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Effects of Growth-Regulators on Cellulase, Polygalacturonase, Respiration, Color, and Texture of Ripening Tomatoes[1]

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Abstract. Changes in firmness, protein, color, respiration, cellulase, and polygalacturonase were followed during maturation and ripening of tomatoes, *Lycopersicon esculentum* L., on the plant and in detached fruit allowed to ripen at 20°C. Cellulase activity in the young fruit increased steadily during the maturation period. Cellulase activity in detached fruit ripened at 20°C increased rapidly during the onset of ripening and reached a higher level than in fruit ripened on the plant. Polygalacturonase activity was not detectable in developing fruit until after the fruit had initiated ripening. Polygalacturonase activity in detached fruit ripened at 20°C did not appear until the onset of the climacteric and then increased rapidly. This corresponded to the polygalacturonase activity in fruit allowed to ripen on the plant.

The changes during ripening appeared to follow a pre-determined pattern. Grow-regulating substances only moderately affected the onset of the climacteric rise, but markedly influenced the time interval to reach the climacteric peak. They also markedly affected the rate of the normal sequence of changes during ripening. Such changes as softening, color formation, and enzyme activities of cellulase and polygalacturonase were accelerated by ethephon and SADH and delayed by gibberellic acid and indoleacetic acid. Gibberellic acid suppressed polygalacturonase activity. After 14 days polygalacturonase activity in the control fruit was 25 times greater than in fruit treated with gibberellic acid. Cellulase activity in gibberellic acid treated fruit increased steadily during this period. The loss of firmness in treated fruit suggests that softening is initiated by action of cellulolytic enzymes and that pectinolytic enzymes are involved in subsequent changes in texture.

The role of specific enzymes in the textural changes of fruits is still unresolved. The use of tomato fruit for studying these enzymatic systems during ripening has several advantages. Tomatoes are characterized by a climacteric rise (a rapid increase in respiration at maturity) which delineates cellular activities associated with growth and maturation and those of ripening and senescence (3, 19). The changes in the fruit after ripening is initiated proceed very rapidly, and these changes occur whether the fruit is left on the plant or removed and allowed to ripen off the plant. Furthermore, a tomato is composed principally of relatively large flesh cells. Whittenberger and Nutting (45) and Cocking and Gregory (7) have observed microscopically that the cells become separated and that their walls become progressively thinner during ripening. The softening of fruit during ripening has been attributed to the solubilization of the pectin materials by pectinases. The implication of a close link between firmness of fruit and polygalacturonase activity has been demonstrated by Hobson with tomatoes (24), Reymond and Phaff with avocadoes (38), and Patterson et al. with cranberries (34).

It has also been suggested by Hall (17) and Dickinson and McCollum (11) that cellulases might contribute to softening of tomato fruit. However, Hobson (25) reported that, although the activity of cellulases increases with normal ripening, the loss in firmness during this period is directly controlled by pectinases.

The recent findings that growth-regulating substances can affect cell wall degrading enzymes are extremely significant, particularly in respect to their roles in the life of plants and plant organs. Several studies (8, 9, 33, 43) have reported the effects of growth-regulating substances on polygalacturonase,

J. Amer. Soc. Hort. Sci. 98(1):77–81. 1973.

77

pectin-esterase, cellulase, β-1,3-glucanase, and amylase activities observed during plant growth. Furthermore, it has been demonstrated that growth-regulating substances can control the normal ripening processes in tomato fruit.

Abdel-Kader et al. (1) found that postharvest treatments with gibberellic acid and indoleacetic acid greatly retarded the normal color development and general quality of tomato fruits. Dostal and Leopold (13) found that the retarding effects of gibberellic acid on the ripening processes in tomato fruits could be overcome by treatments with ethylene. We report here the effects of several growth-regulating substances on the formation of cellulase and polygalacturonase in relation to changes of color, firmness, and respiration of ripening tomato fruit.

