Mode of Action of Pectic Enzymes. I. Purification and Certain Properties of Tomato Pectinesterase*

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ABSTRACT: Tomato pectinesterase (EC 3.1.1.11) was purified 19-fold by precipitation with $(NH_4)_2SO_4$ followed by adsorption on calcium phosphate gel and column chromatography on DEAE-cellulose and Sephadex G-75. The purified enzyme had a uniform specific activity throughout the final chromatographic peak and was free of polygalacturonase activity. Pectinesterase sedimented as a single, homogeneous band in the analytical ultracentrifuge with an $s_{20,w}$ value of 3.08 S. Disc gel electrophoresis, performed both a pH 9.5 and 4.3, indicated the presence of one component which remained at the origin and stained only faintly with Amido-Schwartz or aniline black, but more strongly with sudan black B or oil red O. When purified pectinesterase was subjected to alkaline hydrolysis followed by ether extraction, lipid components were identified in the ether phase. It is tentatively proposed that tomato pectinesterase is a lipoprotein. The enzyme had maximum activity over the broad range of pH values from 6 to 9. With pectin, N.F., as substrate, pectinesterase had a K_m of 4×10^{-3} M and was inhibited competitively by polygalacturonic acid with a K_i of 7×10^{-3} M. These kinetic values were based on the molar concentrations of galacturonide residues. Pectinesterase activity was highest in the presence of either 0.005 M $CaCl_2$ or 0.05 M NaCl.

The work of Solms and Deuel (1955) indicated that orange pectinesterase (EC 3.I.I.II) removes methoxyl groups adjacent to free carboxyl groups at a more rapid rate than those next to esterified residues on pectin molecules. Presumably, enzymatic hydrolysis proceeds linearly as methoxyl groups are removed successively along the pectin chain. The ultimate objective of the present study is to determine if tomato pectinesterase has a mode of action similar to that of the orange enzyme and, if so, to determine the direction of the linear attack in terms of the reducing end of the molecule.

As a prerequisite to this objective, it was necessary to purify highly tomato pectinesterase and to ensure that the accompanying polygalacturonase was removed. Cleavage of the pectin molecule by polygalacturonase would interfere in studies to identify the mode of action of the esterase. This paper presents a procedure for purification of pectinesterase and describes several properties of the purified enzyme.

Materials and Methods

Substrates. Pectin, N.F. (No. 3442), and polygalacturonic acid (No. 3491) were obtained from Sunkist Growers Inc., Corona, California. Polymethyl polygalacturonate methyl glycoside was prepared according to the method of Morell and Link (1933) and Morell *et al.* (1934) by refluxing polygalacturonic acid with 5% dry HCl in absolute methanol. The product had an ash content of 0.37% and a methoxyl content of 17.0% corresponding to 95.8% esterification. This material did not form true crystals and decomposed between 200 and 250°.

Measurement of Enzymatic Activities. Pectinesterase activity was measured at 30° by titration of the carboxyl groups liberated in 0.5% pectin, N.F., containing 0.05 M NaCl in a total volume of 20 ml. The pH was automatically maintained at 7.0 with a pH-Stat which controlled and recorded the amount of 0.02 M NaOH added to reaction mixtures. Nitrogen was bubbled into the reaction vessel to prevent uptake of CO_2 from the air. One unit of pectinesterase is the amount of enzyme that releases 1 μmole of carboxyl groups/min under the above conditions.

Polygalacturonase was assayed at 30° by measurement of the increase in aldehyde groups (Jansen and MacDonnell, 1945). One unit of polygalacturonase is the amount of enzyme that releases 1 μmole of reducing groups/min from 0.5% polygalacturonic acid in 0.1 M sodium acetate buffer (pH 4.5). Trace amounts of polygalacturonase in partially purified pectinesterase preparations were not detectable by the reducing group method and were estimated viscosimetrically as follows. Changes in the flow time of 10 ml of the above reaction mixture were measured in an Oswald–Cannon–Fenske capillary viscosimeter previously standardized against water. Crude pectinesterase containing 0.128 unit of polygalacturonase by the reducing group method took 9 min to decrease the relative flow time by 50%.

