Purification and Pattern of Action of Pectinesterase from *Fusarium oxysporum* f. sp. *vasinfectum**

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ABSTRACT: Pectinesterase (EC 3.1.1.11) from *Fusarium oxysporum* f. sp. *vasinfectum* (fungal pectinesterase) was purified fivefold from the culture broth by a procedure employing column chromatography on DEAE-Sephadex, Sephadex G-75, CM-Sephadex, and CM-cellulose. The purified enzyme was free of contaminating polygalacturonate lyase activity, but a trace of polygalacturonase remained. Only two components were found by disc gel electrophoresis at pH 4.3. No protein bands were detected on gels run at pH 9.5.

The apparent molecular weight of fungal pectinesterase as determined by ascending chromatography on Sephadex G-75, superfine, was 35,000 compared to 27,500 for tomato pectinesterase and 400,000 (by Sephadex G-200) for the apparent esterase lyase complex from *Clostridium multifermentans*. Clostridial exopolygalacturonate lyase was assayed simultaneously in the same reaction mixture with fungal pectinesterase on a highly esterified pectin substrate. Exopolygalacturonate lyase degrades deesterified pectin (polygalacturonic acid) with a terminal action pattern beginning from the reducing end of the chain. When fungal pectinesterase deesterified the pectin chain, the lyase was able initially to degrade the substrate at a rate 57% of the rate found for carboxyl group production by the esterase. By comparison, randomly (nonenzymatically) deesterified substrate was not initially degraded by the lyase. These experiments indicated that over half of the fungal pectinesterase activity occurred at the reducing ends of pectin chains and that the action pattern was similar to that previously found for tomato pectinesterase.

Fusarium oxysporum f. sp. *vasinfectum* (ATCC 7808) produces pectinesterase (EC 3.1.1.11), polygalacturonate lyase (EC 4.2.99.3), and polygalacturonase (EC 3.2.1.15) when grown on media containing pectin (L. Miller and J. D. Macmillan, unpublished data). Pectinesterase catalyzes the hydrolysis of ester linkages on pectin forming free carboxyl groups and methanol. Polygalacturonase degrades the deesterified product formed by the esterase, polygalacturonic acid, by hydrolysis of α1→4-glycosidic bonds. Polygalacturonate lyase cleaves these linkages by a trans elimination reaction rather than by hydrolysis. Previously, we developed methods for determining the action patterns of pectinesterases from tomato (Lee and Macmillan, 1970) and *Clostridium multifermentans* (Lee *et al.*, 1970) which employed partially purified exopolygalacturonate lyase (Macmillan and Vaughn, 1964; Macmillan and Phaff, 1966; Miller and Macmillan, 1970) as a tool to measure the proportion of the total pectinesterase activity occurring at the reducing ends of highly esterified pectin molecules. Exopolygalacturonate lyase, also from *C. multifermentans*, degrades polygalacturonate by sequentially splitting off *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1→4)-D-galacturonic acid (unsaturated digalacturonic acid units) from the reducing ends of the chains (Macmillan *et al.*, 1964). This enzyme can act on highly esterified pectins only after pectinesterase has formed free carboxyl groups at the reducing end.

In this present work we present a method for purifying pectinesterase from *F. oxysporum* f. sp. *vasinfectum* (fungal pectinesterase) and freeing it from the other contaminating pectic enzymes. This enabled us to study the action pattern of the fungal esterase and compare it to those from tomato and *C. multifermentans*.

Materials and Methods

Substrates and Enzyme Assays. Polygalacturonic acid (No. 3491) and pectin N.F. (No. 3442) were obtained from Sunkist Growers, Inc., Corona, Calif. Polymethyl polygalacturonate methyl glycoside (highly esterified pectin) was prepared by refluxing polygalacturonic acid in a solution of anhydrous HCl in methanol (Morell and Link, 1933; Morell *et al.*, 1934). The product was 95.8% esterified (Lee and Macmillan, 1968) and the average number of galacturonides in the chain was 33 (Jansen *et al.*, 1948).

