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Mechanical Stress, Storage Time, and Temperature Influence Cell Wall-degrading Enzymes, Firmness, and Ethylene Production by Cucumbers

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Abstract. **Cucumber (*Cucumis sativus* L.) fruit ('Heinz 3534') subjected to mechanical stress followed by storage for 48 hr exhibited visible degeneration of the mesocarp and endocarp, which was accompanied by several-fold increases in the activity of the enzymes pectin methylesterase, peroxidase, polygalacturonase, and xylanase. The activity of all these enzymes increased in the endocarp, whereas only pectin methylesterase and polygalacturonase increased in the mesocarp, and pectin methylesterase, peroxidase, and polygalacturonase increased in the exocarp. Further, the increase in the activity of pectin methylesterase, peroxidase, and polygalacturonase was less when cucumbers were stored at 0° or 10°C vs. 25° or 38° after mechanical stress. Cucumbers stored for only 8 hr after mechanical stress, or not stressed, and stored for 8 or 48 hr showed no consistent significant increases in enzyme activity. Endocarp firmness of fruit stored at 25° or 38° for 8 hr after mechanical stress was lower than that of unstressed fruit, but this decrease was not evident after 48 hr of storage, and mesocarp firmness was not affected by mechanical stress regardless of storage temperature or time. Ethylene production was stimulated significantly by 8-hr storage at 0°, following mechanical stress, and by 48-hr storage at 0°, but was unaffected by all other treatment and storage regimes. These data indicate that mechanical stress induces biochemical and morphological changes in the major tissues of cucumber fruit, but tissue firmness and/or ethylene production will not serve as indicators of these changes. Moreover, the effects of mechanical stress do not appear to be mediated through the action of ethylene.**

A major consideration of the fresh-pack pickling cucumber industry is the retention of firm fruit texture in the processed product. Tissue softening results in poor-quality processed spears as evidenced by seed and endocarp sloughing and mesocarp deterioration in the jar. This decrease of processed spear quality has been linked to mechanical injury during harvesting and transport (10) and elevated postharvest storage temperatures and times (4, 5), but little work has been done to elucidate the physiological and biochemical changes occurring in softening cucumbers as a result of these stresses. Moreover, a useful indicator has not been found to screen softening cucumbers before processing.

Softening of fruit in general is a result of the combined action of several cell wall-degrading enzymes (9), which may or may not be regulated through the action of ethylene. Maturing cucumbers produce ethylene at very low rates compared to climacteric fruit (1), but still soften due to increases in the activity of pectin methylesterase and polygalacturonase (11). However, after low-temperature stress (18) or mechanical harvest (15), ethylene production by cucumbers is stimulated. Additionally, exogenous ethylene caused a decrease in the firmness of cucumbers (15), but the activity of cell wall-degrading enzymes was not determined.

The aim of this study was to determine the activity of C_x-cellulase (EC 3.2.1.4; CELL), pectin methylesterase (EC 3.1.1.11; PME), peroxidase (EC 1.11.17; PROX), polygalacturonase (EC 3.2.1.15; PG), and xylanase (EC 3.2.1.32; XYL) after mechanical stress and postharvest storage at given temperatures, and relate these data to visible changes within the cucumbers, ethylene production rates, and tissue firmness.

Materials and Methods

Cucumber treatment. Cucumber ('Heinz 3534') plants were grown at the Ohio Agricultural Research and Development Center, Wooster. For each experiment, 36 commercial size three cucumbers (37- to 55-mm diameter) were harvested, washed with cool tap water, and divided into two treatment groups. The first group was divided into four subgroups of four cucumbers each and placed at 0°, 10°, 25°, or 38°C. The second group was mechanically stressed by dropping each cucumber four times (once on each end and twice on the side) from 1 m, then rolling each cucumber for 1 min between a benchtop and a 30-cm square board supporting a 10 kg mass. The second group was then divided into four subgroups of four cucumbers each and placed at 0°, 10°, 25°, or 38°. Relative humidity was maintained at 100% during storage.

The overall sampling protocol was as follows. Two cucumbers from each stress–temperature regime were removed at 0, 8, and 48 hr and individually analyzed for ethylene production and tissue firmness. Specified tissues from these cucumbers were pooled for subsequent enzyme extraction and assay. All data are expressed as the mean values of three replicate experiments.