Analytical Methods. Protein concentration was determined colorimetrically by the method of Lowry *et al.* (1951). Protein in crude and purified enzyme

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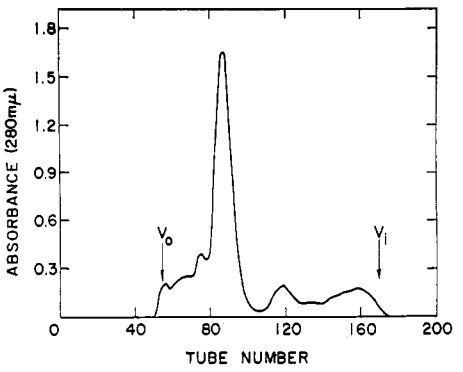

FIGURE 1: Ascending chromatography of pectinesterase on Sephadex G-75 Superfine. The sample was applied to the bottom of a 5 × 82 cm column and eluted with 0.02 M phosphate buffer (pH 8.0). Pressure on the column was maintained at 32 cm of water which gave a flow rate of 60 ml/hr. The volume of each fraction was 9.4 ml.

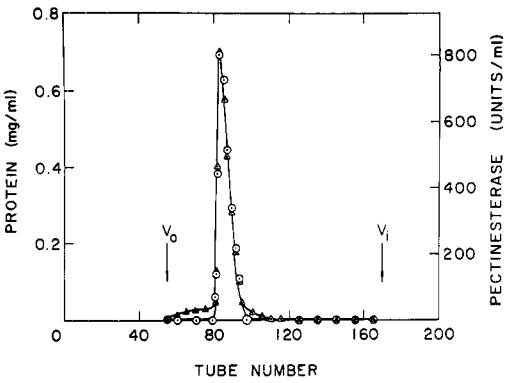

FIGURE 2: Final chromatography on Sephadex G-75 superfine. Conditions are as stated in Figure 1. (⊙—⊙) Pectinesterase; and (△—△) protein.

preparations was subjected to disc gel electrophoresis on 7.5% polyacrylamide gels at pH 9.5 and 4.3 by the methods of Davis (1964) and Ornstein (1964). The reagents used for preparation of gels were obtained from Canal Industrial Corp., Rockville, Md. Protein solutions (0.15 ml), containing 250 or 500 μg of protein, were mixed with 0.1 ml of 20% sucrose and applied directly to the top of each tube of gel. A constant current of 4 mA/tube was maintained during electrophoresis. Protein bands were stained with Amido-Schwartz or aniline black. Lipoprotein was either prestained with sudan black B (Narayan *et al.*, 1966) or stained after electrophoresis with oil red O. Purified preparations of pectinesterase were examined for homogeneity in a Beckman–Spinco Model E ultracentrifuge.

Oligogalacturonides were detected on paper chromatograms by the methods of Demain and Phaff (1954).

Adsorbants. Calcium phosphate gel was prepared by the method of Kunitz (1952). DEAE-cellulose (Mann Research Laboratories, 1475) was suspended in 1 M phosphate buffer (pH 8.0) and the slurry was washed several times with 0.02 M buffer (pH 8.0) before use.

Preparation of Crude Pectinesterase. Pectinesterase was prepared from fresh, fully ripened tomatoes (Heinz variety 135) or from tomatoes which had been frozen immediately after harvest. The methods used to extract the esterase were based on those developed by Patel and Phaff (1960a,b) for tomato polygalacturonase. Tomatoes were washed, sectioned, and squeezed through cheesecloth in a hand press and the serum was discarded. In a typical preparation 115 g of NaCl was combined with 2.3 kg of tomato pulp and 1 l. of 5% NaCl was stirred into the mixture. Thimerosal (Nutritional Biochemicals Corp.), at a concentration of 100 ppm, was added as a preservative. After standing for 30 hr at 5° the liquid was pressed from the pulp and filtered through glass wool and then Hyflo Supercell. Pectinesterase in this crude extract is relatively stable and will remain free of microbial growth for many months when stored at 5°. All of the steps developed for purification of pectinesterase were conducted at this temperature.

Results

Purification of Pectinesterase. Since polygalacturonase would interfere in studies of the properties of pectinesterase, purification of the crude extract was directed toward removal of this contaminating activity. Phosphate buffer (100 ml of 1 M) (pH 8.0) was mixed with 900 ml of crude extract and solid $(NH_4)_2SO_4$ was added to bring the solution to 30% saturation. After stirring for 15 min the precipitate was removed by centrifugation and discarded. Ammonium sulfate was added to bring the supernatant liquid to 70% saturation. The precipitate was collected by centrifugation and dissolved in 250 ml of 0.1 M phosphate buffer at pH 8.0. This solution was treated again with 30 and 70% saturated $(NH_4)_2SO_4$ and the final precipitate was dissolved in 50 ml of phosphate buffer. After dialysis for 4 hr against 0.02 M phosphate buffer (pH 8.0), 5% (v/v) calcium phosphate gel was added and the mixture was stirred for 5 min and then centrifuged. Most of the polygalacturonase was adsorbed on phosphate gel leaving pectinesterase in the supernatant solution.

