Indian J. Plant Physiol., Vol. 9, No. 3, (N.S.) pp. 217-223 (July-Sept., 2004)

CELL WALL HYDROLYSING ENZYMES AND FRUIT SOFTENING IN APPLE AS AFFECTED BY CONTROLLED ATMOSPHERE STORAGE CONDITIONS

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Received on 19 July, 2004, Revised on 20 Sept., 2004

SUMMARY

The fruits of apples cv. 'Golden Delicious' were harvested at preclimacteric and climacteric stages of ripening and stored at 1°C for six months under conventional cold (Cold; 0.03% CO_2 and 21% O_2), controlled atmosphere (CA; 3% CO_2 and 3% O_2) and ultra low oxygen (ULO; 3% CO_2 and 1% O_2) conditions. Maximum retention of fruit flesh firmness was observed under ULO followed by CA and cold storage conditions. The activities of both isolated and cell wall bound preparations of polygalacturonase showed no correlation with fruit firmness. The activity of pectin methylesterase was not affected by various storage conditions. Cellulase activity could not be detected. The activity of ß-D-galactosidase and the amount of soluble polyuronides in the flesh increased during storage and showed an inverse relation with fruit firmness under various storage conditions. Anion exchange chromatography on DEAE-Sephadex A-25 resin yielded only one peak of ß-galactosidase activity. The above results were almost similar in preclimacteric and climacteric apples. The possible mechanism by which the storage conditions affect fruit firmness and cell wall metabolism has been discussed.

Key words : **Apple, cell wall enzymes, controlled atmosphere, flesh firmness, storage .**

INTRODUCTION

Softening of harvested fruits has a major influence on consumers' acceptability. It serves as an important determinant of quality and influences the method by which commodities are handled and stored in market channels. The softening continues after harvest as the fruit ripens. Controlled atmosphere (CA) storage, particularly with ultra low oxygen (ULO) concentrations, diminishes the loss of fruit firmness compared to conventional cold storage (Lidster *et al*. 1981, Yahia 1998).

Softening of fruits occurs largely as a result of the enzyme mediated hydrolysis of cell walls. The activities of exo-polygalacturonases, pectin methyl esterases and galactosidases have been reported to be directly associated with the softening of apples during ripening (Bartley 1976, Huber 1983). Except for a report by Abeles and Takeda (1990), activity of cellulase has not been detected in ripening apples and thus does not seem to be involved in their softening (Huber 1983, Nelmes and Preston 1988).

Information on different O_2 and CO_2 concentrations of controlled atmosphere on apple fruit softening, in particular reference to the cell wall mediated changes, is scanty. The present communication deals with the changes in fruit firmness and activities of cell wall enzymes of 'Golden Delicious' apples harvested at preclimacteric

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and climacteric stages and stored under controlled atmosphere conditions.

MATERIALS AND METHODS

Apples from 8 years old 'Golden delicious' trees grown on M-9 rootstocks, were used in the experiment. The fruits were harvested at preclimacteric and climacteric ripening stages. The preclimacteric fruits had internal ethylene concentrations (in its central cavity) less than 0.05, and climacteric more than 0.6 µl (for details, see Brackmann *et al.* 1993). Immediately after picking, fruits were selected for uniformity and stored at 1°C. For the controlled atmosphere conditions, 40 kg of apples were placed in 240 1 containers. The concentration of CO_2 and O_2 were continuously monitored by gas analysers connected to a process computer. The storage conditions employed were (i) storage at 1°C in air having normal 0.03% CO_2 and 21% O_2, (ii) Controlled atmosphere (CA) storage at 3% CO_2 and 3% O_2 and (iii) ultra low oxygen (ULO) storage having 3% CO_2 and 1% O_2. The temperature under all the storage conditions was 1°C; the relative humidity and ethylene concentrations were maintained around 94% and 100 µ/1, respectively.

During a six month storage period, every second month 2 kg of the samples of apples containing 10-12 fruits from each treatment were randomly removed from storage containers for various estimations. The fruit flesh firmness was measured in $N.cm^{-2}$ by using a penetrometer having a plunger of 11 mm diameter. For enzyme analysis, the fruits were peeled and seeds removed. The flesh was freeze-dried, ground and stored at -20°C. The remaining water content of the freeze dried flesh tissue was around 4.4%, but was termed as dry tissue to express values of the various parameters.