Ethylene measurement. Cucumbers were placed at room temperature (24° to 26°C) for 2 hr then sealed individually in 1.5 liter screw-capped canning jars fitted with a serum stopper. After 1 hr, ethylene was determined by removing a 2-ml headspace sample and injecting this into a Hewlett-Packard 5890A gas chromatograph equipped with a flame-ionization detector and a

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Table 1. The effect of postharvest mechanical stress, storage temperature, and length of time in storage on the firmness of the mesocarp, and endocarp of 'Heinz 3534' cucumber slices.

	Firmness (N)							
	Not stressed				Mechanically stressed			
Storage time (hr)	Storage temp (°C) 0	10	25	38	0	10	25	38
				Mesocarp				
0	---	---	0.4 ± 0.9[z]	---	---	---	9.5 ± 1.0	---
8	9.4 ± 1.7	10.1 ± 0.7	8.3 ± 0.6	7.6 ± 0.4	8.9 ± 0.5	9.2 ± 0.5	8.6 ± 0.4	8.4 ± 0.2
48	9.3 ± 0.5	8.2 ± 1.5	8.4 ± 0.5	8.8 ± 0.4	9.8 ± 0.7	9.9 ± 0.6	9.1 ± 0.4	9.2 ± 0.5
				Endocarp				
0	---	---	1.6 ± 0.2	---	---	---	1.5 ± 0.1	---
8	1.6 ± 0.2	1.5 ± 0.1	1.4 ± 0.2	1.5 ± 0.2	1.4 ± 0.2	1.4 ± 0.3	1.2 ± 0.1	1.1 ± 0.2
48	1.8 ± 0.3	1.3 ± 0.3	1.3 ± 0.2	1.3 ± 0.2	1.4 ± 0.2	1.4 ± 0.2	1.3 ± 0.2	1.4 ± 0.2

[z]Means ± SE (n = three experiments).

Table 2. The effect of postharvest mechanical stress, storage temperature, and length of time in storage in ethylene production by 'Heinz 3534' cucumbers.

	Ethylene production (pmol/hr/per g fresh wt)							
	Not stressed				Mechanically stressed			
Storage time (h)	Storage temp (°C) 0	15	25	38	0	15	25	38
0	---	---	9 ± 1[z]	---	---	---	9 ± 2	---
8	7 ± 1	7 ± 1	5 ± 1	5 ± 1	29 ± 3	10 ± 2	6 ± 2	10 ± 1
48	51 ± 4	6 ± 1	3 ± 1	3 ± 1	39 ± 8	7 ± 1	4 ± 2	5 ± 2

[z]Means ± SE (n = three experiments).

Table. 3. Distribution of specific enzyme activity in the exocarp, mesocarp, and endocarp of unstressed, unstored, size three 'Heinz 3534' cucumbers.

	Enzyme activity				
Tissue	C_x-cellulase (Δ drain time (sec)/ hr per mg of protein)	Pectin methyl-esterase (μmol carboxyl groups released/ min per mg of protein	Peroxidase (OD 470 nm/min per mg of protein)	Poly-galacturonase (μmol reducing groups released/ hr per mg of protein)	Xylanase (μmol reducing groups released hr per mg of protein)
Exocarp	15 ± 3[z]	22 ± 8	166 ± 78	147 ± 20	211 ± 78
Mesocarp	10 ± 3	20 ± 6	78 ± 21	139 ± 7	147 ± 53
Endocarp	5 ± 1	10 ± 6	48 ± 2	85 ± 14	307 ± 72
Ratio (exocarp : mesocarp : endocarp)	3:2:1	2.2:2:1	3.5:1.6:1	1.7:1.6:1	0.7:0.5:1

[z]Means ± SE (n = three experiments).

2.4 m × 2 mm (i.d.) glass column packed with 100/200 mesh Porapak N (Alltech). Nitrogen was used as the carrier gas at 22 ml·min^{-1}. Operating temperatures for the injection, column, and detector ovens were 200°, 50°, and 250°, respectively. An ethylene standard (1 μl·liter^{-1}; Supelco) was used for calibration.

Tissue firmness measurement. Following ethylene measurements, cucumbers were weighed, quartered longitudinally, and a 0.5-cm transverse slice was removed from the mid-region of two quarters for firmness determinations. Firmness of the pericarp and carpel areas of each slice was measured as previously described (17) with an Instron TM equipped with a 20-kg pressure transducer and a 3.2-mm-diameter probe moving at 20 cm·min^{-1}.