It was found that the esterase would not adsorb on either DEAE- or CM-celluloses equilibrated in 0.02 M buffers at pH values of 6, 7, or 8. Passage of enzyme preparations through columns of DEAE-cellulose, however, removed significant amounts of extraneous protein. The following procedure was adopted for routine use. After treatment with calcium phosphate gel, the supernatant liquid was placed on the top of a 2.5 × 36 cm column of DEAE-cellulose and the enzyme was eluted with 0.02 M phosphate buffer (pH 8.0).

Fractions containing pectinesterase were combined and chromatographed on Sephadex G-75, Superfine (Figure 1). Preliminary experiments showed that pectinesterase activity was eluted in a single peak corresponding to the major protein peak. Fractions 81–94 were combined and the enzyme was concentrated by precipitation with 90% saturated $(NH_4)_2SO_4$.

TABLE I: Purification of Pectinesterase.

Fraction	Vol (ml)	Total Pectinesterase (units)	Total Polygalacturonase (units)	Sp Act. (units/mg)	Yield (%)	Purifcn (X)
Crude extract	900	212,000	115	59.1	100	1
$(NH_4)_2SO_4$ fractionation	28.3	181,000	46	320	85	5
Phosphate gel supernatant	39.0	156,000	Nil[a]	320	74	5
DEAE-cellulose eluate	65.4	113,000		430	54	7
Sephadex G-75 superfine eluate (initial)	137	91,200		834	43	14
Sephadex G-75 superfine eluate (final)	145	75,800	Nil[a]	1,150	36	19

[a] No change in reducing value after 1 hr. The reaction mixture contained 10% (v/v) enzyme in 0.5% polygalacturonate.

The precipitate was dissolved in 25 ml of 0.02 M phosphate buffer (pH 8.0) and rechromatographed on Sephadex G-75, Superfine. Fractions containing enzyme activity were collected, precipitated, and chromatographed a third time as above. As shown in Figure 2, the esterase was eluted as a single protein peak. A summary of the complete purification procedure is given in Table I.

Analysis for polygalacturonase by the reducing group method indicated that this enzyme was completely adsorbed by calcium phosphate gel. However, faint spots corresponding to lower oligogalacturonides were detected in polygalacturonase reaction mixtures which had been incubated overnight. This suggested that a trace of polygalacturonase still remained with the pectinesterase. Polygalacturonase could not be detected in the final purified preparation of pectinesterase by either of the methods above. Analysis for polygalacturonase by the viscosimetric method, however, indicated that a trace of activity still remained (Figure 3). The maximum amount of polygalacturonase remaining was estimated from these data as 6×10^{-5} unit/ml. This would correspond to less than 0.01% of the amount of polygalacturonase present in crude extracts.

Analysis of Purified Pectinesterase. Specific activities of pectinesterase in each of the 16 fractions representing the purified enzyme were individually calculated from the data in Figure 2. The values found varied randomly in the range between 1075 and 1250 units of pectinesterase per mg of protein.

Purified pectinesterase was analyzed by ultracentrifugation to determine its homogeneity and sedimentation velocity. Concentrated enzyme solutions were prepared by appropriate dilution of precipitates obtained with 90% saturated $(NH_4)_2SO_4$ which was removed prior to ultracentrifugation by dialysis against 0.02 M phosphate buffer (pH 8.0). At 59,760 rpm the enzyme moved as a single symmetrical peak with an $s_{20,w}$ value of 3.08 S.

Samples of crude pectinesterase containing 250 μg of protein separated into nine components by disc gel electrophoresis at pH 9.5. All of the bands were only faintly visible when stained with either Amido-Schwartz or aniline black. Disc gel electrophoresis of 500-μg samples of purified pectinesterase were not successful. Purified pectinesterase formed only a single faint band at the top of the gels when electrophoresis was run at either pH 9.5 or 4.3. This problem was partially resolved by the discovery that purified pectinesterase gave positive tests for lipid when stained on filter paper with sudan black B or 2′,7′-dichlorofluorescein. This indicated the possibility that pectinesterase is a lipoprotein.