Materials and Methods

Sampling of fruit. Michigan-Ohio hybrid tomato seeds were planted in "jiffy pots" in a heated greenhouse. The seedlings were transplanted after 22 days to individual 12-inch clay pots filled with an artificial soil mix. Equal portions (3 gallon container) of peat moss, pumice, and vermiculite (course) were mixed with 200 g lime, 30 g dolomite, 15 g potassium nitrate, and 15 g super phosphate (commercial grade) to simulate natural soil. An automated hydroponic system was used to water the plants daily. Nutrient stock solutions were formulated according to Hoagland and Arnon (22). The nutrient solution containing the major elements was administered daily to the plants and the nutrient solution containing the minor elements was administered weekly.

The tomato plants were thinned to 1 plant per pot. The plants were trained to a single stem and were topped at the fourth fruit-truss. Individual fruits were tagged at time of pollination and the date recorded. Developing fruit samples were analyzed at intervals from the day of pollination up to 42 days after fruit set. Other samples were harvested when mature (32-34 days after fruit set) and allowed to ripen in respiration chambers at 20°C. Sampling variability was reduced by restricting each harvest to 1 particular truss position.

Each sample consisted of 3 to 5 fruits. Respiration, color, and firmness of each fruit in a sample were determined. An aliquot of a homogenous blend of fruit in each sample was used to determine enzyme activities and pH. All treatments and analyses were performed in triplicate.

Growth-regulating substances. Fruit of uniform size and free from defects were immersed in aqueous solutions of growth-regulating substances at 22°C for 30 minutes and dried. Fruit immersed in distilled water served as the control. The growth-regulating substances used were: (a) (2-chloroethyl)phosphonic acid (ethephon); (b) commercial-grade gibberellic acid (GA, 75% K salt); (c) indole-3 acetic acid (IAA); and (d) succinic acid 2,2-dimethylhydrazide (SADH).

Respiration, color, firmness, protein, pH. Respiration was determined by passing a known volume of air over a weighed amount of fruit enclosed in a sealed container at 20°C (6). The air leaving the container was analyzed for CO_2 by means of a Model No. 29 Fisher-Hamilton Gas Partitioner. Rate of respiration was calculated as milliliters CO_2 evolved per kilogram of fruit per hr. Firmness was measured by the Asco Firmness Meter as described by Garrett et al. (14). Low values indicate that the fruit is firm, while high values indicate soft fruit. Color was measured by a Hunter Color and Color-Difference Meter standarized with a white standard (Rd. of 85.8, a of -0.9, b of 1.7). Readings were determined at the equatorial surface of each fruit and the results were plotted as a/b ratios. Low values of a/b indicate green fruit while high values indicate red fruit. Total protein was determined by the method of Lowry et al. (27) in a sample of the supernatant liquid after centrifugation and filtration. A Beckman Model H-2 pH Meter was used to determine the pH of a blended tomato sample.

Enzyme preparations. After the fruit had been analyzed for color and firmness, the samples were put in polyethylene bags and stored at -29°C to -32°C for not longer than 2 months. For enzyme analysis, the frozen fruit was sliced (after stem and blossom scars were removed) and allowed to thaw overnight (15 to 16 hr) in a walk-in cold room (2 to 4°C). These conditions did not affect enzyme activity[4]. All subsequent extractions were done in the cold room. All of the slices from each sample were blended (Waring blender) for 30 seconds. To 50 g of blended fruit were added and mixed 2.5 g of sodium chloride and 0.5 ml of 1% merthiolate. The pH was adjusted to 9.0 with 1 N NaOH. The blended material was allowed to stand for 40 min prior to centrifugation at 10,000 × G for 5 min. The supernatant liquid was filtered through No. 1 Whatman filter paper under a slight vacuum. This clear filtrate was used to determine cellulase and polygalacturonase activity[4]. The reaction mixture for the determination of cellulase consisted of carboxymethylcellulose (Hercules Co. 7HP) at a final concn of 0.15%; 0.1 M acetate buffer, pH 5.0; and 0.01% merthiolate. Polygalacturonase was determined with a reaction mixture of polygalacturonic acid (Sunkist No. 3491) at a final concn of 2.5%; 0.1 M acetate buffer, pH 4.5; and 0.01% merthiolate. The substrate and enzyme extracts were allowed to come to equilibrium with the water bath temp before they were mixed. Nine ml of the reaction mixture and 1 ml of the enzyme extract were mixed and the time noted. Five ml were immediately pipetted into a standardized Ostwald viscosimeter. The flow times were measured at intervals of 3 min. One unit of enzyme activity is defined as the amount of enzyme causing an increase in the reciprocal of the specific viscosity ($1/\eta_{sp}$) of 0.01 per 10 min (15). In comparing enzyme activities measured by the change in viscosity, care was taken to prepare solutions of very similar initial viscosity.

