AN ENDO-POLYGALACTURONASE IN CUCUMBER FRUIT[1]

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ABSTRACT

A homogenous endo-polygalacturonase (EC 3.2.1.15*) has been isolated from mature pickling cucumbers. This is the first report of an endo-polygalacturonase in cucumbers. The enzyme was purified by three chromatography steps on Sephadex cation exchangers. The molecular weight is* 35,000 *daltons. With polygalacturonic acid as the substrate at an ionic strength (μ) of* 0.15, *the pH optimum is* 5.6. *The enzyme is not active at* $\mu = 0.027$. *Maximum activity occurred at* $\mu = 0.2$. *The half-life of the enzyme is* 1.2 *min at* 70°C *in pH* 5.2, 0.1 *M acetate buffer. An inhibitor, isolated from the sericea lespedeza, which inhibits polygalacturonases from fungal sources, also inhibits the cucumber enzyme. This enzyme may be involved in tissue breakdown during the latter stages of fruit development.*

INTRODUCTION

The retention of firm fruit texture during processing and storage of cucumbers is a major quality consideration in the pickling industry. Fleming *et al.* (1978) and Thompson *et al.* (1979) have reported on factors that affect the softening of brined cucumbers during storage. The reduction

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of bloating defects in brined cucumbers by purging and controlled fermentation techniques (Etchells *et al.* 1973; Costilow *et al.* 1977) has led to increased recognition of soft-centered cucumbers as a significant defect in commercial salt stock that must be controlled to increase product yield. In an effort to better understand the development of softening in cucumbers, investigations have been undertaken to characterize enzymes in the cucumber fruit that may contribute to softening.

Bell *et al.* (1951) reported the presence of a pectinesterase in cucumbers that remained constant during fruit development. Bell (1951) also reported that polygalacturonase activity was low to negative in immature cucumber fruit, but high in ripe cucumbers. Pressey and Avants (1975) found an exo-splitting polygalacturonase in a fresh market cucumber variety.

In this paper, we report on the purification and of an endo-splitting polygalacturonase (EC 3.2.1.15) that was isolated from the seed area of ripe pickling cucumbers.

MATERIALS AND METHODS

Enzyme Source

Cucumbers of the cultivar 'Chipper' were grown at the North Carolina State University Unit 2 Research Farm using standard horticultural practices. Ripe cucumbers that had a pH <4.3 in the seed area were harvested. The seed areas were collected, and the material was frozen at $-20°C$ until use.

Chemicals

Sodium polypectate and pectin were obtained from Sigma Chemical Company or Sunkist Growers, Inc. The commercial polypectate was repurified by three precipitations with ethanol (Pressey and Avants 1973a). An inhibitor of fungal polygalacturonase was prepared from sericea lespedeza (*Lespedeza cuneata* (Dum.) G. Don) according to the procedure of Bell *et al.* (1965b). This inhibitor is believed to be a polymer of leucodephinidin. Ion exchangers, get filtration media, and Ficoll were from Pharmacia Chemical Company. Chemicals for gel electrophoresis and pH 3—10 carrier ampholyte for electrofucusing were from Bio-Rad. Buffers and other chemicals were reagent grade.

Enzyme Assays

Polygalacturonase activity was assayed by the release of reducing

groups at 30°C from sodium polypectate measured by the procedure of Nelson (1944). Galacturonic acid was used as the standard. The reaction mixture contained 5.0 ml of 0.12% repurified polypectate in 0.043 M maleic acid, 0.02% sodium azide, pH 6.2, and 1.0 ml of enzyme solution. One-milliliter samples were taken at five time intervals and mixed with 1.0 ml of copper reagent to stop the reaction. After color developed, the samples were centrifuged for 5 min at 12,000 × g for removal of precipitated substrate. Absorbance measurements were made at 600 nm with a Cary 219 spectrophotometer. Reaction rates were calculated by a linear regression estimate of the slope of the linear portion of the reaction curve. One unit of enzyme activity is defined as the release of 1 μmole of reducing groups/min/ml of reaction mixture.

