Golden Delicious apples that had been stored for 6 months, and heated and unheated Granny Smith apples that had been stored for 5 months. All fruit were stored at 0°C and then ripened at 20°C for two weeks prior to measuring firmness and tissue sampling. CWM from 200 g of cortical tissue from each treatment was prepared by grinding the sample in a Waring blender with 400 ml of -20°C 80% ethanol. The slurry was filtered under vacuum and the residue reground with 200 ml of phenol:acetic acid:water (2:1:1), and centrifuged at 5000 x g for 20 min at 4°C. The pellet was taken up in cold acetone, washed extensively and air dried.

Degree of esterification was determined by a modification of the method of Maness et al. (1990). Duplicate 2 mg samples of CWM were weighed into screw-cap test tubes, and one sample was incubated overnight in 1 ml of 10 mg/ml NaBH₄ in 50% ethanol. This sample was then neutralized with acetic acid, dried, and washed a number of times first with acetic acid:methanol (1:9) and then methanol. Both duplicates were then dissolved in 67% H₂SO₄ and the uronic acid content was determined as described earlier. Incubating with NaBH₄ converts esterified uronosyl residues to galactose, so the difference in uronic acid content between the duplicate samples was the amount of uronic acid which contained methyl esters.

Results and discussion

We have previously found synergistic benefits from dipping heated fruit in Ca (Lurie and Klein, 1992). Such fruit were firmer than either heated or Ca-dipped apples, and did not have the significant loss of acidity and enhanced yellowing of heated fruit. In the present case, by the end of shelf life no effect of Ca could be ascertained in either heated or unheated fruit (Table 1). The well-documented effect of heating on firmness retention (Klein et al., 1990) was again evident. Treatments did not affect total soluble solids (TSS), which averaged 14.8% at removal and 15.5% after shelf life.

Table 1. Firmness (N), titratable acidity (%), and ground color of 'Golden Delicious' apples treated at harvest. Fruit were examined after 4 months storage at 0°C and following 7 days at 20°C.

	Firmness (N)		TA (%)		Color ^z	
	Rem	SL	Rem	SL	Rem	SL
Control	50 b	50 b	0.23 ab	0.20 a	4.5 b	5.1 ab
Calcium	52 b	51 b	0.26 a	0.20 a	4.2 c	4.7 b
Heat	60 a	57 a	0.22 b	0.19 ab	5.1 a	5.5 a
Heat/Ca	58 a	59 a	0.15 c	0.15 b	4.8 b	5.5 a

^zColor was measured with a Techwest Apple Color Meter, where 1=green and 10=yellow. Mean separation within columns by Duncan's multiple range test at the 5% level.

Heated fruit produced 10-20% less CO₂ than unheated fruit during shelf life (Fig. 1), which we have noted before (Klein and Lurie, 1990). Dipping unheated fruit in Ca led to a slower decrease in CO₂ production after the climacteric, but did not affect CO₂ production of heated fruit. Ethylene production by fruit that had been dipped in Ca tended to be higher than that of undipped fruit (Fig. 2). In contrast to previous results (Klein and Lurie, 1990), heating per se did not affect ethylene production.

Fig. 1. CO₂ production by heat and Ca-treated 'Golden Delicious' apples after removal from 4 months storage at 0°C. Means of 4 replicates of 2 fruit each.

Fig. 2. Ethylene production by heat and Ca-treated 'Golden Delicious' apples after removal from 4 months storage at 0°C. Means of 4 replicates of 2 fruit each.

Calcium content of the insoluble material in the cell wall was lower than we had found previously in 'Anna' apples (Lurie and Klein, 1992). Control fruit had 1.28 mg Ca/100 g fresh weight, while heated fruit had 1.41 mg Ca/100 gfw. Unheated fruit that had been dipped in Ca had 1.66 mg Ca/100 gfw, while heated and dipped fruit apparently didn't take up any Ca, since it had only 1.45 mg Ca/100 gfw. The overall lack of Ca effect on ripeness parameters of heated fruit is thus understandable, with the exception of the enhanced production of ethylene (Fig.2). Heated apples are known to take up less Ca than unheated apples (Lurie and Klein, 1992), but this is the first time that we have found no uptake at all.

