

# Activity of Softening Enzymes during Cherry Maturation

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## ABSTRACT

Royal Anne and Bada cherries soften continuously throughout maturation and cold storage. However, major textural changes occurred during a 2-wk period which coincided with sharp increases in weight, volume, soluble solids, polygalacturonase and pectin methyl esterase activity. No difference occurred between cultivars in the activities of softening-associated enzymes at any sampling period. PME activity was detected during the first week of sampling, and PG and  $\beta$ -gal activity were detected following the second and fifth week, respectively. The integrated action of PG, PME and  $\beta$ -gal appear to be required for cherry softening.

Key Words: cherry, enzymes, softening, maturation

## INTRODUCTION

TEXTURE is an important quality factor in both fresh and processed cherries, yet the mechanism by which cherries soften is not fully understood. Decrease in firmness of maturing fruits has been hypothesized to be due to alterations in both the cell wall and middle lamella (Huber, 1983). Structural changes which occur in the middle lamella and primary cell wall during ripening lead to cell separation and softening of tissue (Bartley and Knee, 1982). Such changes are presumably the results of enzymes such as polygalacturonase, pectin methyl esterase,  $\beta$ -galactosidase, cellulase and others.

Plant cell walls consist of cellulose microfibrils embedded in a complex matrix of pectic substances and hemicelluloses. These polysaccharides form the network of the cell wall and depolymerize to some extent during ripening (Huber, 1983; Eskin, 1979). The middle lamella (area between primary cell walls of adjoining cells), forms a continuous intercellular matrix. This layer is high in pectic substances and its solubilization has been correlated with fruit softening during ripening (Eskin, 1979).

The importance of enzymes in fruit softening has been investigated by many, but relatively few have reported on such activity in cherries. Polygalacturonase (PG) activity was related to the loss of firmness in dates (Hasegawa et al., 1969), peaches (Pressey et al., 1971), tomatoes (Wallner and Walker, 1975), apples (Knee, 1978), avocados (Awad and Young, 1979; Zauberman and Schiffmann-Nadel, 1972), pears (Bartley et al., 1982), papaya (Paull and Chen, 1983), green peppers (Jen and Robinson, 1984) and cucumbers (Miller et al., 1987).

Two types of PG have been identified (endo and exo), (Eskin, 1990). Endo-PG randomly hydrolyzes glycosidic bonds while exo-PG hydrolyzes bonds only on the terminal end of the pectin molecule. The activity of both generally increases during ripening, when pectic material in cell walls and middle lamella are hydrolyzed.

Pectin methyl esterase (PME) acts in concert with PG to increase cell wall solubilization (Pressey and Avants, 1981). De-esterification of cell wall galacturonans by PME may be required prior to hydrolysis by PG (Awad and Young, 1979). A close association between textural changes and PME activity

during fruit ripening has been reported. In green peppers, maximum PME activity occurs at the light green stage and then declines during ripening (Jen and Robinson, 1984). Awad and Young (1979) found that avocado PME activity declined from its maximum at the time of harvest to a low level early in the climacteric. Proctor and Miesle (1991) reported increasing PME activity during blueberry development.

$\beta$ -Galactosidase ( $\beta$ -gal), present in cell walls, hydrolyzes galactans (Bartley, 1974). Several studies have identified  $\beta$ -galactosidase activity in fruits and suggested a possible function of this enzyme in fruit softening. Wallner and Walker (1975) reported  $\beta$ -gal in ripe tomatoes. The increase in  $\beta$ -gal activity during tomato ripening was corroborated by Pressey (1983). Perhaps the most convincing evidence for the role of glycosidases in fruit softening has come from studies with apples (Bartley, 1974, 1976). The activity of  $\beta$ -gal increases during apple ripening and probably catalyzes the loss of galactose residues from the cell wall (Bartley, 1977).

Although the presence of PG, PME and  $\beta$ -gal in various fruits has been correlated with softening, reports of such activities in cherries are scarce. Cherries apparently contain no PG (Pilnik and Voragen, 1970). Early studies by Steele and Yang (1960) reported no evidence of the presence of PG in firm ripe cherries, but moldy cherries contained a high concentration of PG. However, Yang et al. (1960) reported that PG was responsible for the severe softening of brined cherries due to pectin degradation.

