Plant Physiol. (1980) 66, 1019–1023
0032-0889/80/66/1019/05/$00.50/0

Polygalacturonase Activity and Ethylene Synthesis during Cucumber Fruit Development and Maturation[1]

Received for publication February 14, 1980 and in revised form June 12, 1980

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ABSTRACT

Polygalacturonase (EC 3.2.1.15) activity in seed cavity tissue from harvested cucumber fruit increased over 20-fold after the fruit had produced a transient burst of ethylene during maturation. This increase was observed in six cucumber cultivars and was present whether polygalacturonase activity was measured at pH 4.6 or 6.2. The seed cavity tissue pH decreased as polygalacturonase activity increased both in ripening fruit and in harvested immature fruit exposed to 10 microliters per liter ethylene in air.

The rate of C_2H_4 biosynthesis is increased in many plants by mechanical injury during harvesting or by environmental stresses (16). Exogenously applied C_2H_4 induces the synthesis of physiologically active compounds in some plants (16). For example, it induces the synthesis of the enzyme chitinase in bean leaves (1) and β-1,3-glucanase in tomato and bean leaves (1, 7). C_2H_4 exposure also enhances Chl loss and softening of harvested cucumbers (8). Polygalacturonases have been found to play an important role in fruit softening (15). The loss of firmness by unheated cucumber slices during storage (3) and the occasional development of soft centers in commercial brine stock may be caused by the presence of PG[3] in cucumbers.

The first report of PG activity in pickling cucumber cultivars was made by Bell (2). Pressey and Avants (10) reported the presence of an exo-splitting PG in a fresh market cucumber variety and in pickling cucumber cultivars. McFeeters *et al.* (5) have observed an endo-splitting PG in mature pickling cucumber fruit.

The purpose here was to evaluate the interrelationship of changes in PG activity rates of C_2H_4 synthesis, and maturation of cucumber fruit.

MATERIALS AND METHODS

Plant Material. Cucumber fruit (cv. Chipper, except as noted below) were hand-harvested from field plots near Raleigh for use in all experiments measuring C_2H_4 and CO_2 production. Commercially harvested fruit of unknown cultivars were also used in specific gassing experiments. Only uniform fruit free of external injuries was used. The peduncle was removed and the fruit was held at room temperature overnight. The fruit then was weighed and C_2H_4 and CO_2 production was analyzed.

Tissue removed from the fruit for analysis was used either fresh or frozen at −10 C. The mesocarp tissue was the fleshy tissue between the green peel and the gelatinous seed-containing tissue. The gelatinous tissue, which consisted of the seeds and associated placental tissue, will be referred to as seed cavity tissue.

Chl, Color, and pH Measurements. Chl was extracted from the peel of individual fruits by first adding 250 ml boiling 80% ethanol to the peel. After cooling, the mixture was homogenized for 3 min at high speed in an Osterizer blender. Next, the homogenate was boiled, cooled, and stored overnight. The mixture then was filtered through Whatman No. 1 filter paper, and the residue was washed with about 200 ml hot 80% ethanol. The solutions were combined and made to 500 ml after they had cooled to room temperature. A 5-ml aliquot was centrifuged at 27,000*g* for 15 min. The clarified green supernatant was decanted and its O.D. was read at 665 nm.

The green color of individual fruit was measured nondestructively with an AgTron color meter, model No. E5-W (Magnuson Engineers Inc., Instrument Division, San Jose, CA), which had been modified to hold cucumber fruit. The pH of the mesocarp and seed cavity tissues was measured after they had been blended with an equal weight of distilled, deionized H_2O.

Gas Analysis and Treatments. Production of C_2H_4 and CO_2 by individual fruit was measured by taking 1-ml gas samples from the head space of 1- or 4-liter glass containers in which the fruit had been enclosed for about 6 h. C_2H_4 and CO_2 were analyzed as previously described (13). Production data of these gases from individual fruit were not used in the statistical analysis if the fruit developed visual symptoms of fungal infection during the experiment.