In determining the degree of pectin methyl esterification from NMR data, the following calculations were made:

- X_i = any i^{th} member, up to X_n , of the X array (a unitless count of Fourier Transformed FID data points (real only))
- $\hat{X}_i = X_i - X_1$
- $\hat{Y}_i = (X_i - X_1) - \left\{ \hat{X}_i \frac{Y_n - Y_1}{X_n - X_1} \right\}$
- $\hat{Y}_i^j = (\hat{Y}_i^j - \hat{Y}_1^j) - \left\{ \hat{X}_i^j \frac{\hat{Y}_n^j - \hat{Y}_1^j}{\hat{X}_n^j - \hat{X}_1^j} \right\}$
- $I_n^j = \int_{i=0}^n \hat{Y}_i^j d\hat{X}_i^j$ whereupon I_n^j is unitless; all I_n^j s were normalized to the largest integral; superscript j designates the different functional groups.
- T_{CH} & $T_{1\rho}$ were calculated, using the Gauss-Newton algorithm, from the relationship shown below; τ_0 is a parameter which corrects for the fact that I_n^j does not approach 0 as the limit $\tau \rightarrow 0$

$$I_n^j = \frac{I_n^{j, \max}}{1 - \frac{T_{CH}}{T_{1\rho}}} * \left[1 - e^{\left\{ \frac{T_{CH}}{T_{1\rho}} \right\} - 1 * \left\{ (\tau - \tau_0) / T_{CH} \right\}} \right] * e^{\left\{ -(\tau - \tau_0) / T_{1\rho} \right\}}$$

The resulting data are presented in Tables 2, 3, and 4. Because of the large error terms in data for the cell wall relaxation terms T_{CH} and $T_{1\rho}$, no real differences were observable in these parameters. A representative set of stacked CPMAS NMR spectra appears in Fig. 3.

Table 2. $I_n^{j, \max}$ for the different treatments.

Treatment	$I_n^{j, \max} \pm \epsilon$			
	Carbonyl	Anomeric	Ring Carbon	Methoxy
C+Ca+7d	0.071 $\pm$ 0.004	0.204 $\pm$ 0.010	1.000 $\pm$ 0.020	0.039 $\pm$ 0.002
C-Ca+7d	0.065 $\pm$ 0.005	0.208 $\pm$ 0.004	1.000 $\pm$ 0.070	0.038 $\pm$ 0.003
H+Ca+7d	0.085 $\pm$ 0.015	0.228 $\pm$ 0.018	1.000 $\pm$ 0.020	0.047 $\pm$ 0.015
H-Ca+7d	0.062 $\pm$ 0.009	0.206 $\pm$ 0.006	1.000 $\pm$ 0.030	0.035 $\pm$ 0.003

Table 3. Calculation of % Methyl Ester, % Uronosyl residues & ratio of anomeric C to other ring Cs from I_n^j s provided in Table 1.

Treatment	% O-CH ₃	% Uronide	$\frac{C \begin{smallmatrix} \nearrow O \\ \searrow O \end{smallmatrix}}{COH}$
C+Ca+7d	54.96	34.66	0.19
C-Ca+7d	58.76	31.02	0.20
H+Ca+7d	54.80	37.52	0.21
H-Ca+7d	55.74	30.03	0.19
Average $\pm\epsilon$:	56.06 $\pm$ 1.84	33.31 $\pm$ 3.44	0.20 $\pm$ 0.01
Expected Value:	[40.0-60.0]	[26.00]*	[0.20]

*assuming that pectin is 80% uronic acid and makes up 1/3 of the cell wall mass

Table 4. Estimates ($\pm \epsilon$) of the cross polarization parameters T_{CH} &