Pectinesterase was assayed by titration of the carboxyl groups produced in 20 ml of 0.5% pectin N.F. containing 0.05 M sodium chloride. Carboxyl groups were automatically titrated in a thermostatically controlled vessel of a Radiometer pH-Stat set at 30° and pH 7 as previously described (Lee and Macmillan, 1968). One unit of activity is that amount of enzyme which releases 1 μmole of carboxyl groups/min under the conditions above. Fungal polygalacturonate lyase was measured spectrophotometrically at 235 nm in a reaction mixture containing 0.5% polygalacturonic acid, 0.002 M $CaCl_2$, and 0.03 M Tris-HCl buffer (pH 9.0). The unit of enzyme activity was based on that employed previously for exopolygalacturonate lyase from *C. multifermentans* (Macmillan and Phaff, 1966). This clostridial enzyme produces unsaturated digalacturonate which absorbs at 235 nm with a molar extinction coefficient of 4600. Although the unsaturated oligogalacturonides produced by the fungal lyase were not identified, for convenience it was assumed that they would have a similar extinction coefficient and, therefore, one unit of activity is that amount of enzyme which produces unsaturated

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bonds equivalent to 1 μmole of unsaturated digalacturonic acid/min formed under the conditions described above.

Polygalacturonase activity was estimated by a modified cup–plate method described previously by Nagel and Vaughn (1961), except that 0.05 M sodium acetate (pH 5.0) was used as the buffer and the plates were incubated for 18 hr at 28°. A clear zone diameter of 12 mm was arbitrarily selected to represent 100 units of polygalacturonase/ml. Trace amounts of polygalacturonase in highly purified preparations of pectinesterase were not detectable by the cup–plate method and were estimated viscosimetrically as follows. Changes in the flow time of a 10-ml reaction mixture containing 0.5 ml of enzyme solution, 0.5% polygalacturonic acid, and 0.04 M histidine–HCl buffer (pH 7.0) were measured at 30° in an Oswald–Cannon–Fenske capillary viscosimeter previously standardized against water. The activity of fungal polygalacturonase was roughly estimated on the assumption that it was an endopolygalacturonase. A 50% reduction in relative viscosity occurs when endo-splitting enzymes catalyze the hydrolysis of approximately 2–3% of the glycosidic bonds present in 0.5% substrate (Nagel and Vaughn, 1961; Edstrom and Phaff, 1964).

Action Pattern Studies. The combined action of purified fungal pectinesterase and clostridial exopolygalacturonate lyase on pectins were monitored simultaneously by the method previously described (Lee and Macmillan, 1970; Lee *et al.*, 1970). The reaction mixture contained 0.125% polymethyl polygalacturonate methyl glycoside or 0.125% pectin N.F., 0.002 M $CaCl_2$, and appropriate amounts of pectinesterase and exopolygalacturonate lyase in a final volume of 10 ml. The combined reactions of the two enzymes took place under a stream of nitrogen in a thermostatically controlled vessel of a Radiometer pH-Stat set at 30°. Pectinesterase activity was calculated from the amount of 0.02 N NaOH required to maintain a constant pH of 7.0. The solution in the reaction vessel was circulated continuously through a 2-mm flow cell in a Gilford Model 2000 recording spectrophotometer. The concentration of unsaturated digalacturonic acid, the product formed by the lyase, was calculated from the increase in absorbance at 235 nm.

Gels and Adsorbants. Sephadex G-75, DEAE-Sephadex A-25, and CM-Sephadex C-50 were obtained from Pharmacia, Inc., Piscataway, N. J. These materials were prepared for use in chromatographic columns by the methods recommended by the manufacturer. Carboxymethylcellulose (Whatman CM-32) was washed with base and acid and then equilibrated in 1 M sodium acetate buffer (pH 5.0). Immediately prior to use the exchanger was equilibrated in 0.05 M sodium acetate buffer (pH 5.0).