In preliminary studies of the inhibition of cucumber polygalacturonase by sericea inhibitor, enzyme activity was measured by the viscometric assay of Bell *et al.* (1955) with the modification that reactions were done at pH 6.0. Enzyme activities for these experiments were expressed in viscosity units (Bell *et al.* 1955).

For this correlation of viscometric assays with reducing sugar assays, 1.2% polypectate or pectin was prepared in 0.043 M maleic acid, 0.02% NaN_3, pH 6.2. One milliliter of dialyzed enzyme solution was mixed with 5.0 ml of substrate in an Ostwald-Fenske MN-300 viscometer. Viscosity measurements were made 1.0 min and 60 min after enzyme was added, and the percent loss of viscosity was calculated. Reactions were run at 30°C.

Chromatography of Reaction Products

Polygalacturonase was added to 0.42% polypectate in 0.10 M sodium acetate buffer, pH 5.6. Samples (1.0 ml) were removed and heated for 2 min in boiling water to inactivate enzyme. Reducing sugar was measured, and 50-μl samples were spotted on cellulose plates and developed according to the procedure of Liu and Luh (1978) for oligogalacturonic acids. Chromatograms were sprayed with a mixture of 0.93 ml aniline and 1.3 ml 85% phosphoric acid in 100 ml of 70% ethanol and heated for 10 min at 105°C.

Protein Determination

Protein determinations were done with the procedure of Lowry *et al.* (1951).

Electrophoresis and Electrofucusing Procedures

The purity of cucumber polygalacturonase was evaluated by disc gel

electrophoresis with 7 × 0.5 cm 15% and 7.5% acrylamide gels with pH 4.5 β-alanine-acetic acid electrode buffer (Gabriel 1971).

Molecular weights were estimated with 10 × 0.5 cm 10% gels according to the procedure of Laemmli and Favre (1973). Protein samples were denatured by heating in sodium dodecyl sulfate (SDS) and mercaptoethanol (Weber *et al.* 1972). Standard proteins used were bovine serum albumin, ovalbumin, aldolase, chymotrypsinogen, trypsin, β-lactoglobulin, myoglobin, and lysozyme.

Electrofocusing was done on 10 × 0.5 cm 7.5% acrylamide gels with ph 3—10 carrier ampholyte according to the procedure of Catsimpoolas (1968). Gels from electrophoresis and electrofocusing were stained with Coomassie Brilliant Blue G250 (Blakesley and Boezi 1977).

Enzyme activity on disc and electrofocusing gels was localized by slicing unstained gels and extracting enzyme into 0.043 M maleic acid, pH 6.2. The activity extracted from each slice was then measured by the standard assay.

Molecular Weight Estimation by Gel Filtration

Gel filtration on Sephadex G-75 under non-denaturing conditions was used to estimate the molecular weight of the cucumber polygalacturonase (Whitaker 1963). A 91.6 × 1.6 cm column was equilibrated with 0.10 M acetic acid, pH 4.0, containing 0.285 M NaCl. The column was calibrated with cytochrome *c*, myoglobin, chymotrypsinogen, ovalbumin, carboxypeptidase, and chymotrypsin.

Effect of pH and Ionic Strength on Enzyme Activity

The pH activity profile for cucumber polygalacturonase was done with 0.05 M sodium acetate, 0.036 M maleic acid buffers containing 0.02% NaN_3 and NaCl such that the ionic strength was 0.15 at each pH. The effect of ionic strength was determined by using the same buffer concentrations for the reactions. The pH was 5.6 for all buffers. The ionic strength was varied by changing the amount of NaCl. For an ionic strength of 0.027, the buffers were used at half their usual concentration.