Results

Cellulase and polygalacturonase are readily eluted from the cellular tissue of tomato fruit with 5% sodium chloride and 1 N NaOH to adjust the pH to 9.0 (Table 1). A comparison of the activities of the 2 enzymes in the extract and subsequent rinses with a solution of 5% NaCl at pH 9.0 revealed little difference in specific activity (units of activity/mg protein) after repeated rinses. For comparative studies involving the presence of these enzymes in tomato fruit, the original filtrate only was used.

The optimum pH for cellulase activity was between 4.8 and 5.0 and the temp optimum was near 30°C. Polygalacturonase[4], was most active near pH 4.5. Since polygalacturonase was found to be unusually stable to heat, enzyme activity was measured at 40°C.

Enzyme activities in the range used in the determination for both cellulase and polygalacturonase were proportional to concn. One ml of extract from ripe tomato fruit was able to reduce the viscosity of a carboxymethylcellulose solution (initial η_{sp}-2) by 35 to 40% and to reduce the viscosity of a

Table 1. Effect of pH and NaCl additives to homogenized tomato tissue on yield of cellulase and polygalacturonase.

Homogenate titrated to pH		Cellulase activity (Units/ml extract)	Polygalacturonase activity (Units/ml extract)
4.3		0.5	5.0
6.0	NaOH	1.5	9.7
8.0	NaOH	6.8	11.0
9.0	NaOH	7.8	11.8
9.0	NaOH + 2.5% NaCl	9.2	19.5
9.0	NaOH + 5.0% NaCl	9.5	21.5
9.0	NaOH + 10.0% NaCl	9.5	- -
10.0	NaOH	1.2	12.1

78

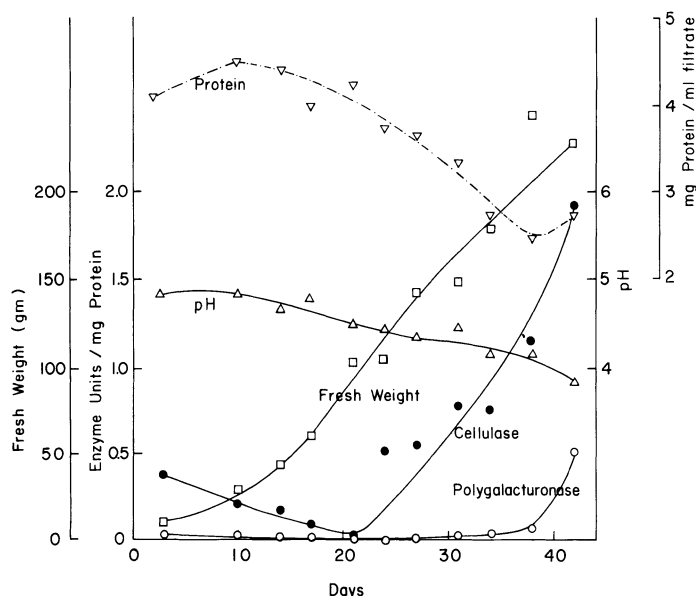J. Amer. Soc. Hort. Sci. 98(1):77–81. 1973.

Fig. 1. Changes in protein, pH, fresh wt, cellulase, and polygalacturonase activity during growth and maturation of tomato fruit. (Each value represents mean of 2 experiments.)

pectic acid solution (initial η_{sp}-2) by 70 to 75% during a 20-min period.