Horticulturists have long sought reliable, simple indicators of fruit maturity. Indices such as color, firmness, removal force, size, weight, and soluble solids are traditionally used to fruit maturity. All hypothesized that the physical changes in cherry firmness, or other maturity indices, may be related to increasing activity of softening enzymes. Our objective was to measure the activity of softening enzymes in cherries during development and storage, and attempt to correlate such changes with physical changes related to maturation.

## MATERIALS & METHODS

ROYAL ANNE and Bada cherries were harvested from the Lewis Brown Farm, Oregon State University at weekly intervals, from June 6 (wk 1) to July 10, 1991 (wk 6), and stored at 1°C for a 2 wk period from July 10 (wk 6) through July 22, 1991 (wk 8). Enzymes were analyzed from the first week, but maturity indices (weight, volume, s.s., firmness and color) began on week two. Cherries were harvested randomly from the interior, exterior and tops of trees. Care was taken to pick only healthy, undamaged fruit. Cherries were placed in plastic bags and stored at 1°C and assays were performed the day of harvest.

Fruit were evaluated for changes in weight, volume, color, firmness, soluble solids and enzyme activities. Mean weight and volume of 100 cherries from each harvest period were determined. All characteristics were analyzed by one way analysis of variance with the date of harvest as the variable. Means were separated by the method of LSD at  $p < 0.05$ .

### Soluble solids and firmness

Soluble solids were determined by the official method of analysis of the AOAC, using a Bausch & Lomb refractometer with a temperature compensator (VWR model 1160). For firmness, 10 cherries were selected from each sample. Flesh firmness was measured with a Hunter Spring mechanical force gage (Ametek Inc., Hatfield, PA), using a flat

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**Table 1**—Maturity indices for Royal Anne cherries

Harvest week	Weight (g)	Volume (mL)	S.S. (%)	Firmness (g)	Color a(*)	Hue
2	47.80 <sup>a</sup>	48.0 <sup>a</sup>	5.2 <sup>a</sup>	>2000	—	-0.05 <sup>b</sup>
3	68.90 <sup>b</sup>	70.2 <sup>b</sup>	8.4 <sup>b</sup>	1691.2 <sup>a</sup>	-2.44	-8.08
4	106.42 <sup>c</sup>	107.4 <sup>c</sup>	9.0 <sup>b</sup>	675.0 <sup>b</sup>	-0.50	0.83 <sup>c</sup>
5	105.50 <sup>c</sup>	103.6 <sup>c</sup>	10.6 <sup>c</sup>	506.5 <sup>c</sup>	-0.31	-3.75
6	123.60 <sup>d</sup>	120.8 <sup>d</sup>	14.3 <sup>d</sup>	300.7 <sup>d</sup>	0.74 <sup>b</sup>	-0.33
<b>Storage</b>						
7	123.68 <sup>d</sup>	123.0 <sup>d</sup>	15.8 <sup>c</sup>	285.4 <sup>d</sup>	2.80 <sup>c</sup>	0.09 <sup>b</sup>
8	121.30 <sup>d</sup>	119.2 <sup>d</sup>	16.8 <sup>c</sup>	360.2 <sup>d</sup>	2.70 <sup>c</sup>	0.89 <sup>c</sup>

\*\* Means within a column separated by LSD = 5%. Means in columns with same letters not significantly different ( $p < 0.05$ ).

head attachment tip. Both cheeks of each cherry were tested after removal of the skin with a razor blade. Mean firmness was calculated from 20 readings.

### Color

Hunter L\* a\* b\* values were measured in the reflectance mode using a HunterLab D25-PC2 tristimulus colorimeter (Hunter Assoc. Inc., Reston, VA) equipped with optical sensor and IBM computer. Ten fruit were evaluated on both cheeks, those values were averaged and then averages of 10 cherries were recorded. Two indices of color change during maturation (a\* scale and hue angle) were calculated. The a\* scale indicates redness when positive, gray when zero, and greenness when negative. Hue angle, calculated from the arctangent of b\*/a\* (Clydesdale, 1991; McGuire, 1992), is a measure of the visual property normally regarded as color (red, yellow, green, etc.). In order to usefully evaluate hue angle, the method developed by McGuire (1992) for conversion of calculated values to those which remain positive between 0° and 360° was implemented. Means were separated by LSD at  $p < 0.05$ .