To observe the effects of C_2H_4 on PG activity, uniform green cucumber fruit were placed in 4-liter glass containers and gassed with air ± 10 μl l^{-1} C_2H_4 at a flow rate of 500 ml min^{-1}. The air had been passed through a column of $KMnO_4$-covered Perlite to reduce contaminating C_2H_4 to below 1 nl l^{-1}. C_2H_4 was added to the air streams with a diffusion apparatus, as previously described (12).

PG Assay. Seed cavity tissue was removed from cucumber fruit, dry NaCl was added to give a concentration of 0.2 M, and the mixture was homogenized in a Waring Blendor or Sorvall Omni-Mixer at room temperature. The homogenate was centrifuged at 17,000*g* for 10 min. The supernatant was decanted and dialyzed at 4 C against a 36 mM maleic acid buffer (pH 6.2) containing 0.33 M NaCl and 0.02% sodium azide to control microbial growth.

PG activity measurements were done at pH 4.6 and 6.2 because preliminary results had suggested the possibility of two forms of PG in cucumber fruits. Assays of ripe cucumbers showed higher PG activity when measured at pH 6.2, whereas PG from immature

[1] This is paper No. 6299 of the Journal series of the North Carolina Agricultural Research Service, Raleigh, North Carolina. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service, or by the United States Department of Agriculture of products named, nor criticism of similar ones not mentioned.

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[3] Abbreviation: PG, polygalacturonase.

fruit had higher activity when measured at pH 4.6. Therefore, all samples were analyzed at both pH values.

For pH 4.6 measurements, the substrate, 0.12% repurified sodium polypectate (11), was dissolved in a pH 4.5 solution of 0.10 M Na acetate and 0.02% sodium azide. The pH of the reaction mixture increased to 4.6 when the 1.0-ml enzyme sample, at pH 6.2, was added. For pH 6.2 reactions, the substrate was dissolved in a pH 6.2 buffer of 43 mM maleic acid and 0.02% sodium azide. Reactions were done at 30 C. One-ml samples of dialyzed extract were added to 5.0 ml substrate solution. Measurements were made at 0 and 20 h if the enzyme preparation was from immature fruit or at 0 and 1 or 2 h if the enzyme preparation was from mature or C_2H_4-treated fruit. Previous studies had shown that the reactions were linear within these times. Release of reducing groups was measured from 1.0-ml samples of reaction mixtures at 600 nm on a Cary 219 spectrophotometer using the Nelson (6) procedure. One unit of enzyme activity was defined as the release of 1 μmol of reducing equivalents min^{-1}. Galacturonic acid was used as the standard.

Purification of C_2H_4-induced PG. Freshly harvested cucumbers, ranging in weight from 120 to 170 g/fruit, were gassed with air containing 10 $\mu l\ l^{-1}$ C_2H_4 at a flow rate of 500 ml min^{-1} for 1 week. The seed cavity tissue was removed and dry NaCl was added to give a concentration of 50 mM. The lower NaCl concentration was used because it facilitated adsorption of the enzyme on the Sephadex CM-50 column. The enzyme was extracted and chromatographed on a 2.5- × 30-cm Sephadex CM-50 column and then on a 1.6- × 30-cm Sephadex SP-C25 column. The enzyme was eluted from the CM-50 column with 2 liters of a 0.10 to 0.50 M linear NaCl gradient in 43 mM maleic acid buffer (pH 6.2) and 0.02% sodium azide. A 1-liter pH 4.0 to 5.0 gradient in 0.1 M Na-acetate and 0.25 M NaCl was used to elute the enzyme from the SP-C25 column. These procedures were also used during the purification of PG from ripe cucumber fruit (5).

Disc-gel electrophoresis was done in 7- × 0.5-cm 15% and 7.5% acrylamide gels with pH 4.5 β-alanine-acetic acid electrode buffer (4).

RESULTS

Generally, C_2H_4 production increased and CO_2 production, mesocarp pH, and seed cavity tissue pH decreased as peel Chl content decreased (Table I). Since peel Chl is lost during cucumber fruit maturation, the above noted changes appear to be characteristic of the maturation process.