The exo-D-polygalacturonases (EC.3.2.1.67), henceforth referred as PG, were extracted by a slightly modified method of Nevins (1970). In brief, one g of freeze dried flesh powder was suspended in 15 ml of deionized water, centrifuged, residue washed further 2 times with 15 ml of water and suspended in 10 ml of 50 mM Na-acetate buffer pH 6.5 for 30 min. The suspension was centrifuged and further washed with the buffer. The washings were discarded and the residue resuspended in 10 ml of 100 mM Na-acetate buffer (pH 6.5, containing 2.4 M NaCl and 0.3 ml of 1% Thimerosal). After 1 h, the suspension was centrifuged to use as a source to determine PG activity. All extraction procedures were carried out at 2 - 4°C and centrifuged at 10,000 g for 10 min. The PG activity was estimated according to the method of Bartley (1976), by measuring the hydrolytic release of hexuronic reducing groups from the substrate 'Orange polygalacturonic acid' (Sigma), at 30°C. The hexuronic acid residues were measured using Cu-reagent (Milner and Avigad, 1967) and taking galacturonic acid as the standard. The amount of hexuronic acid present in the assay mixture at 0-time was determined and subtracted from the final value. The total activity of the enzyme was expressed as µg galacturonic acid equivalents h^{-1} g^{-1} dry weight.

Pectin methyl esterases (EC.3.1.1.11), henceforth referred as PME, were extracted according to the method by Awad and Young (1979). In brief, 1 g freeze dried flesh powder was extracted with 10 ml of cold 0.4 M NaCl and adjusted to pH 7.5 with 3 N NaOH. After 1 h (desorption interval), the mixture was centrifuged at 6,000 g for 20 min and the supernatant used to determine PME activity. The assay mixture consisted of 3 ml of enzyme extract and 5 ml of 0.5% of apple pectin (Sigma) in 0.1 M NaCl and brought to pH 7.5 with 3 N NaOH. It was allowed to incubate for 30 min at 25°C. Care was taken to avoid the development of a low pH during incubation period by adding 1 ml of 0.01 N NaOH after every 10 min and computing this amount later on in final titration. After the end of the incubation period, the reaction was stopped by addition of 1 ml of teepol. The liberation of carboxyl groups by the action of PME on the substrate, was measured by titration with 0.01 N NaOH, and using 0.1% methyl red as an indicator. The total activity was expressed in meq of ester hydrolysed h^{-1} g^{-1} dry weight.

ß-D-galactosidases (EC.3.2.1.23) were extracted with cold 100 mM Na-acetate buffer, pH 6.5 and containing 2.4 M NaCl. After 1 h desorption interval, the mixture was centrifuged at 6,000 g for 20 min and the supernatent used to determine galactosidase activity. The assay mixture contained 0.25 ml of enzyme, 2.5 ml of 50 mM Na-acetate buffer (pH 4.5) containing (3.6% (w/v) bovine serum albumin and 1 mg of *p*-nitrophenyl-ß-

D-galactoside as substrate. After incubation of 30 min at 30°C, the reaction was stopped by adding 2 ml of 0.2 M Na_2CO_3, and the liberated *p*-nitrophenol was measured at 400 nm. The total activity was expressed as mg of *p*-nitrophenol liberated h^{-1} g^{-1} dry weight.

For cellulase (EC 3.2.1.4) activity in apples, several extraction and assay methods were tried, including viscometry (Abeles and Takeda 1990) and CM-Cellulose-RBB dye (Blue substrates e.V., Goettingen, Germany), but no activity could be detected.

At the last sampling stage (6 months), the following determinations were also made in both preclimacteric and climacteric apples.

For PG activity, an autolysis experiment, as described by Rushing and Huber (1984) was conducted. Enzymatically active cell walls were prepared by washing freeze dried flesh powder thoroughly with deionized cold water (4°C) to free it from all soluble materials. The residue which was the freshly prepared cell wall material, was placed in 10 ml of acetate buffer (50mM, pH 4.5, 150 mM NaCl and 0.2% Thimerosal) and incubated in a shaking water bath at 30°C. Aliquots of 0.5 ml were removed from the reaction mixture at the start and after 22 h of incubation and analysed for hexuronic acid by the method already described above. At the end of the incubation period, the reaction mixture was centrifuged at 10,000 g for 10 min and dried to a constant weight for cell wall dry weight determination. Uronides (µg equivalents) released during autolysis were added to obtain an estimate of initial dry weight. The activity was expressed as µg galacturonic acid equivalents released h^{-1} mg^{-1} cell wall dry weight.

Soluble polyuronides in the freeze dried flesh were estimated by the procedure of Blumenkrantz and Asboe-Hansen (1973) and expressed in terms of µg galacturonic acid equivalents g^{-1} dry weight.