### Enzyme assays

**Extraction.** The method described by Buescher and Furmansk (1978) was used to extract all enzymes. Pectolytic enzymes were extracted by homogenizing cherry flesh in an aqueous solution containing 0.5M NaCl and 1% polyvinylpyrrolidone at 1°C. The homogenate was centrifuged at 12,000 × g for 15 min at 2°C in a RC-5 Superspeed refrigerated centrifuge (Sorvall, Dupont Instruments) and filtered through 4 layers of cheesecloth. This step was repeated once and the filtrate was used for enzyme assays.

**Polygalacturonase.** PG activity was assayed according to Pressey and Avants (1981). Enzyme extract (100 µL), 400 µL of 0.15M NaCl, and 100 µL of 1% polygalacturonic acid (PGA) in 0.1M sodium acetate buffer, pH 4.5, were incubated at 37°C for 30 minutes. After incubation, 500 µL of the mixture were analyzed for reducing groups (Milner and Avigad, 1967). A unit of activity was defined as the amount that catalyzed release of 1 micromole of reducing groups in 30 min.

PG activity was also tested by a modified procedure based on the "cup plate" technique developed by Dingle et al. (1953). A PGA substrate medium was prepared by slowly suspending 0.25% (w/v) of PGA (Sigma, St. Louis, MO, from orange) in distilled water with constant stirring. Then 0.25% ammonium oxalate, 1% potassium biphthalate and 0.1% thymol preservative were added and dissolved. The pH was adjusted to 4.0 with 0.1N sodium hydroxide. The medium was transferred to a bottle and 2% bacto agar medium (Difco) was added, then the mixture was sterilized by autoclaving for 15 min at  $\approx 1000\text{g}/\text{cm}^2$ . Aliquots (25 mL each) were transferred to 15×150mm Petri dishes and allowed to solidify on a level surface. Wells were cut into the solidified agar with a sterile 6 mm internal diameter cork borer. Enzyme extract (60 µL) was dispensed into the wells, with one well designated as a negative control containing boiled enzyme extract, and one as a positive control containing a standard PG. After inoculation, plates were incubated for 24 hr at 35°C. PG activity was measured by a clear zone after flooding the plate with 6N HCl.

**Pectin methyl esterase.** PME activity was measured using a modification of the method of Rouse and Atkins (1955). The rate of citrus pectin demethylation was measured at room temperature ( $\approx 23^\circ\text{C}$ ) by titration with 0.025 N NaOH using a pH meter with a glass electrode and an automatic temperature compensator (Corning Model 125). Fifty mL of 1% pectin in 0.1M NaCl were the substrate, adjusted to pH 7.0 before addition of 25 mL of enzyme extract. Alkali was added at the

**Table 2**—Maturity indicators for Bada cherries

Harvest week	Weight (g)	Volume (mL)	S.S. (%)	Firmness (g)	Color (a*)	Hue (a/b)
2	67.0 <sup>a</sup>	66.0 <sup>a</sup>	5.7 <sup>a</sup>	—	—	-2.86
3	99.0 <sup>b</sup>	93.2 <sup>b</sup>	11.2 <sup>b</sup>	752.3 <sup>a</sup>	-2.34	-19.49
4	121.6 <sup>c</sup>	125.4 <sup>d</sup>	10.2 <sup>b</sup>	391.0 <sup>b</sup>	2.45 <sup>b</sup>	-0.26
5	116.9 <sup>c</sup>	115.6 <sup>c</sup>	13.4 <sup>c</sup>	277.0 <sup>c</sup>	3.88 <sup>c</sup>	0.90 <sup>b</sup>
6	126.0 <sup>d</sup>	122.2 <sup>d</sup>	17.3 <sup>d</sup>	245.3 <sup>d</sup>	4.18 <sup>d</sup>	4.92 <sup>d</sup>
<b>Storage</b>						
7	127.0 <sup>d</sup>	122.4 <sup>d</sup>	16.7 <sup>d</sup>	215.0 <sup>d</sup>	4.91 <sup>d</sup>	1.50 <sup>c</sup>
8	121.1 <sup>d</sup>	118.6 <sup>d</sup>	16.3 <sup>d</sup>	268.4 <sup>c</sup>	3.96 <sup>c</sup>	1.60 <sup>c</sup>

\*\* Means within a column separated by LSD = 5%. Means in columns with same letters not significantly different ( $p < 0.05$ ).

rate required to keep the pH at 7.0 for 10 min. One unit of PME activity was defined as the amount of enzyme capable of catalyzing the consumption of 1 micromole of base/10 min under assay conditions.