When C_2H_4 production from individual harvested fruit was monitored for 30 days, it was observed that each fruit produced a burst of C_2H_4 during its maturation (Fig. 1). The burst of C_2H_4 production occurred from 6 to 25 days after the harvest of fruit ranging in size from 350 to 550 g/fruit. This transient increase in C_2H_4 production decreased in intensity as the interval after harvest increased. There was no statistically significant correlation between fruit weight and either the timing or intensity of the burst of C_2H_4 production.

The relationship between the decrease in green color and C_2H_4 production was investigated by arranging data from another experiment with 20 fruit so that the date of maximum C_2H_4 production was defined as day zero (Fig. 2). Green color was slowly lost before the maximum rate of C_2H_4 production was attained. However, after the burst had occurred, green color was lost at a much faster rate. Fruit which had not produced a burst of C_2H_4 had an

Table I. *Analysis of Harvested Cucumber Fruit*

Fruit were harvested and visually segregated on the basis of peel color, and 12 fruit representing a range of color were selected for analysis.

Chlorophyll	Fresh Wt	C_2H_4 Production	CO_2 Production	Mesocarp	Seed Cavity
O.D.$_{665}$/*g*	*g*	*nl/kg·h*	*ml/kg·h*	*pH*	
16.34	410	32.9	28.4	5.6	6.2
10.16	374	34.9	28.4	5.6	6.1
7.57	398	32.8	27.8	5.5	6.2
6.93	330	48.7	35.6	5.4	5.8
6.48	451	69.4	17.6	5.6	5.4
6.00	421	107.8	26.6	5.6	5.7
5.44	479	89.4	26.8	5.5	5.7
2.13	539	90.6	19.0	5.4	4.9
0.86	522	183.1	11.9	5.1	4.3
0.55	577	250.7	16.5	5.2	4.2
0.52	591	190.1	11.1	5.1	4.5
0.41	702	279.2	10.5	5.0	4.6

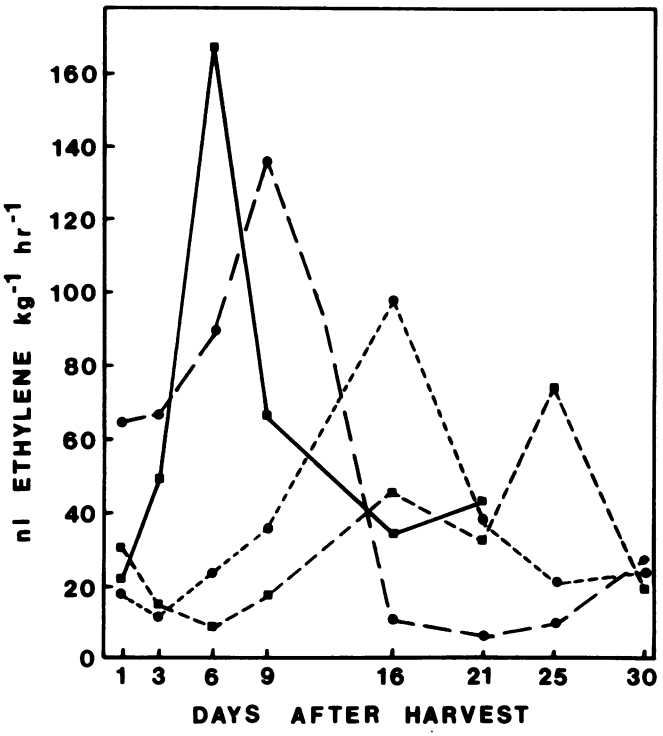

FIG. 1. C_2H_4 production from four harvested cucumber fruit.