Treatment	$T_{1\rho}$		$T_{1\rho}$	
	C=O	O-CH ₃	C=O	O-CH ₃
C+Ca+7d	433 $\pm$ 130 μ s	6.1 $\pm$ 0.2 ms	445 $\pm$ 200 μ s	6.0 $\pm$ 0.2 ms
C-Ca+7d	271 $\pm$ 81 μ s	7.2 $\pm$ 1.4 ms	328 $\pm$ 289 μ s	6.1 $\pm$ 0.9 ms
H+Ca+7d	491 $\pm$ 196 μ s	6.6 $\pm$ 3.6 ms	644 $\pm$ 483 μ s	5.2 $\pm$ 3.5 ms
H-Ca+7d	538 $\pm$ 237 μ s	4.5 $\pm$ 1.1 ms	129 $\pm$ 86 μ s	6.9 $\pm$ 1.7 ms

Fig. 3. Sample CPMAS NMR spectra.

Heat and calcium treatments did not result in differences in degree of methylation in cortical tissues of 'Golden Delicious' apples. The degree of methylation averaged approximately 58% in all treatments and pectic fractions (except for the CDTA-extractable fraction, which is relatively small). This was observed using both 'wet chemistry' and NMR techniques, with remarkably (and encouragingly) similar results despite the great differences in method (Table 5). NMR measurements did not detect differences between fruit upon removal from storage and after shelf-life (data not shown).

Table 5. Degree of methylation in critically-dried tissues (NMR) and in water- or CDTA-soluble and insoluble pectin fractions (colorimetric).

Treatment	Degree of methylation (%)			
	NMR	Colorimetric		
	(total)	<u>H₂O</u>	<u>CDTA</u>	<u>Insol</u>
Control	59	58	10	60
Calcium	55			
Heat	56	60	15	57
Heat/Ca	55			

Despite differences in cultivar, length of storage, treatment, and firmness, there was no difference in degree of methyl esterification in 'Golden Delicious' and 'Granny Smith' apples, as measured by the method of Maness et al. (1990) (Table 6). Degree of methylation averaged 60%, which is comparable to the data presented in Table 5. This is further confirmation of the soundness of the methylation values obtained, despite the fact that each of the three techniques employed is based on a different method of detecting the presence of methyl esters.

Table 6. Degree of methylation in CWM from treated apples.

<u>Cultivar</u>	<u>Heated</u>	<u>Storage time</u>	<u>Firmness (N)</u>	<u>Degree of methylation (%)</u>
Golden				
Delicious	no	6 mos.	46	60
Granny				
Smith	no	5 mos.	61	60.5
	yes	"	67	59.5

Percent total uronides, as measured by NMR, did not vary by treatment, although there was a trend for a slightly higher figure in Ca-treated tissues (Table 3). These data are indicative of uronides in the critically-dried apple cell wall solid. However, when the cell wall was alcohol-extracted and fractionated, the amount of soluble polyuronides (measured as galacturonic acid) in AIS from heated apple tissue was proportionally lower than that in unheated tissue. Total galacturonic acid residues in both heated and unheated tissues averaged 398 mg / 100 g fresh weight cortical tissue. We have obtained similar results previously (Klein et al., 1990).

Table 3. Uronide concentration in critically-dried tissues (NMR) and in water- or CDTA-soluble and insoluble pectin fractions (colorimetric).

Treatment	<u>Percent uronides</u>			
	NMR (total)	Colorimetric		
		<u>H₂O</u>	<u>CDTA</u>	<u>Insol</u>
Control	31	17	10	73
Calcium	35			
Heat	30	4	8	88
Heat/Ca	37			

We originally hypothesised that heat treatments could delay softening of apple fruits by interfering with methyl deesterification of pectin chains. Data presented in this report reinforce the notion that degree of methylation of the pectins is not a factor associated with apple fruit softening (Knee, 1978). The heat treatment does decrease the solubilization of the pectic material, but not sufficiently to explain the retention of firmness observed. Processes other than cleavage of polyuronide chains appear to be involved, which means that the mechanism of softening in apple fruits remains incompletely-understood.