**$\beta$ -Galactosidase.**  $\beta$ -Gal activity was assayed using the method described by Labavitch and Rae (1977). The reaction mixture consisted of 1.0 mL of 50 mM sodium acetate buffer, pH 5.0, containing 0.05% p-nitrophenylgalacto-piranoside substrate (Sigma, St. Louis, MO), and 0.25 mL of enzyme extract. After 1 hr incubation at 37°C, the reaction was stopped by adding 2 mL of 1 N ammonium hydroxide containing 2 mM EDTA. The amount of liberated p-nitrophenol was determined by measuring the optical density at 400 nm. One unit of enzyme was defined as the amount of enzyme capable of catalyzing the release of 1 micromole of p-nitrophenol/hr under assay conditions.

**CMC-Cellulase.** CMC-cellulase activity was measured in a mixture containing 0.4 mL of a 0.5% CM-cellulose (CMC 7HOF, Aqualon Co., Hopewell, VA) solution in 50 mM sodium acetate, pH 5.0 and 0.1 mL of cherry extract. After incubation at 30°C for 1 hr, the reaction was stopped and reducing sugars were measured by addition of the Nelson Somogyi reagent. Samples were centrifuged and absorbance at 510 nm was read.

## RESULTS & DISCUSSION

**HARVEST DATE** had a significant relationship to all fruit quality factors measured, many of which are typically used as maturity indices. Mean values for weight, volume, soluble solids, firmness and color (Table 1) were compared for Royal Anne and (Table 2) for Bada cherries. The major growth period for both cultivars occurred between wk 2 and 4. Neither weight nor volume changed during storage. Soluble solids levels in both Royal Anne and Bada cherries increased at a fairly constant rate during maturation and storage. Soluble solids content in fruit depends on the stage of maturity (Drake and Fellman, 1987), and Bada may have been more mature than Royal Anne cherries. By week 6, s.s. levels in Bada cherries were 17.3%, while in Royal Anne they were 14.3%.

Cherries softened continuously throughout maturation and harvest, but the most notable softening occurred between wk 2 and 4 (Tables 1 and 2). The firmness of samples harvested on June 12 was too great to be measured with the available penetrometer. Bada cherries softened faster than Royal Anne, another indicator that Bada matured earlier. Note that after 2 wk of refrigerated storage, firmness increased in the Bada cultivar to a level greater than that at harvest. Dehydration following harvest may have caused a toughening of the skin, hence higher firmness. This increase was only significant in Bada cherries and probably reflects earlier maturity.

Cherry firmness has been used as an indicator of fruit maturity by several investigators (Brown and Bourne, 1988; Fac-teau, 1982). Losses in firmness have been attributed to both enzyme activity and transpirational water loss. Drake and Fellman (1987) correlated the loss of sweet cherry firmness with increases of soluble solids. In our study, firmness may also be correlated to changes in soluble solids. Note that firmness changes were at their peak when major growth occurred.

In both cultivars, fruit color changed from green, as indicated by "a\*" values and hue angle, at a steady rate during maturation. As noted by McGuire (1992), a hue angle between 0° and 45° indicates a red color, while the range of 135° to

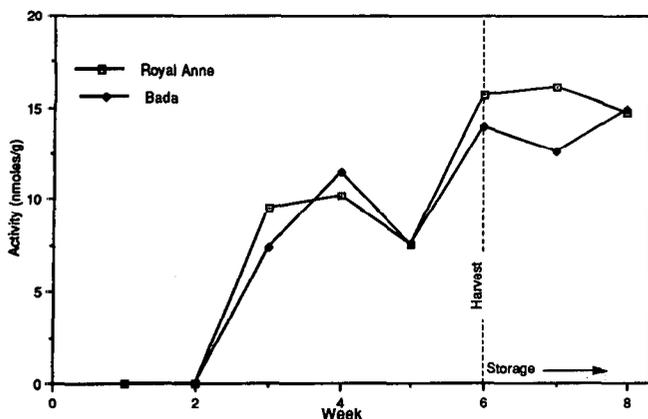


Fig. 1—Polygalacturonase activity in Bada and Royal Anne cherries.