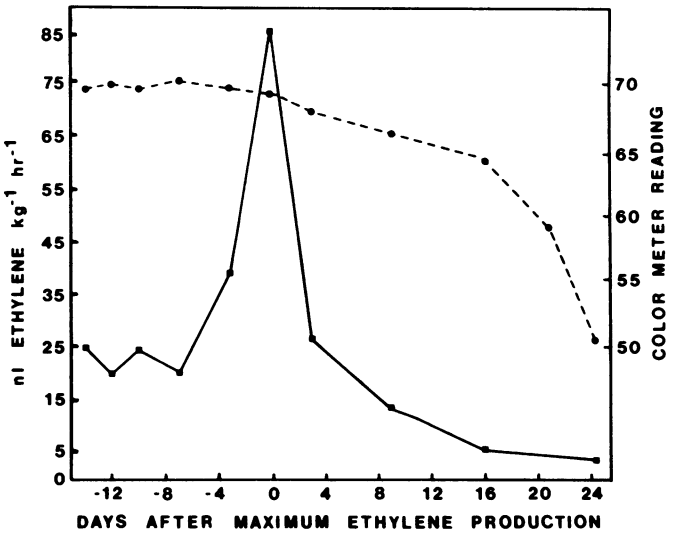

FIG. 2. Green color of cucumber fruit in relation to the timing of maximum C_2H_4 production. (——), C_2H_4 production; (- - -) color meter reading.

average seed cavity tissue pH of 5.3 and average PG activities of 0.83 and 0.68 × 10^{-3} units g^{-1} at pH 6.2 and 4.6 respectively. In comparison, fruit which had produced a burst of C_2H_4 had an average seed cavity tissue pH of 4.2 and average PG activities 36 (*i.e.* 30.4 × 10^{-3} units g^{-1}) and 25 (*i.e.* 16.9 × 10^{-3} units g^{-1}) times higher than the control values pH 6.2, and 4.6, respectively.

If endogenous C_2H_4 stimulated the production of PG and the maturation of cucumber fruit, then gassing fruit with C_2H_4 in air should also induce higher PG activity. CO_2 production was stimulated over 2-fold, from 35.7 to 73.3 ml kg^{-1} h^{-1}, within 24 h of gassing fruit with 10 μl l^{-1} C_2H_4 in air. The pH of seed cavity tissue was lower in C_2H_4-treated fruit than in air-treated controls (Fig. 3). Also in C_2H_4-treated fruit, PG activity increased significantly before the pH of the seed cavity tissue changed significantly. PG activity assayed at pH 6.2 remained constant at about 2.9 × 10^{-3} units g^{-1} in fruit during 8 days of gassing with air, but it increased progressively after 2 days in fruit gassed with C_2H_4, reaching 6.7 × 10^{-3} units g^{-1}, or 2.3 times the control level, after 8 days. Although PG activity assayed at pH 4.6 increased in both control and C_2H_4-treated fruit during the experiment, it increased more rapidly in the latter. After 8 days, the C_2H_4-treated fruit had enzyme activity over twice that of the control fruit. The PG activity at pH 4.6 remained higher than that at pH 6.2 throughout the period of C_2H_4 treatment.

Two cultivars of cucumbers were harvested in sizes ranging from 10 to 350 g/fruit. These fruit showed no evidence of ripening, either by visual appearance or by a decrease in the pH of the seed cavity tissue. PG activity was determined on seed cavity tissue from individual fruit. Linear regression of PG activity as a function of cucumber weight showed that the size of the 'Pixie' fruit was not related to PG activity in the seed cavity tissue at either pH (data not shown). The mean PG activity of 52 individual fruit was 0.74 ± 0.31 and 0.14 ± 0.10 × 10^{-3} units g^{-1} at pH 4.6 and 6.2, respectively. There was also no relationship between fruit weight of the Addis cultivar and PG activity at pH 4.6; however, there was a slight decrease in PG activity measured at pH 6.2 as cucumber fruit size increased. The mean PG activity of 61 'Addis' fruit was 0.91 ± 0.39 and 0.22 ± 0.09 × 10^{-3} units g^{-1} at pH 4.6 and 6.2, respectively. These values show the considerable variation in PG activity among fruit.

To evaluate PG changes during ripening of cucumber fruit, the seed cavity pH was used as an index of the extent of ripening. Seed cavity tissue from ripening 'Chipper' cucumbers was collected and classified according to pH in 0.2-pH unit increments. As the fruit ripened, as indicated by a decrease in pH from 5.2 to 3.3, the PG activity increased 20-fold when measured at pH 6.2, and 10-fold when measured at pH 4.6 (Fig. 4). Similar results were obtained with 'Addis' cucumbers in which the PG activity was measured by viscometry at pH 6.0 (data not shown).