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II. Polyuronide motions in apple cell walls

We had intended to use electron paramagnetic resonance (EPR) and electron spin resonance (ESR) spectroscopy to study the relative molecular motions of apple cell wall pectic polysaccharides in response to heat and Ca treatments. Unfortunately, the tissue samples sent from Israel to the US proved unsuitable for ESR and EPR experiments, despite efforts by the US collaborator to dry the samples from Israel sufficiently so as to perform the multiple quantum work originally proposed. Fortunately, we were able to accomplish some of the originally proposed research goals using untreated apple tissue samples already at hand in Dr. Irwin's laboratory. The report follows.

F. Description of cooperation

Fruit samples were sent several times from Israel to the US. J.D. Klein visited P.L. Irwin in August 1991 to observe NMR analysis first-hand and to discuss where research emphasis should be placed in light of continuing equipment and sample difficulties. Contact was maintained throughout by computer mail, fax, and even regular post.

G. Evaluation of research achievement with respect to original research proposal and the benefit to agriculture.

We have demonstrated that changes in the degree of methylation are not involved in the maintenance of firmness of heat-treated apples. This has been shown using both colorimetric methods and 'less invasive' NMR techniques. Our data also show that despite the increased solubility of pectin in softening fruit, the disruption of cohesion in apple cortical tissues seems to result from processes other than the cleavage of the polyuronide backbone. Although a number of agrotechnical techniques, including Ca treatment and prestorage heating, are useful in maintaining fruit firmness, the mode of action of such treatments must be studied further to enable the development of even more effective methods for maintenance of apple quality during storage.

H. List of publications.

- 1) Klein, JD, J Hanzon, PL Irwin, S Lurie, and N Ben Shalom. 1992. Studies on the mechanism of firmness retention of apples heat-treated before storage. HortScience 26:189 (abst.) [Appendix 1]
- 2) Irwin, PL, MD Sevilla, W Chamulitrat, AE Hoffman, and J Klein. 1992. Localized, internal, and supramolecular polyuronide motions in cell wall matrices: a comparison of solid state NMR and EPR relaxation behavior. Journal of Agricultural and Food Chemistry 40:21045-2051
- 3) Klein, JD, J Hanzon, PL Irwin, S Lurie and N Ben-Shalom. 1993. Studies on the mechanism of firmness retention in heat-treated apples. Acta Horticulturae (in press) [Appendix 2]
- 4) Irwin, P, G King, T Kumosinski, P Pfeffer, J Klein, and L Doner. 1993. Solution state NMR and molecular modeling evidence for the entrapment of water in a simple carbohydrate complex. ACS Symposium Series (manuscript in preparation).
- 5) Klein, JD, PL Irwin, J Hanzon, and S Lurie. 1994. Prestorage heat treatment of apples does not affect degree of pectin methyl esterification. (manuscript in preparation)

Appendix 1

STUDIES ON THE MECHANISM OF FIRMNESS RETENTION OF APPLES HEAT-TREATED
BEFORE STORAGE

JD Klein, J Henson, PL Irwin¹, S Lurie* and N Ben Shalom

Volcani Center-ARO, Bet Dagan 50250 ISRAEL

¹ USDA-ERRC, Philadelphia, Pennsylvania

Prestorage heating of apples lead to enhanced retention of fruit firmness of as much as 10 N upon removal from storage, compared to unheated fruit. Further enhancement of firmness retention was obtained by dipping fruit in 2-3% calcium chloride after heating prior to storage. Cortical tissue of heated fruit had more insoluble and less water soluble pectin than unheated fruit, although the total pectin content was similar in both treatments. During the heat treatment neutral sugars were lost from the pectic fractions, with no accompanying decrease in galacturonic acid. No effect of heat treatment on degree of methyl esterification was observed in pectic fractions or in critical-dried whole tissues, using colorimetric and NMR techniques respectively. Treatment differences in dissolution of the middle lamella were not observable in electron micrographs. We suggest that the loss of neutral sugar side chains during the heat treatment may have lead to closer packing of the pectic strands and in turn hindered enzymatic cleavage during and after storage.