Figure 1 shows that pH and protein levels were highest at the time of fruit set (0 days) or soon thereafter. Protein levels decreased until the fruit attained maturity (35 days after fruit set) and then increased during ripening. The presence of polygalacturonase in the developing fruit was negligible until the

Table 2. Changes in color, firmness, cellulase, and polygalacturonase during ripening of tomato fruit on the plant.[z]

Days after color inception	Cellulase activity (Units/ml extract)	Polygalacturonase activity (Units/ml extract)	Firmness meter units	Color a/b values
0	1.87	1.72	3.18	-0.32
2	1.92	9.15	3.98	0.25
4	1.55	13.64	4.43	0.84
6	1.92	21.24	4.99	1.06
8	1.73	23.83	5.40	1.13
10	1.98	32.45	5.85	1.21

[z]Each value represents the mean of 2 experiments.

fruit initiated ripening (first appearance of color changes at the blossom end of the fruit). Cellulase activity was present in very young fruit, decreased to a low point midway through development, and then increased during maturation.

To determine the sequence of changes in color, firmness, cellulase, and polygalacturonase during ripening of tomato fruit on the plant, the date when the fruit started to initiate a color change was recorded. Then 5 fruit for each daily interval after initiation of color were removed for sampling. After the tomato fruit began to ripen on the plant, polygalacturonase activity increased steadily to the over-ripe stage at 8-10 days (Table 2). Cellulase activity remained relatively constant during this period. Although color inception may be a useful index to determine when ripening is initiated in fruit on the plant, it cannot be used as an indicator to determine the physiological state of the fruit. All the fruit used in Table 2 regardless of whether or not the fruit had initiated a color change ranged in age after fruit set from 47-51 days. We found that the tomato fruit reached maturation 32-34 days after fruit set. The fruit had the capacity to ripen within a few days after harvest (see control fruit in Fig. 2).

The respiratory behavior of ripening tomatoes followed the

same pattern as that reported by Biale (2). The respiratory rate decreased to a pre-climacteric min at maturity (green-mature), increased to a climacteric peak, and again decreased gradually as the fruit approached the red-ripe stage 12-14 days after detachment (Fig. 2). Color began to change 3 days after detachment, lagging behind the onset of the climacteric rise. Cellulase activity in the fruit increased rapidly during the onset of ripening while polygalacturonase activity was not detected until 6 days after detachment. Over-all softening in the fruit occurred continuously during ripening.

Ethephon (1,000 ppm) accelerated over-all ripening while GA (1,000 ppm) retarded ripening (Fig. 2). Ethephon at concn of 100 ppm and 1,000 ppm caused a more rapid increase in the climacteric rise indicated by accelerated red coloration and softening of tomato fruit (Fig. 2). Color formation associated with ripe tomatoes was completed 7 to 8 days earlier in ethephon treated fruit than in the control fruit. The rapid increase in the activity of cellulase in ethephon treated fruit during the first 6 days was accompanied by an initial decrease in firmness. After 6 days, cellulase activity gradually decreased while the activity of polygalacturonase steadily increased (Fig. 2).

The stimulatory effects of SADH were similar to ethephon but not as great. SADH (1,000 ppm and 10,000 ppm) enhanced cellulase activity but had no effect on polygalacturonase activity. Color formation was accelerated, but the max color developed after 14 days was the same as that of the control fruit. Softening of the fruit was accelerated by SADH and the fruits were much softer than the control fruit after 14 days.

The respiratory rates of fruit treated with GA were lower than the control fruit (Fig. 2) and the climacteric rise was delayed 2 to 3 days. Color development in fruit treated with GA was delayed and altered (Fig. 2). GA (100 ppm as well as 1,000 ppm) suppressed lycopene synthesis, but chlorophyll degradation did occur. Breakdown of chlorophyll in the fruit was completed by 14 days. Polygalacturonase activity was suppressed by GA (Fig. 2). Cellulase activity was temporarily slowed by GA but it then accelerated and was similar to the control fruit after 14 days. Firmness of fruit treated with GA (100 as well as 1,000 ppm) at 14 days was similar to that of the control fruit at 9 days. Polygalacturonase activity was almost completely absent following either GA treatment; only the latter is shown in Fig. 2.