Heat Inactivation

The heat stability of cucumber polygalacturonase was determined by heating the enzyme in 0.10 M sodium acetate buffer, pH 5.2. Samples (50 μl) of the heated enzyme were transferred to 0.95 ml of cold 0.043 M maleic acid buffer, pH 6.2, to stop denaturation. Reactions were then run in the usual way to determine the remaining enzyme activity.

Enzyme Purification Procedure

Frozen seeds from cucumbers with a seed-area pH of 3.7 to 4.3 were thawed. The seeds and connective tissue were removed by passing the juice through a hand-operated food mill. The juice was centrifuged for 20 min at 14,500 × g in a refrigerated Sorvall RC-2 centrifuge. The supernatant was collected, and the precipitate discarded. Usually, purification was begun with about 3 kg of seeds from 10 kg of fresh cucumbers. This yielded 2 kg of juice after centrifugation.

The juice was mixed with Sephadex CM-50 cation exchanger that had been equilibrated with 0.043 M maleic acid buffer, pH 6.2, containing 0.02% NaN_3 (5 g juice/g ion exchanger). The pH of the slurry was adjusted to pH 4.1 with HCl. After standing for about 1 h with occasional stirring, the slurry was filtered on Miracloth to collect the ion exchanger. The ion exchanger was washed first with 5 ml of 0.043 M maleic acid buffer, pH 6.2, per gram of ion exchanger, and then with an equal volume of the same buffer containing 0.10 M NaCl. The ion exchanger was resuspended in more of the second buffer and deaerated. This slurry was packed at room temperature in a 4.4 × 30 cm column. Enzyme was eluted from the column with a 2-liter linear salt gradient. The initial buffer was 0.043 M maleic acid, 0.10 M NaCl, 0.02% NaN_3, pH 6.2. The final buffer contained 0.50 M NaCl.

Fractions with enzyme activity from the Sephadex CM-50 column were combined and dialyzed in 0.05 M acetic acid, pH 4.0. This dialysate was pumped into a 1.6 × 30 cm Sephadex SP-C25 cation exchange column. After adsorption of the enzyme, the column was washed with a solution of 0.10 M sodium acetate, 0.25 M NaCl, pH 4.0. When the column had equilibrated, a 1-liter pH gradient was run. The first chamber contained 500 ml of pH 4.0 equilibration buffer. The second chamber contained an equal volume of the same buffer adjusted to pH 5.0.

The final purification step was to combine fractions from the pH gradient column, dialyze at pH 4.0 and adsorb the enzyme to a 1.6 × 15 cm Sephadex SP-C25 column. The column was equilibrated with 0.10 M sodium acetate, 0.25 M NaCl, pH 4.0 buffer. Enzyme was eluted with a 500-ml linear salt gradient from 0.25 to 0.45 M NaCl.

Enzyme samples for electrophoresis and other analytical procedures were concentrated by use of Ficoll. The purified enzyme was stable for several months when stored under refrigeration in pH 4.0 acetate buffer.

RESULTS AND DISCUSSION

This is the first report of the presence of an endo-polygalacturonase in

cucumber fruit. Pressey and Avants (1975) found only an exo-splitting polygalacturonase in a fresh market cucumber variety. Only one polygalacturonase was observed in chromatograms of extracts from the juice of ripe pickling cucumbers. However, about 20% of the total polygalacturonase activity present in the juice failed to bind to Sephadex CM-50. The possibility that another polygalacturonase may be present in this fraction has not been excluded.

Inhibition of Polygalacturonase

Bell *et al.* (1965b) found that extracts from a number of plants inhibited polygalacturonase and cellulase from fungal sources. A leucodelphinidin polymer with a molecular weight of about 15,000 daltons was isolated from sericea (Bell *et al.* 1965b) and was shown to improve the firmness of salt-stock cucumbers when added to cucumber fermentations (Bell *et al.* 1965a). The inhibition of cucumber polygalacturonase by this inhibitor was tested in preliminary studies prior to purification of the enzyme (Table 1). Ninety-nine percent inhibition occurred with 100 ppm of inhibitor.