Ripe fruit from the cultivars Addis, Model, and SMR-18, and a gynecious breeding line, which were from diverse genetic backgrounds, were harvested when mature seeds could be recovered from the fruit. PG activity ranged from 19 to 31 × 10^{-3} units g^{-1} at pH 4.6 and from 19 to 26 × 10^{-3} units g^{-1} at pH 6.2. These data suggest that the amount of PG formed during ripening may be similar in different pickling cucumber varieties.

In 15% acrylamide gel, PG purified from C_2H_4-treated cucumbers (gel b) had the same mobility as homogenous PG prepared from mature cucumbers (gel a) (Fig. 5). At the same stage of purification, the major band from C_2H_4-treated cucumbers corresponded with the major protein band from vine-ripened cucumbers (gel c). This same pattern also was observed in 7.5% acrylamide gels. McFeeters *et al.* (5) had previously found, by slicing gels and doing PG activity measurements on the slices, that the major protein band in purified PG preparations, like that used in gel c, corresponded with PG activity.

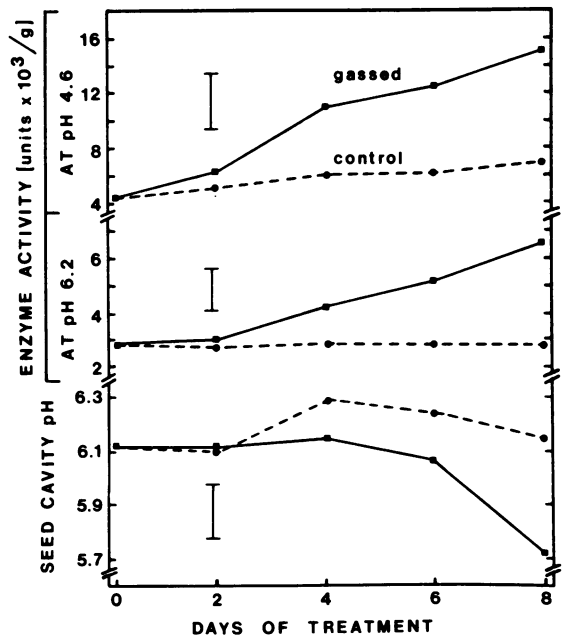

FIG. 3. Effect of the length of time immature harvested cucumber fruit were gassed with air ± 10 μl l^{-1} C_2H_4 on the seed cavity tissue pH, and on the PG activity of the seed cavity tissue measured at pH 4.6 and 6.2. Vertical bars represent the LSD values at 0.05.