The effects of IAA on respiration, softening, and color development were similar to the delaying effects of GA. Polygalacturonase activity decreased with each increase in concn of IAA (10 ppm - 1,000 ppm) while cellulase activity was not appreciably affected.

Discussion

Of the methods available for the estimation of cellulase and polygalacturonase activity, it is accepted that viscometry is a very sensitive indication of even traces of enzyme activity (15). Speiser and Eddy (42) have shown that the rapid decrease in viscosity during early stages of hydrolysis is due to the sharp drop in the wt-average molecular wt. When activity is assayed viscometrically, units of activity are often based on the initial rate of change of the reciprocal of the specific viscosity ($1/\eta_{sp}$) (32). The change in $1/\eta_{sp}$ with time reflects the presence of very small amounts of enzyme and presumably the cellulase and polygalacturonase values in the figures indicate the actual rate of dissolution of cellulose and pectin in tomato fruit.

In other studies it was found that cellulase and polygalacturonase activities increased while firmness decreased during ripening of tomatoes (18, 23, 25). Unlike those workers, we used tomato fruit of known ages after fruit set because age is a better indication of maturity than size or color of fruit (7, 16, 29). Over 1,000 fruits of known age after fruit set were used.

Difficulty may be encountered in interpreting papers referring to stages of maturation and ripening. A mature tomato
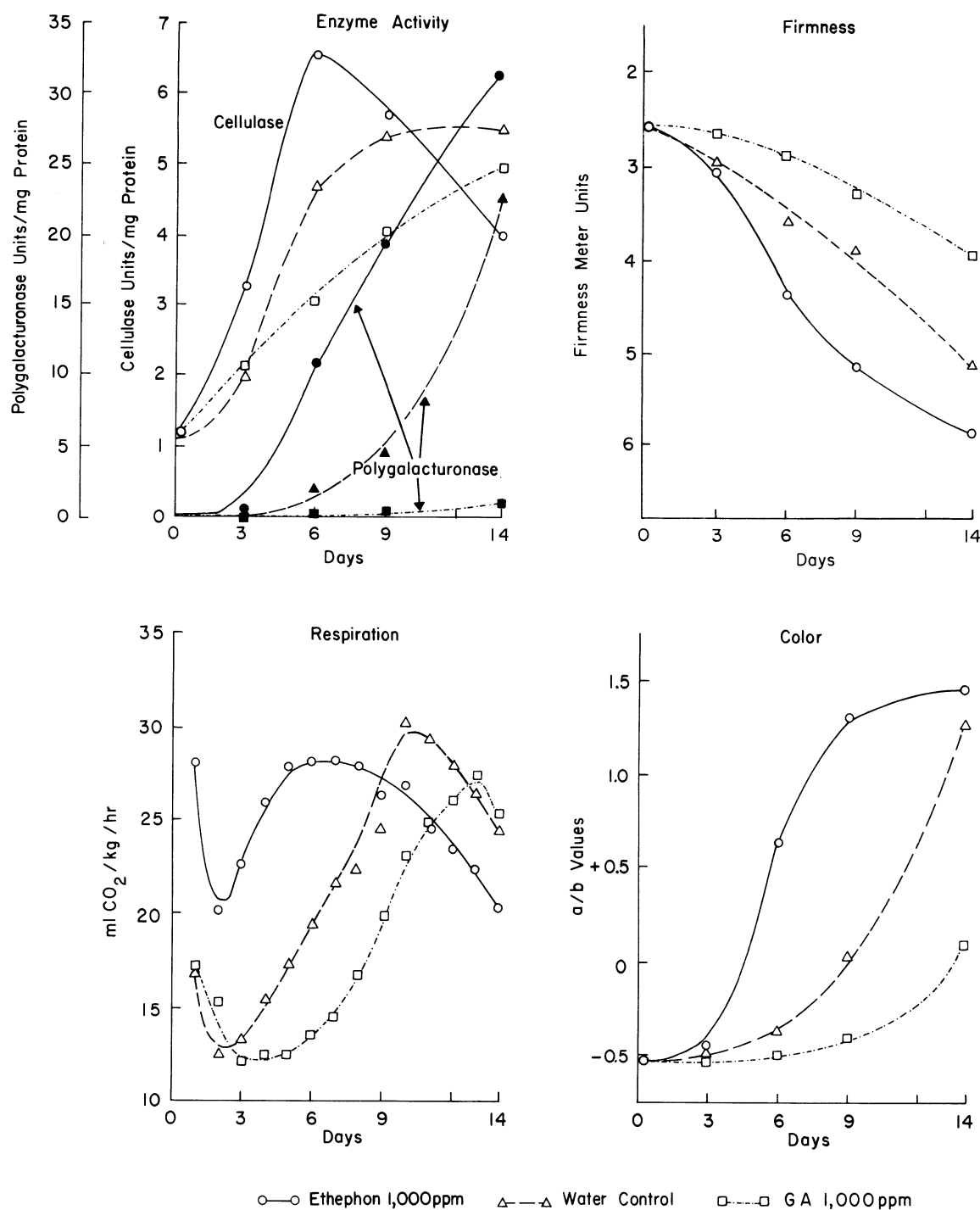
Fig. 2. Effect of ethephon and gibberellic acid (GA) on tomato enzyme activity, firmness, respiration, and color in comparison to control.