Samples of purified pectinesterase, prestained with sudan black B, were subjected to disc gel electrophoresis for analysis of lipoproteins. A single dark band which barely penetrated the top of the gel was observed after electrophoresis at both pH 9.5 and 4.3. A band in the same location was also detected by staining with

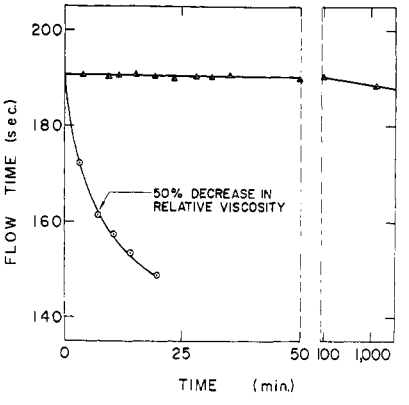

FIGURE 3: Viscosimetric analysis of polygalacturonase in crude (○) and purified (△) preparations of pectinesterase. Reaction mixtures contained 1 ml of enzyme, 0.5% polygalacturonic acid, and 0.1 M sodium acetate buffer at pH 4.5 in a total volume of 10 ml. Flow times were measured periodically in an Oswald-Cannon–Fenske 75 capillary viscosimeter maintained at 30°. The flow time for 10 ml of distilled water was 133.8 sec.

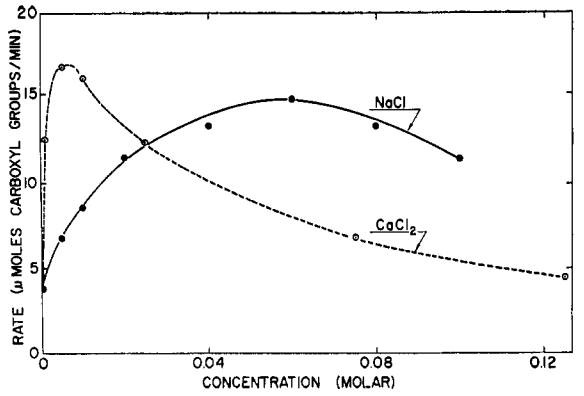

FIGURE 4: Effect of NaCl and $CaCl_2$ on activity of purified pectinesterase. Reaction mixtures contained 15 units of pectinesterase, 0.5% polymethyl polygalacturonate methyl glycoside, and varying amounts of the two salts. All reactions were at pH 7.0 and 30°.

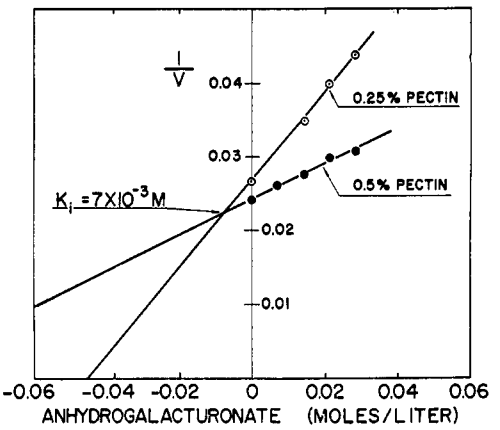

FIGURE 5: Inhibition of pectinesterase by polygalacturonate. Reaction mixtures contained either 0.5 or 0.25% pectin, N.F., 0.05 M NaCl, varying amounts of polygalacturonate, and 40 units of pectinesterase in a total volume of 20 ml. The pH was 7.0 and the temperature was 30°.

oil red O after electrophoresis. Further attempts to move the enzyme deeper into gels or to separate it into other components were not successful under a variety of experimental conditions.

Another indication of the presence of lipid in purified pectinesterase was obtained by extraction with 35% (v/v) ethanol in ether by the method of Harwalkar and Brunner (1965). Lipid was identified in both the aqueous and ether phases. A sample of pectinesterase was digested overnight at 110° in 1 N KOH and the digest was extracted with ether. Preliminary examination of this extract by gas chromatography indicated the presence of several volatile components.