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Appendix 2

STUDIES ON THE MECHANISM OF FIRMNESS RETENTION IN HEAT-TREATED APPLES

JD Klein¹, J Henzon¹, PL Irwin², S Lurie¹ and N Ben-Shalom¹

¹ Agricultural Research Organisation-Volcani Center, Bet Dagan ISRAEL

² USDA-ERRC, Philadelphia, Pennsylvania

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ABSTRACT

Prestorage heating (38°C for 4 days) of apples leads to enhanced retention of fruit firmness upon removal from storage, compared to unheated fruit, but the mechanism of action is unknown. Cortical tissues of heated fruit had more insoluble and less water soluble pectin than unheated fruit, although the total pectin content was similar in both treatments. During the heat treatment, neutral sugars were lost from the pectic fractions, with no accompanying decrease in galacturonic acid. No effect of heat treatment on degree of methyl esterification was observed in pectic fractions or in critical-dried whole tissues, using colorimetric and NMR techniques, respectively. We suggest that loss of neutral sugar side chains during the heat treatment may have lead to closer packing of the pectin strands and in turned hindered enzymatic cleavage during and after storage.

INTRODUCTION

Prestorage heating of apples leads to enhanced retention of fruit firmness upon removal from storage, compared to unheated fruit. We investigated whether changes in the cell walls of heat-treated apples could be responsible for the increased firmness. We also compared colorimetric assays with nuclear magnetic resonance spectroscopy techniques. The former require extensive extraction of cell wall material, which may result in artifacts, while the latter only requires less-invasive critical-point drying of the tissue.

MATERIALS AND METHODS

'Golden Delicious' fruit were stored immediately at 0C after harvest, or were held at 38C for 4 days prior to storage. Cell wall components of the fruit were examined at harvest, after treatment, and after 3 months of storage at 0C.

Alcohol insoluble solids (AIS): cortical tissue was homogenized in 100% ethanol (100 g tissue/200 ml EtOH), filtered, reground twice in 70% EtOH, again in 100% EtOH and left to dry. AIS was serially extracted in water, 0.02M CDTA pH 6.5, and 0.05M Na₂CO₃ and used in colorimetric determinations of uronic acids (2) and degree of methylation (4), and for gas chromatography of neutral sugars (1).

Cell wall material (CWM): Blocks (2 x 3 x 5 mm) of cortical tissue were serially dried in 50-70-80-95-100% EtOH, which was then exchanged off with liquid CO₂. CWM was used in determining degree of methyl esterification by NMR (3).

RESULTS AND DISCUSSION

Heated fruit measured 60 N in firmness upon removal from storage, as opposed to 50 N for control fruit. This confirmed previously reported results (5).

Tissues from heated and control fruits did not differ in degree of methylation, which averaged 58% in all treatments and in both water-soluble and insoluble pectic fractions. This was observed using both 'wet chemistry' and NMR techniques, with similar results despite the great differences in method.

Immediately after treatment, heated fruit had less neutral sugars in soluble pectic fractions than did unheated fruit (Table 1). After storage, however, there was no difference between heated and unheated fruit in neutral sugar distribution. There was no difference between heated and unheated fruit in uronic acid distribution in pectic fractions immediately after treatment. After storage, however, heated fruit had less uronic acids in water- and CDTA-soluble pectic fractions than did heated fruit.

Table 1. Neutral sugar (NS) and galacturonic acid (UA) content (mg/100 gfw) of pectic fractions of 'Golden Delicious' apples.

Fraction	At harvest	Post heating	Control + 3 mos	Heat + 3 mos
Water				
NS	39	19	21	17
UA	18	12	49	16
CDTA				
NS	5	4	4	2
UA	34	34	47	29
Carbonate				
NS	62	41	40	42
UA	257	253	200	260
Insoluble				
NS	150	288	134	121
UA	21	17	20	18
Total				
NS	256	352	199	182
UA	330	316	316	323

CONCLUSIONS

Heat treatment causes a transient change in neutral sugar distribution in the cell wall. This change may lead to closer packing of pectic strands, in turn hindering enzymatic cleavage fruit softening. However, data on degree of methylation and total uronide concentration indicate that disruption of cohesion in apple cortical tissues may result from processes other than cleavage of the polyuronide backbone. The mode of action of heat treatments in retaining fruit firmness remains a subject for further study.

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