180° would imply a yellow-green to green color. Bada apparently matured faster, with red color appearing much earlier. Red color in Bada fruit appeared to decline somewhat after 2 wk storage, while red color development in Royal Anne continued to proceed after harvest. Color is one of the most widely used maturity indicators, but it can vary tremendously among cherries on the same tree.

Cherry PG, PME, and  $\beta$ -gal activities were compared at different stages of maturation and storage (Fig. 1, 2, and 3). Cellulase activity was not detected at any time during maturation or storage. PG activity was not detected in either cultivar until wk 3 (Fig. 1). In general, PG activity increased during maturation and storage. No difference occurred in activity of the two cultivars until harvest and storage, but both showed the greatest increase in activity between June 12 and 26, 1991. PG activity in stored samples remained constant at about the same level as that of harvest, with activity in Royal Anne cherries slightly higher than Bada (Fig. 1).

Note that from wks 2 to 4 (the period of greatest increase in PG activity), firmness values showed the sharpest changes. Many investigators have associated textural changes with PG activity (Pressey et al., 1971; Wallner and Walker, 1975; Milner and Avigad, 1967). However, Pressey et al. (1971) also reported that peaches softened considerably prior to appearance of PG.

The level of PG activity in cherries was relatively low compared to that reported in other fruits. Previous studies have reported the following units/g fresh weight: avocado 0.8 (Awad and Young, 1979), peaches 4 to 6 (Pressey et al., 1971), tomatoes up to 30 (Gross and Wallner 1979), pears 20 to 70 units/mg protein (Ben-Arie and Sonogo, 1979). We found from zero to  $\approx 0.32$  units/g fresh weight. Steele and Yang (1960) used the diffusion assay to evaluate cherry PG and reported activity in only a few samples. They did not report specific values for PG activity, but indicated that the enzyme was present. When the diffusion assay was used for our study, PG was not detectable.

PME activity (Fig. 2) was detected throughout maturation and storage, increased at a fairly constant rate and reached a maximum at harvest in both cultivars. PME was detectable even in the first 2 wk of sampling, before PG activity was detected. This supports the hypothesis that PME action is required before PG can be fully effective. No significant difference occurred in the activity of the two different cultivars at any time. PME activity was relatively constant after harvest and during storage.

Awad and Young (1979) also reported that PME activity was detectable long before that of PG in harvested avocados. They reported cellulase activity increasing at about the same time as that of PG. Note that both PG and cellulase activity were associated with the climacteric, whereas PME was not.

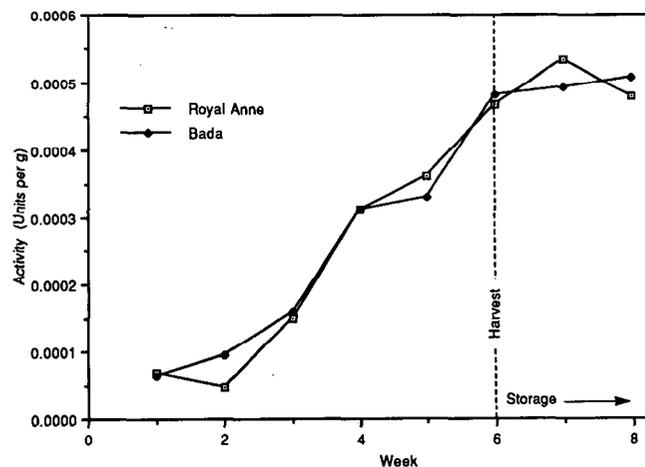


Fig. 2—Pectin methyl esterase activity in Bada and Royal Anne cherries.