Anion exchange chromatography was performed for galactosidases from climacteric apples. The column used was of 1.5 x 10 cm, packed with DEAE-Saphadex A-25 and equilibrated with 0.05 M Na-acetate (pH 6.0) buffer containing 100 mm NaCl. The galactosidase enzyme extract (as prepared earlier) was applied to the column and eluted with equilibriation buffer. Fractions of 1 ml were collected at a flow rate of 15 ml h^{-1}. The elution was carried out at 4°C. The fractions obtained on elution were assayed for galactosidase activity by the procedure already described.

The data presented in the tables are the means of three replications, separated by least significant difference (LSD) at P = 0.05.

RESULTS AND DISCUSSION

Fruit flesh firmness decreased with storage time in both preclimacteric and climacteric apples (Tables 1 and 2). The decrease in flesh firmness was more in apples stored in air than in CA and ULO storage conditions. The ULO conditions resulted in maximum retention of fruit firmness. The fruit firmness is affected by a loss of cell turgor pressure and structural and compositional changes in the cell wall. Since the humidity in all the storage chambers was maintained high (94 %), the major factor responsible for the decreased fruit firmness may be the changes in cell wall composition and structure. The decreased flesh firmness in apples under storage has earlier been reported by many workers (Lidster *et al*. 1981, Siddiqui and Bangerth 1996).

The activity of PG in preclimacteric apples (Table 1), throughout the storage period, was higher in fruit from air storage than in fruit from CA and ULO storage. However, in climacteric apples (Table 2), the PG activity was not significantly different among storage conditions. There was an apparent lack of relationship between flesh firmness and PG activity under various storage conditions. This lack could be either due to a lack of influence of PG on the process of softening or the factors such as incomplete extraction, lability, inhibition and multiplicity of the enzyme. In the present case, the addition of PVP, dithio-threitol and Ca^{2+} in the extraction media and the use of comparable amounts of fresh tissue or the increased amount of freeze dried tissue, did not improve the activity (data not shown).

The ability of PG to release hexuronic acid residues, from the enzyme rich cell wall preparations, i.e. autolysis

Table 1. Effect of storage conditions on fruit flesh firmness (N cm^{-2}) and total activities of polygalacturonase (µg galacturonic acid equivalents g^{-1} dw h^{-1}), pectin methyl esterase (meq. ester hydrolysed g^{-1}dw h^{-1}) and ß-galactosidase (µM of nitrophenol liberated g^{-1}dw h^{-1}) of *preclimacteric apples* stored for different periods.

Storage condition	Period of storage (month)		
	2	4	6
		Flesh firmness (0 day = 78.3[a])	
Cold	46.6[b]	38.3[b]	33.3[b]
CA	73.3[c]	67.4[c]	61.6[c]
ULO	76.9[d]	77.4[d]	77.4[d]
		Polygalacturonase (0 day = 66.7[a])	
Cold	90.7[b]	114.7[b]	111.7[b]
CA	72.7[a]	84.7[a]	87.7[a]
ULO	66.7[a]	78.7[a]	75.7[a]
		Pectin methyl esterase (NS) (0 day = 16.0)	
Cold	16.9	16.2	19.0
CA	16.9	16.9	19.6
ULO	17.6	16.9	19.0
		ß-D-Galactosidase (0 day = 35.8[a])	
Cold	49.9[b]	50.2[b]	65.7[b]
CA	40.2[c]	49.3[c]	54.1[c]
ULO	42.5c	44.4[d]	49.7[d]

Mean separation : Within columns of a parameter values having different letters indicate significant differences at P = 0.05

Table 2. Effect of storage conditions on fruit flesh firmness (N cm^{-2}) and total activities of polygalacturonase (µg galacturonic acid equivalents g^{-1}dw h^{-1}), pectin methyl esterase (meq. ester hydrolysed g^{-1}dw h^{-1}) and ß-galactosidase (µM of nitrophenol liberated g^{-1}dw h^{-1}) of *climacteric apples* stored for different periods.