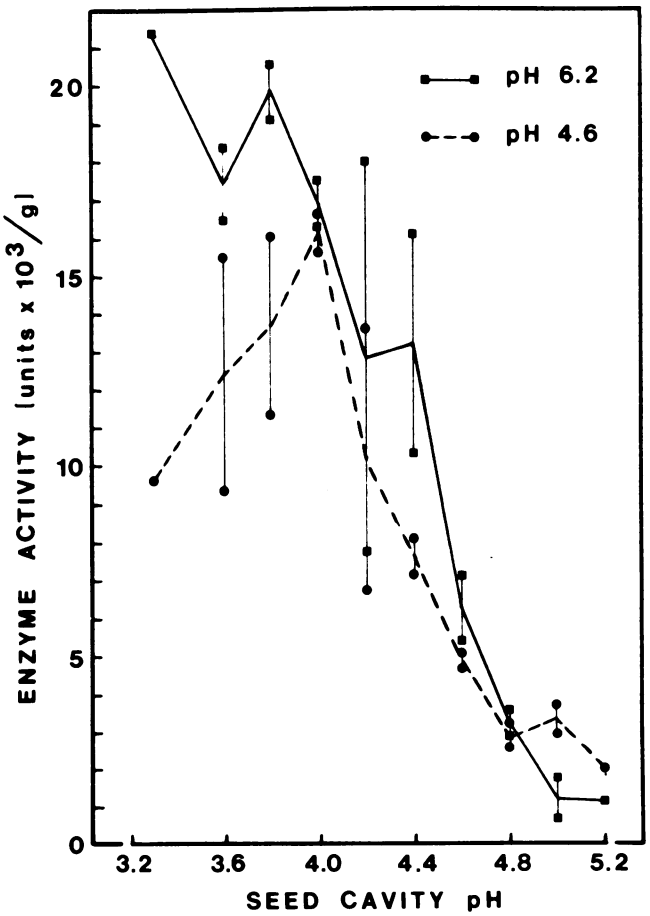

FIG. 4. PG activity of seed cavity tissue measured at pH 4.6 and 6.2 as a function of the initial pH of the tissue. Vertical bars represent the LSD values at 0.05.

Table II. *Purification of Polygalacturonase Activity from Ethylene-treated Cucumbers*

Purification Step	Volume	Enzyme Activity	Protein	Total Enzyme Activity	Total Protein	Yield	Specific Enzyme Activity	Purification
	ml	*units × 10³/ml*	*μg/ml*	*units × 10³*	*mg*	*%*	*units/mg protein*	*-fold*
1. Initial enzyme extract	1000	7.73	748	7.73	748	100	0.0103	1.0
2. Absorption to CM-50 (by difference)	1000	7.13	379	7.13	379	92.2	0.019	1.9
3. After CM-50 fractions, 33–51	283	15.80	75	4.47	21.2	57.8	0.21	20.0
4. After SP-C25 pH gradient	148	19.6	7.4	2.90	1.1	37.5	2.65	257

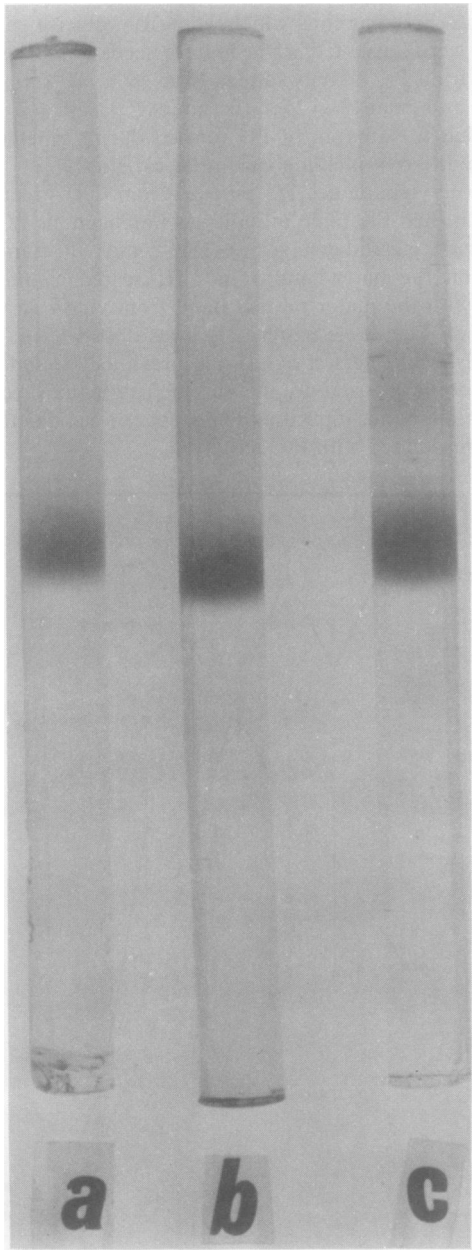

FIG. 5. Gel electrophoresis in 15% acrylamide gel of homogeneous PG prepared from vine-ripened mature cucumbers (a) and partially purified PG from C_2H_4-treated immature fruit (b) and from vine-ripened mature fruit (c).