Enzyme extraction and assay. Cucumber sections used for enzyme analyses were immediately adjacent to the slices removed for firmness measurements, and were further divided into exocarp, mesocarp, and endocarp tissues with a knife. All tissues were then lyophilized and stored at −40°C for subsequent analysis.

Soluble and ionically bound enzymes were extracted by grinding 1 g of lyophilized tissue at 0° to 4°C in 10 ml of 1 M NaCl with a mortar and pestle and 10 g of acid-washed sea sand. The homogenates were incubated at 4° for 1 hr to complete the extraction, then filtered through two layers of cheesecloth and cleared by centrifugation at 20,000× *g* for 20 min. The resulting supernatant was assayed directly for PME, PROX, and CELL activity. A 1.5-ml aliquot of the supernatant was further purified by applying it to a 2.5 × 20 cm gel chromatography column packed with Sephadex G-25-300 and eluting with 0.25 M NaCl.

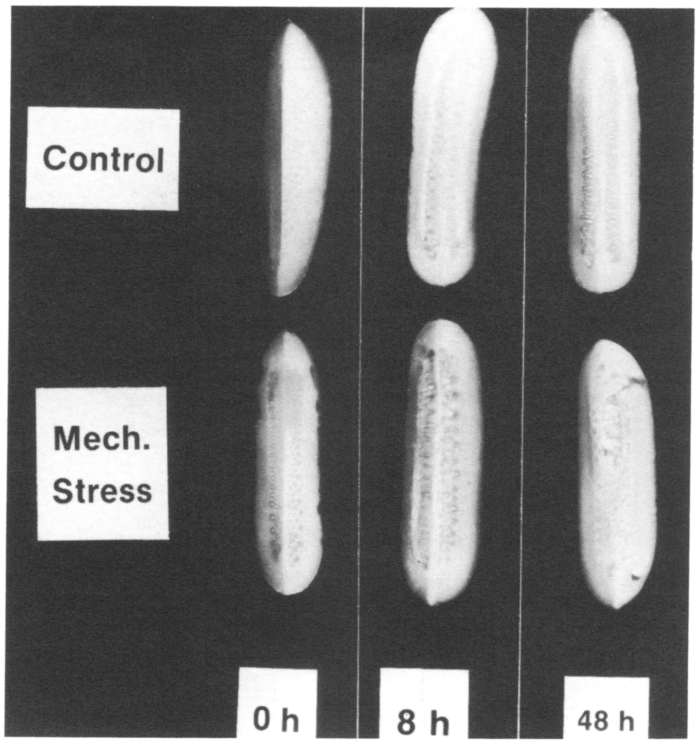

Fig. 1. Unstressed and mechanically stressed cucumber slices after storage for 0, 8, or 48 hr at 25°C.

The protein-containing fractions were pooled and assayed for PG and XYL activity.

C_x-cellulase was assayed (14) at 30°C by incubating 0.75 ml of the cleared homogenate with 2 ml of 1% (w/v) carboxymethylcellulose (Sigma) in 0.03 M sodium phosphate buffer (pH 6.5), and determining the time required for 0.5 ml of the reaction mixture to drain from a 1- ml serological pipet.

Peroxidase activity was assayed (13) at 25°C by incubating 50 µl of cleared homogenate with 1 ml of 120 mM guaiacol (Sigma) 0.3 ml of 1% (v/v) H_2O_2, and 1 ml of 0.06 M sodium phosphate buffer (pH 6.5). The reaction was initiated by the addition of the H_2O_2 and the absorbance at 470 nm was determined after 1 min using distilled H_2O as the blank.

Pectin methylesterase was assayed spectrophotometrically according to Hagerman and Austin (7). Two milliliters of pectin substrate (pH 6.5) containing 0.5% (w/v) pectin (Sigma), 0.02 M sodium phosphate, 0.3 M KCl, 3 mM NaN_3, and 0.03 mM bromthymol blue (Matheson, Coleman and Bell) were incubated with 0.5 ml of cleared homogenate at 25°C and the absorbance at 616 nm was determined after 5 min using distilled H_2O as the blank.

Polygalacturonase was assayed (14) at 30°C by incubating 1 ml of purified extract with 2 ml of 0.5% (w/v) polygalacturonic acid (Sigma) in 0.03 M sodium phosphate buffer (pH 6.5). After 8 hr, the mixtures were boiled for 10 min to stop the reaction, centrifuged for 5 min at 10,000× *g*, and the reducing groups in 0.5 ml of the supernatant were determined by the potassium ferricyanmide method (8) using galacturonic acid as the standard.