Table 1. Cucumber polygalacturonase versus sericea enzyme inhibitor

Inhibitor Concentration (ppm)	Polygalacturonase Activity (viscosity units)	Inhibition (%)
0	286	—
10	125	56
100	1	99
1000	0	100

Purification of Polygalacturonase

Polygalacturonase activity was found to be present in highest concentration in the seed area (endocarp) of ripe cucumbers. Therefore, the enzyme was purified from the juice obtained from the seed area of the fruit.

Table 2 summarized purification of cucumber polygalacturonase. A major difficulty in the purification was the removal of the enzyme from the viscous juice that was obtained from ripe cucumbers. Attempts to precipitate the enzyme by ammonium sulfate or ethanol resulted in the concomitant precipitation of polysaccharide material, making the resuspended enzyme solution too viscous for chromatography. Batch absorp-

Table 2. Purification of polygalacturonase from cucumber seed areas

Purification Step	Volume (ml)	Total Protein (mg)	Activity (units)	Yield (%)	Specific Activity (units/mg)	Purification (fold)
1. Cucumber juice	2240	1350	43.2	100	0.032	1
2. Adsorption to Sephadex CM-50	2240	765	35.1	81	0.046	1.4
3. Sephadex CM-50 chromatography	686	16.1	26.9	62	1.7	53
4. Sephadex SP-C25, pH 4.0—5.0 gradient, chromatography	212	1.84	9.5	22	5.2	161
5. Sephadex SP-C25, 0.25—0.45 M NaCl gradient, chromatography	89	1.04	6.1	14	5.9	186

tion of polygalacturonase to Sephadex CM-50 was a convenient method to remove enzyme from the juice. Enzyme was eluted in all three chromatography steps as a broad peak. Maximum enzyme activity eluted at 0.25 M NaCl from the Sephadex CM-50 column, at pH 4.55 from the Sephadex SP-C25 column with a pH gradient, and at 0.36 M NaCl from the Sephadex SP-C25 column with a salt gradient.

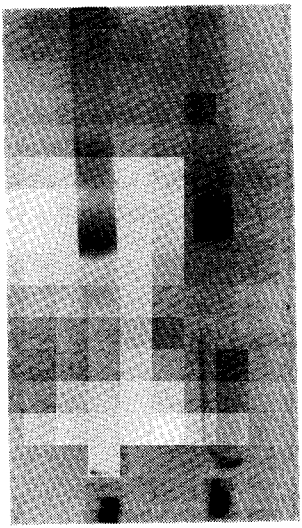

FIG. 1. DISC GEL ELECTROPHORESIS OF CUCUMBER POLYGALACTURONASE AFTER SEPHADEX SP-C25 CHROMATOGRAPHY

A. Eluted with a pH 4.0—5.0 gradient.
B. Eluted with a 0.25—0.45 M NaCl gradient.

Figure 1 shows 15% acrylamide disc gels of cucumber polygalacturonase. Gel A shows the enzyme after elution of the pH gradient SP-C25 column. Comparison of protein bands with enzyme activity in gel slices showed that the location of polygalacturonase activity coincided with the location of the major protein band. Gel B shows that after elution from Sephadex SP-C25 with a NaCl gradient, a homogenous enzyme was obtained. A single band was also observed when electrophoresis was performed with a 7.5% acrylamide gel. Experiments on the properties of cucumber polygalacturonase were done with enzyme preparations comparable in purity to the preparation shown in gel A (Fig. 1). The only previous report of a homogenous endo-polygalacturonase from higher plants was the enzyme purified from tomatoes by Takehana *et al.* (1977). Several homogenous endo-polygalacturonases have been prepared from a yeast (Phaff and Demain 1956) and from molds (Rexová-Benková and Markovic 1976; Cervone *et al.* 1977).