Storage condition	Period of storage (month)		
	2	4	6
		Flesh firmness (0 day = 69.3[a])	
Cold	44.4[a]	39.8[b]	37.4[b]
CA	67.4[c]	47.4[c]	44.9[c]
ULO	70.8[a]	58.3[d]	59.1[d]
		Polygalacturonase (0 day = 51.7[a])	
Cold	69.7[b]	120.7[b]	135.7[b]
CA	66.7[b]	117.7[b]	144.7[b]
ULO	60.7[b]	126.7[b]	129.7[b]
		Pectin methyl esterase (0 day = 18.5[a])	
Cold	11.9[b]	11.3[b]	9.9[b]
CA	16.3[c]	16.0[c]	12.7[c]
ULO	14.1[c]	14.3[c]	13.8[c]
		ß-D-Galactosidase (0 day = 40.0[a])	
Cold	49.4[b]	52.4[b]	57.9[b]
CA	45.6[c]	51.7[c]	52.4[c]
ULO	44.0[c]	47.9[d]	48.3[d]

Mean separation : Within columns of a parameter values having different letters indicate significant differences at P = 0.05.

of cell wall (Table 3), though was slightly higher under cold storage conditions but failed to show any significant difference under other storage conditions. Thus, there was a lack of clear cut relationship of PG activity and softening of apples under various storage conditions. The activity of exo-PG has been reported to be responsible for fruit softening in apples and other fruits (Bartley 1976, Huber 1983). However, some suggests this is unlikely (Fischer and Bennett 1991, Yoshioka *et al*. 1992). Apples lack endo-PG (Bartley 1976) but its presence during ripening has been reported by Wu *et al*. (1993). The total free soluble polyuronides of both preclimacteric and climacteric apples were found to be higher after storage in air at 1°C than in other storage conditions (Table 3). The ULO conditions showed minimum quantity of soluble polyuronides in the fruits. The trend of soluble polyuronide content fits well to the observed effect of various storage conditions on fruit firmness, thereby showing that solubilization of pectin is associated with fruit softening in apples.

Table 3. The content of total soluble polyuronides (µg galacturonic acid equivalents g^{-1}dw h^{-1} present and wall autolysis of cell wall by polygalacturonases (µg galacturonic acid equivalents released g^{-1}dw h^{-1}) in preclimacteric and climacteric apples stored for 6 months.

Treatment	Ripeness at harvest	
	Preclimacteric	Climacteric
	Soluble polyuronides	
0-day	0.76[a]	0.88[a]
Cold	132[b]	1.52[b]
CA	1.21[c]	1.16[c]
ULO	1.09[d]	1.08[d]
	Wall autolysis	
0-day	7.2[a]	7.0[a]
Cold	11.3[b]	10.8[b]
CA	8.3[a]	7.9[a]
ULO	7.9[a]	6.4[a]

Mean separation, within columns of a parameter having different letters indicate significant differences at P = 0.05.

The presence of maximum content of free soluble polyuronides in cold stored fruits and minimum in ULO conditions, does not appear to be mediated by the hydrolysis of wall pectins by the enzyme PG, because the differences in PG activity, particularly in climacteric apples, were not significant under these conditions. It has been suggested in apples by Bartley (1977), that an another depolymerising enzyme ß-galactosidase hydrolyses a cross linking galactan and thereby causes a release of wall polyuronides. The evidence for such a possibility in the present investigation will be discussed a little later in this paper. The other possibility may be either an increased *de novo* synthesis of soluble polyuronides or non enzymatic degradation of insoluble pectins in cold storage and to a lesser extent in CA and ULO storage conditions. There is some evidence that *de novo* synthesis may partially account for the quantities of soluble polymers in ripening fruits, however, degradative changes are in predominance (Brady 1987). Non enzymic hydrolysis of cell walls, as reported for other fruits (Brady 1987), may also account for the solubilization of wall polyuronides, however, there is no evidence for it in apples.

The activity of PME was not significantly affected by various storage conditions in preclimacteric apples. In climacteric apples the activity decreased with storage time and the fruits under cold storage showed minimum activity. The fruits under CA and ULO conditions did not show significant differences in PME activities but differed in flesh firmness (Tables 1 and 2). Thus, PME activity can not account for the observed fruit firmness under various storage conditions. The possibilities, however, exist for changes in the relative activities of various isozymes, the total activity remaining unchanged. Alonso *et al.* (1996) reported four different pectinesterase isoforms present in sweet cherries.

Abeles and Takeda (1990) have reported that the slow and steady loss of flesh firmness in apples may be caused by the continued action of cellulase present in the fruits. On the other hand, on the basis of our own efforts, by several methods, and reports by others (Nelmes and Preston 1988, Huber 1983), ripening apples had no cellulase activity. Therefore, the involvement of cellulase in the softening of fruits under various storage conditions

seems to be questionable. However, the lack of detailed information regarding multiplicity and specificity of fruit cellulases renders any attempt to assign a specific role to this enzyme in ripening, purely speculative (Huber 1983).