Xylanase was assayed (14) similar to PG, except that 0.5% (w/v) larch xylan (Sigma) was substituted for polygalacturonic acid and xylose was used as the standard.

Protein was determined (3) using bovine serum albumin as the standard. Enzyme assays of each extract were duplicated.

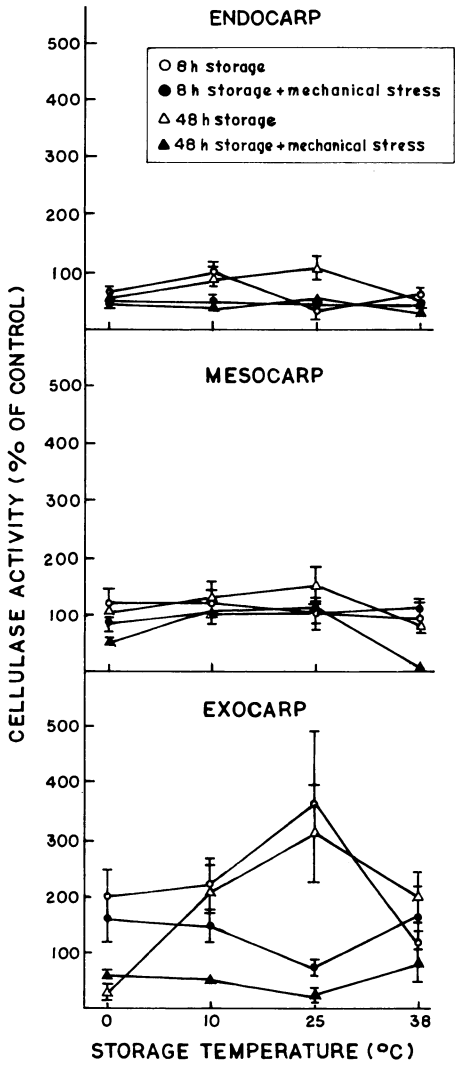

Fig. 2. C_x-cellulase activity in the exocarp, mesocarp, and endocarp of unstressed and mechanically stressed 'Heinz 3534' cucumbers stored for 8 or 48 hr at 0°, 10°, 25°, or 38°C. Data is presented as percentage of the enzyme activity in the respective tissues from the unstressed, unstored control cucumbers. Vertical bars represent the SE (n = three experiments).

Results and Discussion

Tissue morphology and firmness. Regardless of mechanical stress, cucumbers stored for 48 hr at 25° or 38°C were "rubbery" compared to unstored fruit, those stored for 8 hr at any temperature, or fruit stored for 48 hr at 0° or 10°. The cause of this rubbery characteristic is unknown.

The exocarp of cucumbers subjected to mechanical stress, which simulated mechanical harvesting and grading, was not visibly injured (split, punctured, etc.) compared to unstressed fruit. It is possible that damage to membranes, cell walls, or organelles occurred as a result of mechanical stress.

The endocarp and mesocarp, likewise, exhibited no visible damage immediately after mechanical stress (Fig. 1). However, after 48 hr of storage at 25°C, the endocarp of mechanically stressed cucumbers had degenerated markedly. This breakdown was evidenced by the separation of the seeds from the carpel wall and, in some cucumbers, the seed cavity appeared to have been degraded (Fig. 1). Cucumbers stored at 25° for only 8 hr