The specific activity of the cucumber enzyme was 5.9 units/mg protein at pH 6.2. Takehana *et al.* (1977) found a specific activity of 47 units/mg for the tomato enzyme, but the reactions were run at 37°C. The reported specific activities of enzymes isolated from yeasts and

or molds (Phaff and Demain 1956; Kaji and Okada 1969; Ishii and Yokotsuka 1972; English *et al.* 1972; Cervone *et al.* 1977) were all from 20 to 200-fold the specific activity of the cucumber enzyme.

Properties of Polygalacturonase

The molecular weight of cucumber polygalacturonase was estimated to be 35,000 daltons by both gel filtration and SDS gel electrophoresis. Figure 2 shows the relationship between protein size and mobility in SDS

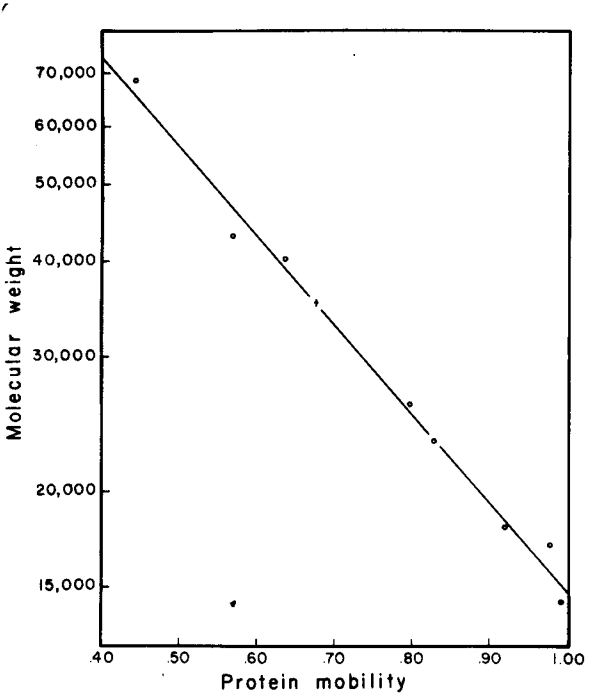

FIG. 2. RELATIONSHIP OF PROTEIN MOBILITY AND MOLECULAR WEIGHT ON SDS GEL ELECTROPHORESIS

Cucumber polygalacturonase (+). Standards (0) in order of decreasing molecular weight are bovine serum albumin, ovalbumin, aldolase, chymotrypsinogen, trypsin, β-lactoglobulin, myoglobin and lysozyme.

gel electrophoresis. Takehana *et al.* (1977) found a molecular weight of 52,000 daltons for the tomato enzyme. Peaches have an endo-polygalacturonase with a molecular weight of 41,000 daltons (Pressey and Avants 1973a). Therefore, this cucumber enzyme is smaller in size than

other endo-polygalacturonases from higher plants but is of the same size as some endo-polygalacturonases from molds (Ishii and Yokotsuka 1972; Rexová-Benková and Slezarik 1968; Cervone *et al.* 1977). It is also smaller than the exo-polygalacturonase from cucumbers, which has a molecular weight of 55,000 daltons (Pressey and Avants 1975).

The isoelectric point of the enzyme was found to be pH 10 or greater by isoelectric focusing in pH 3—10 ampholyte.

Whether this polygalacturonase was an endo- or an exo-splitting enzyme was determined by comparison of the release of reducing sugar endgroups to viscosity loss. Reactions were run in 0.5% sodium polypectate in 0.024 M maleic acid, pH 6.2 at 30°C. Under these conditions, the viscosity of the solution decreased 50% while only 0.06% of the polypectate was hydrolyzed.