has been defined as one that has reached nearly full external and internal development and has the capacity to ripen within a few days after harvest (31). The period of active ripening is considered by some authors to begin with the onset of the climacteric rise and to include the various processes involved in the fruit attaining an edible condition (3, 19).

Our treatments with GA and IAA delayed ripening, but they did not change the normal pattern of ripening in the tomato fruit. This response is in agreement with recent reports that GA and IAA delay maturation and ripening (1, 13, 40, 41). Supplementing the endogenous GA or IAA by dip-treatments to add more GA or IAA may have affected the threshold level at which ethylene initiates ripening (4, 12, 35), thus accounting

for the observed delay in ripening.

Although ethephon and SADH promoted ripening, postharvest softening, color changes, and accelerated respiration sequence appeared to follow a pre-determined pattern. Other workers (30, 39, 44) have given evidence indicating that ethephon hastens ripening and senescence by increasing the concn of ethylene in the fruit. The response of SADH appears to enhance the ethylene system by stimulating ethylene or suppressing regulators counteracting ethylene (10, 26, 28, 36).

The mechanism of ethylene action in inducing ripening of fruits has not been resolved (37). Hansen (20) studied the effect of ethylene on pear fruit and considered ripening to be a sequential phase in the life of the fruit which requires induction

80

J. Amer. Soc. Hort. Sci. 98(1):77–81. 1973.

in order to become active. Once ripening is induced, it proceeds according to a pre-determined pattern.

Our data suggest that although fruit tissues undergo many changes during ripening, a highly organized biochemical system must be present to maintain respiratory control and protein synthesis. Ripening in the tomato fruit could be induced or delayed, yet most sequential changes during ripening were not greatly altered. Two systems that were altered by growth-regulating substances were lycopene synthesis and formation of polygalacturonase. Hayward (21) did not find a direct relationship between chlorophyll formation or decomposition and lycopene content in tomato fruit. Indeed, GA suppressed lycopene synthesis, but chlorophyll degradation did occur in our experiments.

Furthermore, GA suppressed polygalacturonase activity. There was only a small detectable amount of polygalacturonase in fruit treated with 1,000 ppm of GA after 14 days. Cellulase activity in the GA treated fruit increased steadily during this period which was accompanied by a decrease in the over-all firmness of the fruit. Perhaps softening is initiated by the action of cellulases on the interwoven cellulose fibrils of the cell walls, thereby enabling the pectin enzymes to penetrate into the middle lamella. The subsequent breakdown of the pectin adhesive that binds the cells together would cause the rapid changes in texture we observed during ripening.

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J. Amer. Soc. Hort. Sci. 98(1):77–81. 1973.

81