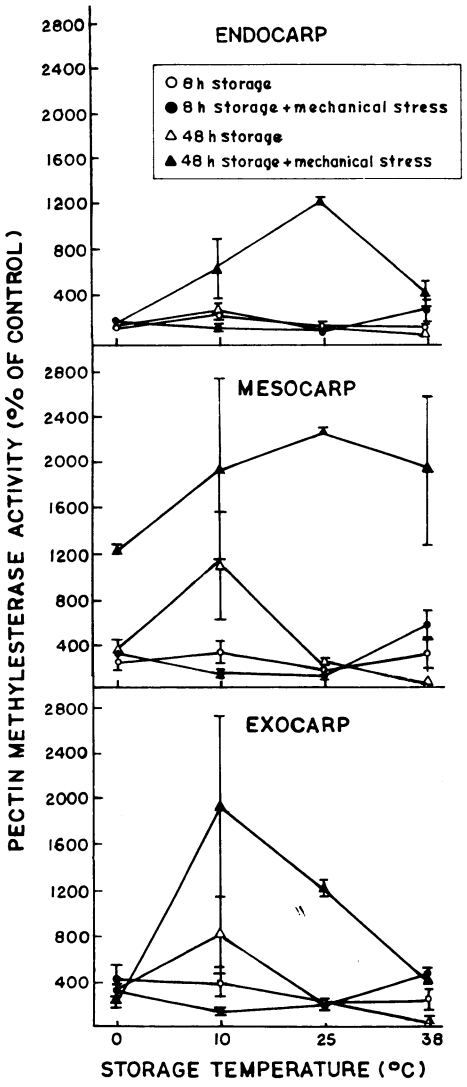

Fig. 3. Pectin methylesterase activity in the exocarp, mesocarp, and endocarp of unstressed and mechanically stressed 'Heinz 3534' cucumbers stored for 8 or 48 hr at 0°, 10°, 25°, or 38°C. Data is presented as percentage of the enzyme activity in the respective tissues from the unstressed, unstored control cucumbers. Vertical bars represent the SE (n = three experiments).

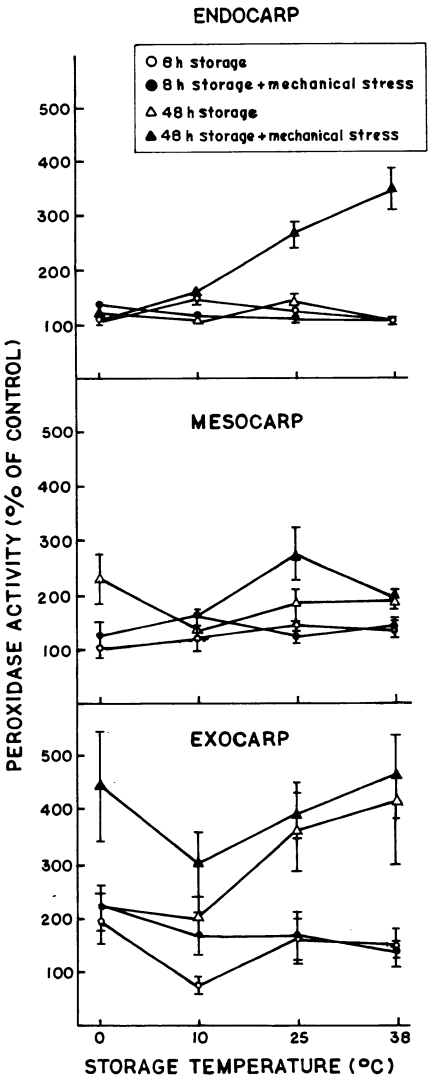

Fig. 4. Peroxidase activity in the exocarp, mesocarp, and endocarp of unstressed and mechanically stressed 'Heinz 3534' cucumbers stored for 8 or 48 hr at 0°, 10°, 25°, or 38°C. Data is presented as percentage of the enzyme activity in the respective tissues from the unstressed, unstored control cucumbers. Vertical bars represent the SE (n = three experiments).

after mechanical stress (Fig. 1) and those stored at 0° or 10° for 48 hr after mechanical stress (data not shown) exhibited less degeneration of the endocarp than those stored for 48 hr at 25°. These observations indicated that this phenomenon was inducible as degeneration increased with storage time and was not evident immediately after mechanical stress. Thus, it was unlike carpel separation, which has been reported to occur during mechanical harvesting and grading (10).

Mesocarp and endocarp firmness of mechanically stressed fruit was not different from that of unstressed fruit when measured immediately after treatment (Table 1). After storage for 8 hr at 38°C, the mesocarp firmness of both unstressed and mechanically stressed cucumbers decreased, but this change was not evident after 48-hr storage and may have been related to the development of the rubbery character of these fruit. In contrast to the mesocarp, the firmness of the endocarp decreased after mechanical stress, but this change was evident only after 8-hr storage at 25° or 38°. Endocarp firmness after 48-hr storage was not different from firmness at time zero, regardless of storage temperature or mechanical stress.