In addition, cucumber polygalacturonase hydrolyzed a maximum of 10.9% of the bonds in polygalacturonic acid subjected to extended hydrolysis. Thin-layer chromatography of the reaction products showed no detectable galacturonic acid present at any time during the hydrolysis. If cucumber polygalacturonase were exo-splitting, galacturonic acid would be the primary reaction product. The viscosity data and analysis of reaction products both indicated that cucumber polygalacturonase was an endo-splitting enzyme (Rexová-Benková and Markovic 1976).

There was no increase in absorbance at 235 nm when the enzyme was incubated with substrate at pH 5.6. This indicated that the enzyme was not a lyase (Rexová-Benková and Markovic 1976).

Figures 3 and 4 show the relationship of enzyme activity and viscosity loss for polypectate and citrus pectin substrates. Polypectate was degraded so that over 80% viscosity loss occurred. However, the viscosity loss did not go beyond 25% when pectin was the substrate. Polypectate has been found to be a better substrate than pectin for most other endo-polygalacturonases (Rexová-Benková and Markovic 1976).

The ionic strength of the reaction medium had a considerable effect on the rate of enzyme activity under conditions in which the enzyme was stable (Fig. 5). No activity was observed at $\mu = 0.027$. The activity increased rapidly up to an ionic strength of 0.2. At ionic strength levels above 0.2, precipitation of the polypectate substrate was visible. This precipitation was associated with loss of all measurable enzymatic activity by $\mu = 0.4$. Pressey and Avants (1973b) observed this same pattern for tomato polygalacturonase II. Because cucumber polygalacturonase had an isoelectric point at pH 10 or higher, it would have a positive charge at pH 5.6. Therefore, the suggestion (Pressey and Avants 1973b) that at low ionic strength polygalacturonase may form relatively stable complexes with the negatively charged substrate would be reasonable in the case of cucumber polygalacturonase.

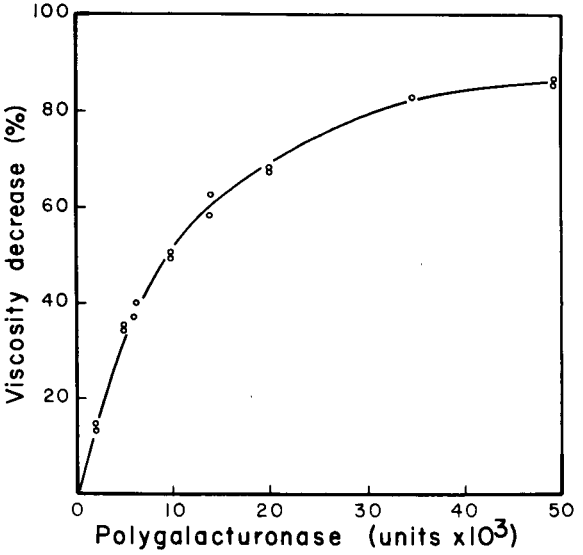

FIG. 3. RELATIONSHIP OF CUCUMBER POLYGALACTURONASE CONCENTRATION AND VISCOSITY DECREASE AS MEASURED AFTER 60 MIN IN 1.0% POLYPECTATE SUBSTRATE (μ = 0.15), 0.043 M MALEIC ACID AND 0.05 M NaCl, pH 6.2.