Effects of Cations on Activity of Pectinesterase. Pithawala *et al.* (1948) reported that Ca^{2+} ions were necessary for activity of tomato pectinesterase. We initially observed that $CaCl_2$ did not stimulate activity of purified pectinesterase. The reaction mixture used in these experiments also contained NaCl which was routinely included to prevent precipitation of the enzyme as reported by McColloch *et al.* (1946). Later it was found that no visible precipitate formed even after extensive dialysis of purified enzyme against deionized water. This dialyzed preparation had lost, however, over 95% of its activity. Up to 50% of the activity could be restored by addition of $CaCl_2$ to reaction mixtures. These observations led to further investigations on the effect of $CaCl_2$ and NaCl on enzyme activity. A highly esterified pectin (polymethyl polygalacturonate methyl glycoside) was employed as substrate in these experiments because it had an ash content of 0.37% as compared with 1.76% for pectin, N.F., and presumably contained less of the two salts. As shown in Figure 4, maximum stimulation of pectinesterase occurred at 0.005 M $CaCl_2$. Higher concentrations were inhibitory. In contrast, 0.05 M NaCl was required to produce a comparable amount of stimulation. This amount of NaCl was subsequently included in all routine assays for pectinesterase.

Product Inhibition of Pectinesterase. Pectinesterase was found to be inhibited by addition of polygalacturonate to reaction mixtures. The rate of deesterification in the presence of various concentrations of polygalacturonate was determined on both 0.25 and 0.5% pectin, N.F. The results, plotted by the method of Dixon (1953), are shown in Figure 5. The inhibition is competitive with a K_i value of 7×10^{-3} M anhydrogalacturonate residues.

Effect of Substrate Concentration on Activity of Pectinesterase. The initial rates for pectinesterase activity were measured at various concentrations of pectin, N.F. The results are shown as a double-reciprocal plot in Figure 6. The value for K_m is 4×10^{-3} M anhydrogalacturonate residues. In the calculation of this value an appropriate correction was made for the weight of methoxyl groups assuming that the pectin, N.F., was 70% esterified.

Effect of pH on Activity of Pectinesterase. The activity of purified pectinesterase was assayed at various pH values (Figure 7). Measurement of pectinesterase activity above pH 7.0 is subject to error because pectin undergoes alkaline deesterification in addition to enzymatic deesterification. Rates for alkaline deesterification were determined at each pH value in the absence of pectinesterase and corrections were applied to the rates obtained enzymatically. It was observed, however, that measurements of alkaline deesterification are difficult to reproduce, particularly at higher pH. For example, at pH 9.0 the rates found in identical experiments for alkaline deesterification ranged from 3.25 to 9.5 μmoles per min. The enzymatic activity peak at 8.5, illustrated in Figure 7, therefore, may not be significant.

Discussion

The purification procedure reported here has been repeated on numerous occasions and is highly reproducible. Although there was no apparent increase in specific activity by treatment with calcium phosphate gel, this step was included since it is essential in removing contaminating polygalacturonase.

The largest increase in specific activity was produced

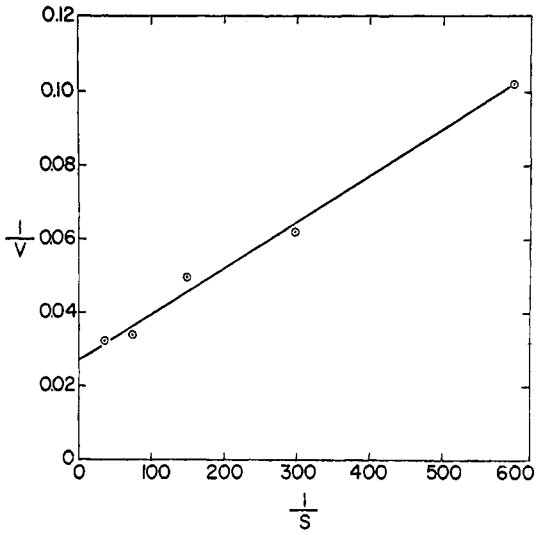

FIGURE 6: Effect of substrate concentration on pectinesterase. Reaction mixtures contained varying amounts of pectin, N.F., 0.05 M NaCl, and 30 units of pectinesterase in a total volume of 20 ml. $K_m = 4 \times 10^{-3}$ M.