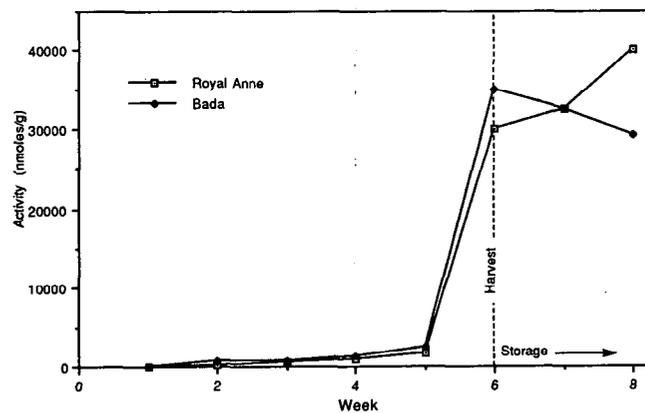


Fig. 3— $\beta$ -Galactosidase activity in Bada and Royal Anne cherries.

$\beta$ -Galactosidase activity was also detected at the early stages of cherry ripening (Fig. 3). The  $\beta$ -gal activity from June 6 through July 3, 1991 was relatively low. However, activity was considerably higher in cherries picked on July 10. Values for  $\beta$ -gal at harvest and during storage were about nine times higher than values prior to July 10, 1991. Neither Royal Anne cherries nor Bada cherries showed any changes in  $\beta$ -gal during storage.

The sharp increase in  $\beta$ -gal activity occurred at a later date in both cultivars than the peaks in either PG or PME. There may be some association between the dip in PG activity on July 3 and the immediate burst of  $\beta$ -gal activity. Bartley (1977) reported that an increase in  $\beta$ -gal was not necessarily required for initiation of galactose residue hydrolysis from the cell walls of stored apples. Those results also indicated that  $\beta$ -gal activity increased after the decline in firmness, and suggested that either an increased rate of enzyme synthesis, decreased rate of degradation, activation of the enzyme or disappearance of an inhibitor may have been responsible. Some have suggested that  $\beta$ -galactosidase activity may be associated with the amount of soluble solids present, since it is involved with hydrolysis of galactans. The involvement of glycosidases in fruit softening has probably not been given enough consideration (Pressey, 1983).

In another study, the appearance of PG, PME and  $\beta$ -gal in blueberry fruit coincided with pectin solubilization (Procter and Peng, 1989). Peak PME activity occurred in red berries and preceded peak PG activity, which was observed in blueberry fruit (Procter and Miesle, 1991). In our study, both PME and  $\beta$ -gal activity were detectable before that of PG. The max-

imum activity of all 3 enzymes correlated with harvest, when cherries were most mature and had the greatest red color. The suggested mechanism for pectin metabolism in ripening blueberries (Proctor and Peng, 1989) involves at least two enzymes, PME and PG. Demethylation by PME results in a greater number of carboxyl groups which may facilitate PG activity. PG preferentially degrades deesterified pectic substances (Huber, 1983). Our results further support the proposed mechanism by which pectic substance changes occur at different stages of maturity.

Fruit ripening appears to be accompanied by a decrease in cell wall galactose content. This loss of galactose may represent a separate process of cell wall degradation. Declines in cell wall galactose content have been reported in tomatoes (Pressey, 1983) and apples (Bartley 1974, 1977). Our results indicated that  $\beta$ -gal is present in cherries and that its activity increases during ripening.

Koch and Nevins (1989) showed that the extent of tomato uronide release by PG depended on the stage of fruit development. When cell wall preparations from tomatoes of different maturities were pretreated with PME the release of uronides by PG was equivalent. Wallner and Walker (1975), evaluating tomato softening, suggested that the major modifications of the cell wall had already occurred by the turning stage, prior to the onset of greatest PG activity. They indicated that possibly the pronounced increase in PG activity was a consequence of the disruption of cellular organization and control. The correlation between onset of increased PG activity and ripening and softening has been noted by several investigators in tomatoes (DellaPenna et al., 1986; Grierson and Tucker, 1983).

In our study, changes in the physical state of cherries appeared to occur at about the same time that at least PG and PME activity were increasing. In cherries, as in tomatoes, PME activity increases precede and may be required for the most effective PG-initiated softening changes.

The integrated action of PG, PME and  $\beta$ -gal, and possibly other enzymes, appear to be required to break down the closely packed structure of fruit cell walls.

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