DISCUSSION

The results here agree with the observation of Poenicke *et al.* (8) that harvested cucumber fruit produce a burst of C_2H_4. However, in the study presented here, the burst of C_2H_4 production peaked 6 to 25 days after harvest rather than within 60 h of harvest, and the maximum rates of C_2H_4 production observed here were only 1.4% of their reported values. The higher rates of C_2H_4 biosynthesis reported by Poenicke *et al.* (8) may have been due to their use of either younger fruit or fungus-infected cucumber fruit. It was found here that the timing of the C_2H_4 burst varied among the harvested fruit and that the amount of C_2H_4 produced during the burst declined as the time after harvest increased. However, there was no statistically significant correlation between fruit weight and either the timing or the intensity of the peak of C_2H_4 production. Although the transient increase in C_2H_4 production is reminiscent of that found during the climacteric in some fruit, an examination of data presented in Table I shows that, as C_2H_4 production increased, CO_2 production decreased.

It was also found that the burst of C_2H_4 production was followed by a loss of Chl, a decline in the seed cavity tissue pH, and an increase in PG activity in the seed cavity tissue. Poenicke *et al.* (8) reported that cucumbers lost Chl after exposure to C_2H_4. The results here confirm this observation and, moreover, show that PG activity increased in response to C_2H_4 exposure. A significant increase in PG activity was measured in harvested immature cucumber fruit which were exposed to C_2H_4 for 4 days. The fruit also yellowed during this period. However, neither a similar increase in PG activity nor a loss of Chl was observed in control fruit.

Changes in PG activity of cucumbers during fruit development on the vine were investigated. Fruit had low, but measurable, levels of PG activity at the earliest stages of fruit development measured, *i.e.* 10-g fruit. This is in contrast to the report by Bell (2) that pectolytic activity was difficult to measure in green cucumber fruit. Prior to ripening, no clear change was found in PG activity as fruit size increased from 10 to over 350 g. However, during ripening of both 'Chipper' and 'Addis' cucumber fruit, PG activity increased markedly. This increase was correlated with a decrease in the pH of the seed cavity tissue during ripening. Such a decrease in pH was first reported by Bell (2).

Because two different PG enzymes have been reported in cucumbers (5, 10), an attempt was made to determine whether different forms of PG were present at various stages in the development of fruit by examining PG activity at pH 4.6 and 6.2 in all of the experiments described. Differences in the ratio of PG activity at these pH values were observed. Because of considerable variability, changes in the ratio from 0.9 to 1.3 did not constitute reliable evidence for the presence of different PG enzymes in cucumber fruit.

Pressey and Avants (10) have reported an exo-splitting PG in fresh market and pickling cucumbers. Therefore an attempt was made here to determine if the C_2H_4 treatment induced an exo-splitting PG or a different endo-splitting PG than that found in vine-ripened cucumbers by McFeeters *et al.* (5). However, the PG activity from C_2H_4-treated cucumbers had the same chromatographic properties as PG from vine-ripened fruit on two different ion exchange columns (Table II). A 250-fold purification of PG

activity from C_2H_4-treated fruit was obtained after the final ion-exchange step. This enzyme had a specific activity of 2.65 units mg^{-1} protein, which was about half the specific activity found in a homogenous preparation of PG from ripe cucumbers (5). The major protein in this preparation had the same electrophoretic mobility as the homogenous PG from vine-ripened cucumbers at two acrylamide concentrations. These data indicate that the PG isolated from vine-ripened and C_2H_4-treated cucumbers are the same.

Poovaiah and Nukaya (9) extrapolated their preliminary data on tomato ripening to suggest that "PG plays a key role in fruit ripening," that C_2H_4 is not evolved unless PG activity increases, and that "ethylene has no effect on PG activity." In the study presented here, PG activity in cucumber fruit increased after the burst of C_2H_4 production, and PG activity in harvested immature fruit could be induced by exogenous C_2H_4. The data obtained here support the conclusion of Samamura *et al.* (14), based on results from tomatoes, that increased PG activity is most likely a consequence of the primary ripening process, rather than the cause of ripening.

Acknowledgments—The authors wish to thank Dr. D. Mason Pharr for his help in planning and performing this research and for his critical review of this manuscript. The technical assistance of Mr. Don Batot and Mrs. Karen Clark are also appreciated.

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