Ethylene production. Since mechanical stress stimulates ethylene production by numerous plant tissues (1) and exposure of cucumbers to ethylene decreases tissue firmness (15), endocarp deterioration observed here may have been regulated by stress-induced ethylene. To test this hypothesis, we measured ethylene production by cucumbers stored at 0°, 10°, 25°, or 38°C for 8 or 48 hr after mechanical stress. As shown in Table 2, mechanical stress had no effect on ethylene production by cucumbers when measured immediately after treatment, and stimulated ethylene production after 8 hr only when the cucumbers were stored at 0°. Both unstressed and mechanically stressed cucumbers exhibited increased ethylene production when stored for 48 hr at 0°. Low-temperature stress has been shown previously to stimulate ethylene production by cucumbers (18). In this study, mechanical stress may have promoted the synthesis of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC),

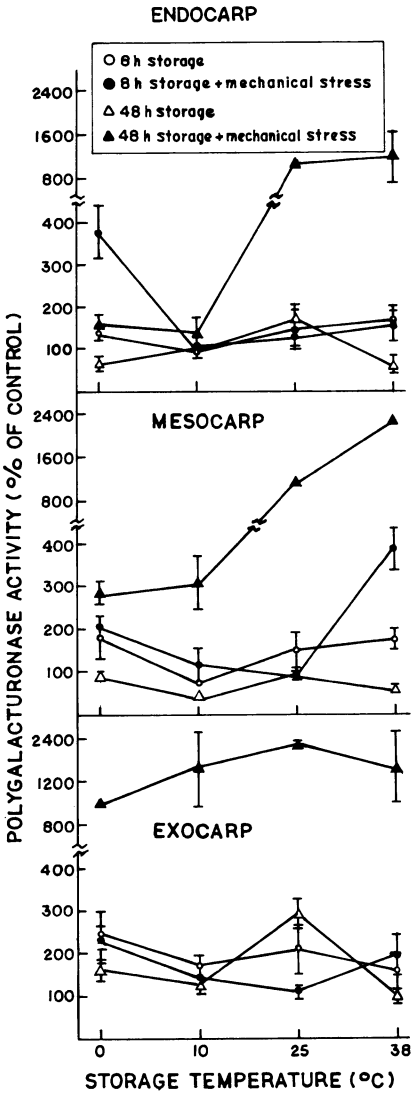

Fig. 5. Polygalacturonase activity in the exocarp, mesocarp, and endocarp of unstressed and mechanically stressed 'Heinz 3534' cucumbers stored for 8 or 48 hr at 0°, 10°, 25°, or 38°C. Data is presented as percentage of the enzyme activity in the respective tissues from the unstressed, unstored control cucumbers. Vertical bars represent the SE (n = three experiments).

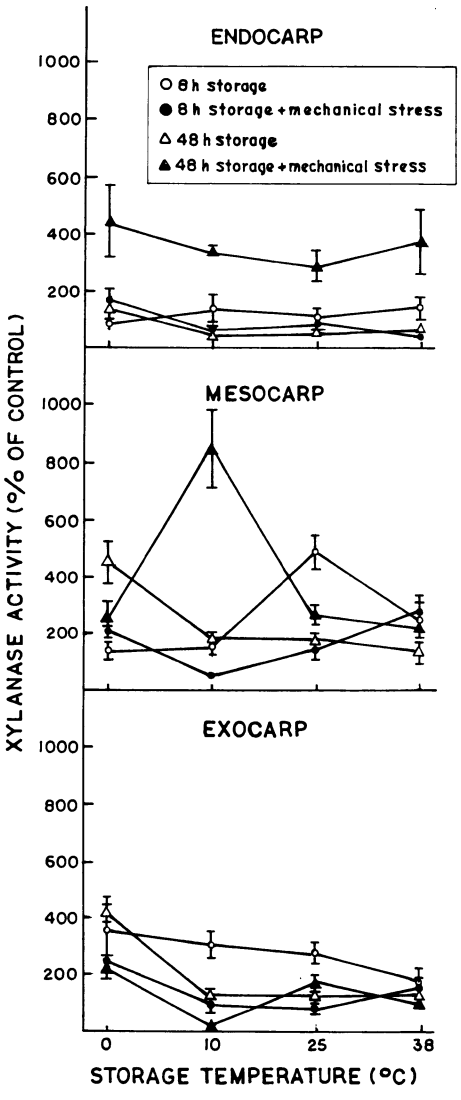

Fig. 6. Xylanase activity in the exocarp, mesocarp, and endocarp of unstressed and mechanically stressed 'Heinz 3534' cucumbers stored for 8 or 48 hr at 0°, 10°, 25°, or 38°C. Data is presented as percentage of the enzyme activity in the respective tissues from the unstressed, unstored control cucumbers. Vertical bars represent the SE (n = three experiments).

but possible deterioration of cellular membranes during storage at 10°, 25°, or 38° may have prevented its conversion to ethylene. Oxidation of ACC to ethylene requires membrane integrity and is inhibited by elevated temperatures (19). Low-temperature (0°) storage may have stabilized membrane structure, thereby allowing increased ethylene production after mechanical stress.