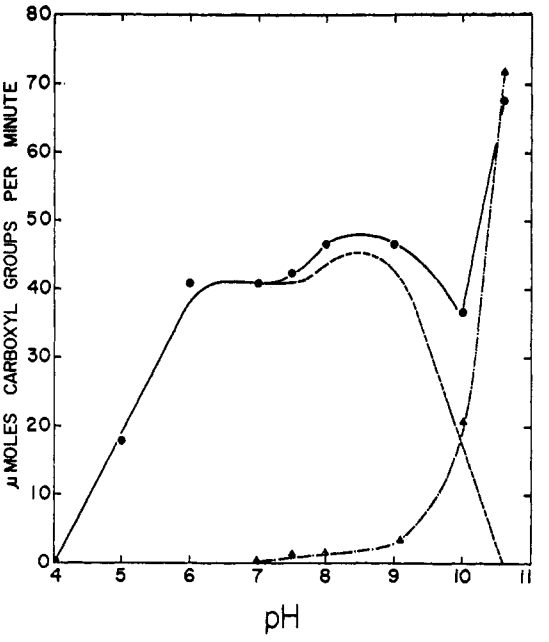

FIGURE 7: Effect of pH on enzymatic and nonenzymatic deesterification of pectinesterase. Enzymatic reaction mixtures (●) contained 0.5% pectin, N.F., 0.05 M NaCl, and 40 units of pectinesterase. Nonenzymatic reaction mixtures (△) were the same except no pectinesterase was added. Constant pH values were maintained in a pH-Stat. The broken line represents the difference between enzymatic and nonenzymatic deesterification at pH values above 7.0.

by Sephadex gel chromatography. Best results were obtained when pressures on Sephadex columns were maintained between 30 and 40 cm of water. In this pressure range the flow rates were about 60–80 ml/hr. Pressures much in excess of the higher value caused packing of the gel which resulted in decreased flow rates and eventually complete stoppage.

Occasionally the gel filtration steps were combined in a recycling chromatography procedure. Fractions containing extraneous protein were discarded and that portion of the effluent which contained pectinesterase was fed directly back into the bottom of the column. Although this method produced a comparable amount of purification it did not offer any particular advantage and required constant supervision.

All of the pectinesterase fractions which represent the chromatographic peak obtained on Sephadex G-75 in the final purification step have approximately the same specific activity. Enzyme in the pooled fractions sedimented as a single homogeneous band in the analytical ultracentrifuge. These facts suggest that the purified enzyme preparation is homogeneous with respect to molecular size and sedimentation properties. Tomato pectinesterase is not excluded from Sephadex G-75 and has a $s_{20,w}$ value of 3.08 S. These observations indicate that the molecular weight of this enzyme is considerably smaller than the pectinesterase produced by *Clostridium multifermentans* (Macmillan and Vaughn, 1964). This bacterial enzyme was excluded from Sephadex G-200 and was retained and separated on Sepharose-4B (L. Miller and J. D. Macmillan, unpublished data).

Purification of the esterase resulted in a 19-fold increase in specific activity and in complete removal of all polygalacturonase activity as measured by the reducing-group method. The viscosimetric data in Figure 3, however, indicates that a trace of polygalacturonase still remains in purified pectinesterase. If it is assumed that the slight change in viscosity was actually caused by polygalacturonase, it is evident from the following discussion that the amount of activity is small and has little practical significance.

Hydrolysis of glycosidic bonds by crude extracts from tomatoes is caused by two different enzymes. There is some speculation that one of these enzymes is an exopolygalacturonase (an end-group splitting enzyme). It is generally agreed that a randomly splitting enzyme, endopolygalacturonase, is by far the most predominant activity (Patel and Phaff, 1960b; Hobson, 1964). On the basis of analogy with other randomly splitting pectic enzymes, it can be assumed that the activity from tomatoes will cause relatively large decreases in viscosity after hydrolyzing only a few glycosidic bonds. For example, endopolygalacturonate lyase (Nagel and Vaughn, 1961) and endopectin lyase (Edstrom and Phaff, 1964) caused a 50% reduction in viscosity of their respective substrates when only 2–3% of the total bonds were broken. If the data in Figure 3 is extrapolated as a straight line to the 50% level, it can be seen that the purified enzyme would take at least 11 days to cause 2–3% hydrolysis. Furthermore, the ratio of pectinesterase to polygalacturonase on an activity basis is approximately 9×10^6. Therefore, it is ulikely that polygalacturonase, if present, would interfere in studies on the mode of action of pectinesterase.

It was found that the values for K_i and K_m for tomato pectinesterase are of the same order of magnitude. This indicates that at pH 7.0 this enzyme has a similar affinity for both pectin, N.F., and polygalacturonate.

The inability to initially detect dark bands after disc gel electrophoresis of the purified preparation suggested that the enzyme had not migrated into the gels. No pectinesterase activity could be detected, however, in the buffer solutions above the gels. The observation that a single band could be visualized with lipid stains may partially resolve this problem. There is no experimental data which can account for the failure of the enzyme to migrate deeper into the gels. This apparent lack of polarity at various pH values is supported by the observation that pectinesterase will not absorb on either DEAE- or CM-celluloses.

Acknowledgments

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