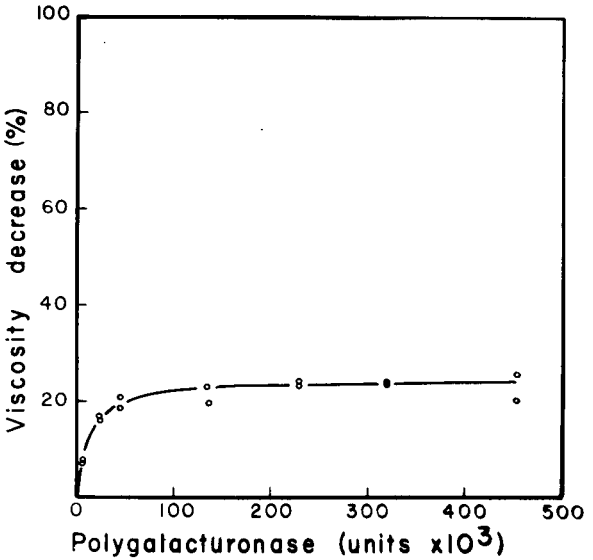

FIG. 4. RELATIONSHIP OF CUCUMBER POLYGALACTURONASE CONCENTRATION AND VISCOSITY DECREASE AS MEASURED IN 1.0% CITRUS PECTIN SUBSTRATE IN 0.043 M MALEIC ACID, pH 6.1, μ = 0.10.

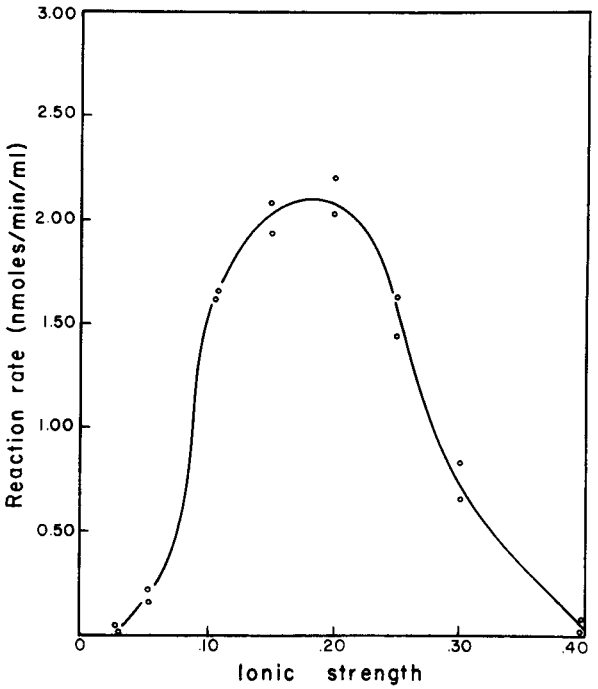

FIG. 5. EFFECT OF IONIC STRENGTH ON THE REACTION RATE OF CUCUMBER POLYGALACTRUONASE

Reactions were run in 0.12% polypectate dissolved in 0.05 M sodium acetate, 0.036 M maleic acid and 0.02% NaN_3, pH 5.6.

The relationship of pH to the activity of cucumber polygalacturonase with polypectate as a substrate is illustrated in Fig. 6. In a buffer of 0.05 M sodium acetate and 0.036 M maleic acid with NaCl added to give an ionic strength of 0.15, the enzyme had a pH optimum of 5.6, the same value as was found for avocado (Raymond and Phaff 1965), but higher than the value found for the enzyme from tomato (Patel and Pharr 1960; Takehana *et al.* 1977), cranberry (Arakji and Yang 1969), peaches (Pressey and Avants 1973a), and pears (Pressey and Avants 1976). Pressey and Avants (1975) found that the exo-polygalacturonase from cucumbers had a pH optimum of 5.5.

The enzyme was stable at room temperature between pH 4.0 and 6.2. Column chromatography was carried out within this pH range without any large loss in enzyme activity. When the enzyme was heated at 60°C in 0.10 M sodium acetate buffer, pH 5.2, with $\mu = 0.10$, less than 25%