Enzyme activity. With the exception of XYL, enzyme specific activity decreased from the exocarp to the endocarp of unstressed, unstored cucumbers (Table 3). Enzyme total activity showed similar trends (data not shown). The distribution of enzyme activity within cucumbers has not been reported previously, and it may be related to level of cellular activity in the respective tissues. The seeds as the source of cell wall-degrading enzymes in the endocarp, however, is unlikely, since developing seed would be synthesizing cell walls. The specific activity of all enzymes did not change immediately after mechanical stress (data not shown).

In general, enzyme activity in the exocarp, mesocarp, and endocarp after 8-hr storage, regardless of mechanical stress, and in unstressed cucumbers after 48-hr storage was not different from that in untreated, unstored cucumbers (Figs. 2–6). Moreover, C_x-CELL activity in all three tissues was unaffected by mechanical stress even after 48-hr storage (Fig. 2). In fact, the largest change of C_x-CELL activity was exhibited by the exocarp of unstressed cucumbers after 8- or 48-hr storage at 25°C.

The most significant changes in the activity of PME, PROX, PG, and XYL were exhibited by cucumbers that had been mechanically stressed and then stored for 48 hr. In these fruit, PME activity increased in all three tissues, with activity in the mesocarp showing the greatest increase (Fig. 3). This increase was also temperature-dependent, as both the endocarp and mesocarp exhibited the greatest increase of PME activity in fruit stored at 25°C and less change in those stored at 0°, 10°, or 38°. The change in activity of PME in the exocarp was greatest in fruit stored at 10°.

Peroxidase activity exhibited marked increases in the exocarp

across all storage temperatures (Fig. 4). Increased PROX activity was also evident in the endocarp and appeared to be linearly dependent on storage temperature. The mesocarp exhibited no significant variation in PROX activity. Interestingly, PROX activity also increased in the exocarp of unstressed cucumbers that were stored for 48 hr at 25° or 38°C. Hence PROX would not serve as an indicator of mechanical stress in these fruit.

Polygalacturonase exhibited very large increases in activity in all tissues, especially in response to storage at 25° or 38°C after mechanical stress (Fig. 5). Further, the activity of PG in the exocarp showed a marked increase after storage at 0° or 10° following mechanical stress. Storage at 38° for 8 hr after mechanical stress also increased PG activity in the mesocarp. A central role for PG during the softening of cucumber tissues has been suggested by several authors (2, 11, 16).

Xylanase was the only enzyme whose activity exhibited a preferential increase in the endocarp in response to mechanical stress and storage for 48 hr (Fig. 6). The percentage of increase of XYL activity in the endocarp was not as high as other enzymes (e.g., PG and PME); however, XYL activity in this tissue was already higher than either the mesocarp or exocarp (Table 3). Since the endocarp showed the greatest deterioration in response to mechanical stress and storage (Fig. 1), XYL may play a key role in the degradation of this tissue. Xylanase activity in cucumbers, to our knowledge, has not been reported previously.

Mechanically stressed processing cucumbers exhibited a temperature- and time-dependent degeneration of the endocarp that was associated with increased activity of the cell wall-degrading enzymes PME, PG, and XYL. These changes of enzyme activity occurred before 48 hr, since the endocarp was visibly degraded by this time. The induction of these morphological and biochemical changes was not related to increased ethylene production and, hence, appears to be regulated by some endogenous factor other than ethylene. The identity of this regulator is presently unknown, but may be another natural growth regulator or possibly a cell wall polysaccharide fragment (12).

At present, there is no viable physiological indicator of mechanical injury to cucumber fruit. Attempts have been made to use ethylene production (ref 15; Table 2) and tissue firmness (Table 1) for this purpose, but they have been quite variable and unreliable. In other plants subjected to mechanical stress, changes in the activity of PROX are well established (6). In this study, however, PROX activity increased during storage regardless of mechanical stress (Fig. 4), indicating the nonspecific nature of this proposed enzymatic marker.

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