The activity of ß-galactosidases increased with storage period (Tables 1 and 2). Similar increase in ß-D-galactosidase activity during softening of apples has been reported by other workers (Bartley 1977, Wallner 1978). Both in preclimacteric and climacteric apples, and throughout the storage period, maximum activity was observed in fruits under cold storage and minimum in ULO conditions. This trend inversely fits well with the observed effect on fruit firmness, thereby showing that this enzyme may have a primary role in apple fruit softening under various storage conditions.

Anion exchange chromatography of various galactosidases in apple on DEAE-Sephadex A-25 resin yielded only one peak (Fig. 1), corresponding to peak A of Pressey (1983), who reported two peaks A and B in tomatoes. The peak height corresponded inversely with the observed fruit firmness under various storage conditions. However, existence of only one peak at the same position (fractions 10-12) indicates that there were few changes in electrostatic properties of the enzyme proteins during storage.

As is evident from the present investigation, storage of apples at low O_2 and high CO_2 concentrations of the atmosphere have favourable effect on fruit softening by reducing cell wall hydrolysis. The well documented enzyme PG does not seem to play a primary role in cell wall hydrolysis. Involvement of PME and cellulases is also doubtful. ß-galactosidase activity is likely the possibility. The physiological mechanism by which storage conditions affect ß-galactosidase activity is still not clear. Whether this is a direct effect of low O_2 and/or high CO_2 could not be deduced from this experiment. For other reasons (see Brackmann *et al.* 1993), ethylene in this experiment was kept constant above a saturating level. Since the effect of ethylene is largely reduced or almost eliminated under CA and ULO conditions (Bangerth 1984, Knee 1985), the manipulation of fruit softening and cell wall metabolism by O_2 and CO_2 could well have been mediated by the elimination of ethylene action. Experiments need to be conducted to investigate these possibilities by changing the concentrations of O_2, CO_2 and ethylene individually.

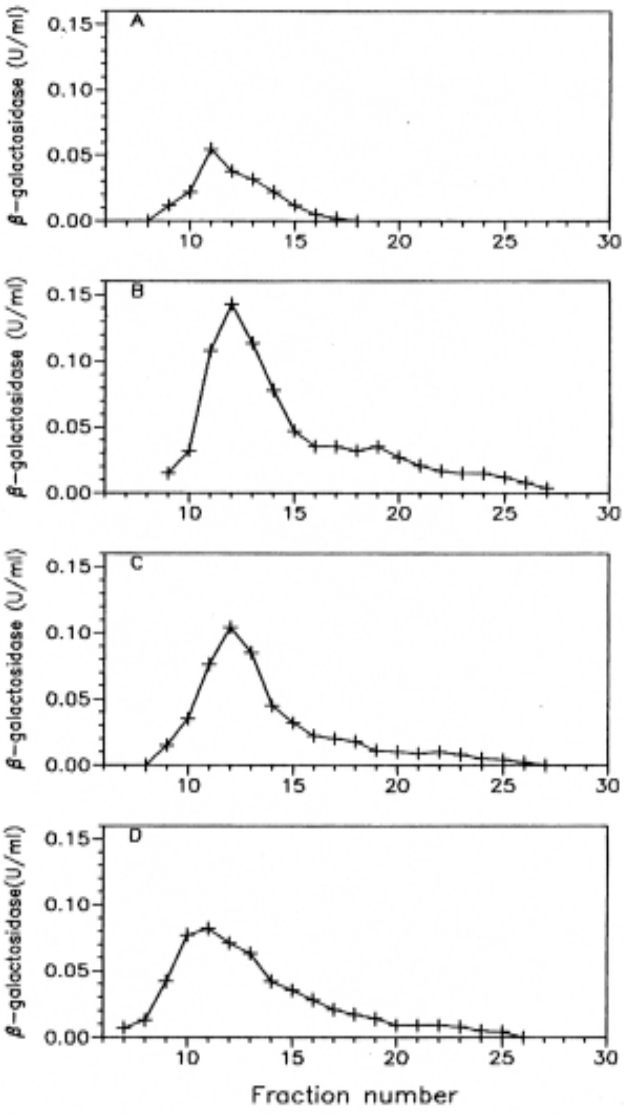

Fig. 1. DEAE-Sephadex A-25 gel chromatography of galactosidases extracted from the climacteric apples at 0-day (A) and after six months of storage under cold (B), controlled atmosphere (C) and ultra low oxygen (D) conditions

ACKNOWLEDGEMENT

This work was supported by a grant to S.S. from German Academic Exchange Service (DAAD).

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