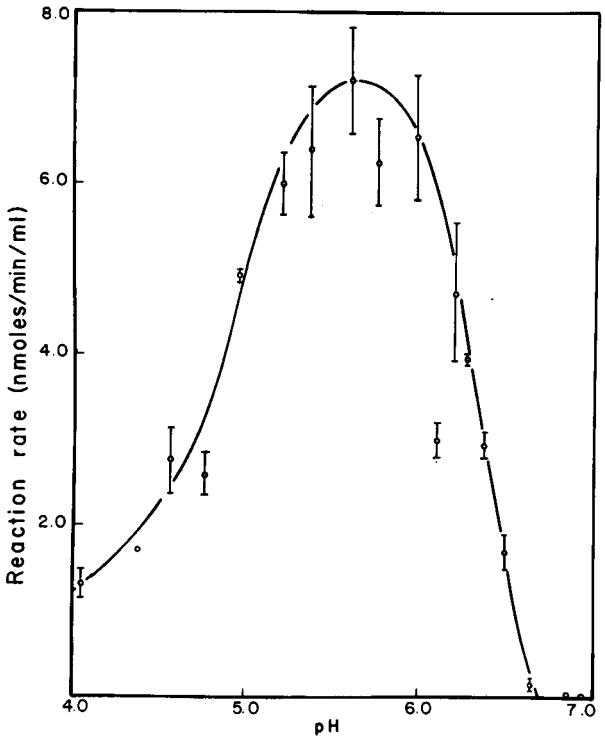

FIG. 6. pH-ACTIVITY PROFILE FOR CUCUMBER POLYGALACTURONASE

Reactions were run with 0.10% polypectate substrate in 0.05 M sodium acetate, 0.036 M maleic acid and 0.02% NaN_3 buffers with NaCl added to give $\mu = 0.15$.

loss in activity occurred in 30 min. At 70°C under the same incubation conditions, a first-order denaturation was observed (Fig. 7). The enzyme had a half-life of 1.2 min at 70°C. Thus, a heat treatment of 77°C for 3.5 min, which was used prior to fermentation of sliced cucumbers by Fleming *et al.* (1978), would greatly reduce polygalacturonase activity.

CONCLUSIONS

An endo-polygalacturonase was isolated from ripe cucumber fruit and purified to homogenity. The size and catalytic properties of the enzyme were similar to those of other endo-polygalacturonases that have been found in molds and higher plants.

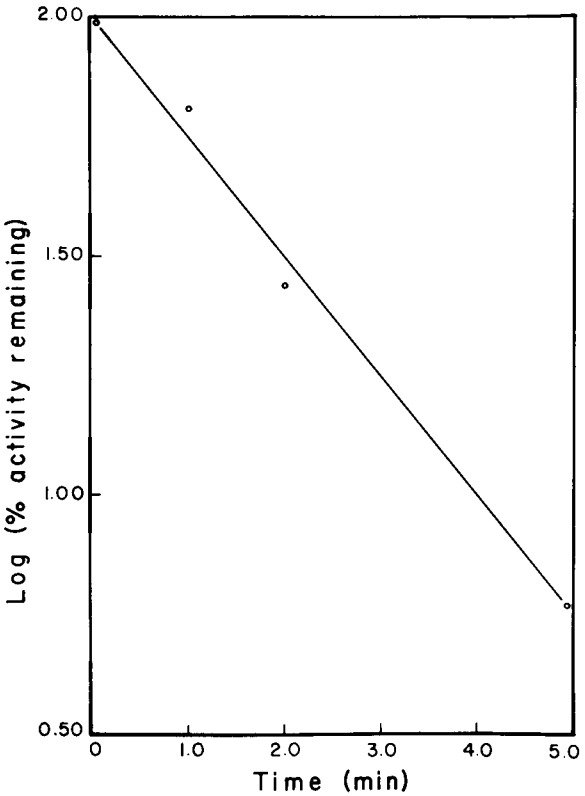

FIG. 7. HEAT INACTIVATION OF CUCUMBER POLYGALACTURONASE AT 70°C IN 0.10 M SODIUM ACETATE, pH 5.2, $\mu = 0.10$

The large increases in polygalacturonase activity observed during fruit ripening (Bell 1951; Saltveit and McFeeters unpublished) were accompanied by softening of the seed area of the fruit. Therefore, it is likely that this enzyme plays a significant role in tissue breakdown during the latter stages of fruit development.

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