Determination of Molecular Weights by Gel Filtration. The molecular weights of fungal pectinesterase, fungal polygalacturonate lyase, and tomato pectinesterase were determined by ascending chromatography on a 2.5 × 82.4 cm column of Sephadex G-75, superfine. The gel was washed and equilibrated with 0.05 M sodium acetate buffer (pH 5.0). The void volume as determined with blue dextran on three separate runs was 154, 153, and 155 ml. Samples (10 mg) of the standards (ovalbumin, 45,000; chymotrypsinogen A, 24,000; and ribonuclease A, 13,700) were used to calibrate the column. Peaks for the standards were located by absorption at 280 nm. Elution volumes for each of the enzymes of unknown molecular weight were determined in separate runs; peaks were located by absorbance at 280 nm and verified with enzyme assays.

The molecular weight of the apparent complex of pectinesterase and exopolygalacturonate lyase from *C. multifermentans* (Miller and Macmillan, 1970) was determined in a similar manner on a 2.5 × 78 cm column of Sephadex G-200 equilibrated with 0.02 M potassium phosphate buffer (pH 6.0). The void volumes found for this column were 138, 138, and 139 ml. Aldolase (158,000) and thyroglobulin (669,000) were used in addition to the other standards for calibrating the column.

Analytical Methods. Protein concentration was determined colorimetrically by the method of Lowry *et al.* (1951). Proteins in crude and purified enzyme preparations were analyzed by disc gel electrophoresis on 7.5% polyacrylamide gels at pH 4.3 and 9.5 by the methods of Davis (1964) and Ornstein (1964). The reagents used for preparation of the gels were obtained from Canal Industrial Corp. (Rockville, Md.). The separating gel (0.8 ml) was polymerized in tubes (5-mm i.d.) and then layered with 0.2 ml of stacking gel. The sample was mixed with stacking gel and applied as a third layer (0.2 ml). A constant current of 4 mA/tube was maintained during electrophoresis. Protein bands were stained with coomassie blue.

Oligogalacturonides produced by fungal polygalacturonase were detected by descending chromatography on Whatman No. 1 filter paper. Chromatograms were developed for 20 hr at 25° in ethyl acetate–acetic acid–water (9:2:2), air-dried, and sprayed with 1% *p*-anisidine hydrochloride in methanol–1-butanol (1:9, v/v). Spots were visualized after heating for 5 min at 105°. For comparative purposes the products formed by yeast endopolygalacturonase prepared by the method of Phaff (1966) were also chromatographed.

Preparation of Enzymes. Exopolygalacturonate lyase was prepared from culture broth of *C. multifermentans* and freed of pectinesterase activity by heating for 30 min at 38°, pH 7.0, in 0.001 M $CaCl_2$ (Miller and Macmillan, 1970; Lee *et al.*, 1970). After inactivation of the esterase the preparation was passed through a 5 × 86 cm column of Sephadex G-200. The preparation had 6 units of exopolygalacturonate lyase/ml and no pectinesterase activity. The heat treatment was omitted for preparing the sample used to determine the molecular weight of the apparent complex of clostridial exopolygalacturonate lyase and clostridial pectinesterase.

Purified tomato pectinesterase was prepared by the method of Lee and Macmillan (1968).

Crude fungal pectinesterase was prepared as follows. Stock cultures of *F. oxysporum* f. sp. *vasinfectum* were maintained in tubes containing 5 ml of potato dextrose broth. After growth for 7 days at 25° stock cultures were stored at 5° and used as required to prepare inoculum for pectinesterase production. Stock culture (5 ml) was used to inoculate a 500-ml erlenmeyer flask containing 50 ml of a medium composed of 3% pectin N.F. in yeast nitrogen base (Difco). After 7 days at 25° the broth and mycelium in the flask were homogenized for 1 min in a blender and the entire homogenate was used to inoculate a 2.5-l. fernbach flask containing 500 ml of the pectin medium. A heavy mycelial mat formed within a few days and after 4 weeks the broth was harvested leaving the mat intact within the flask. The broth contained 8.6 units of pectinesterase and 0.17 unit of polygalacturonate lyase per ml. Fresh sterile medium (500) ml was then introduced under the mycelial mat and the culture was incubated for an additional 6 days before the second batch of broth was harvested and assayed. The amount of pectinesterase was 6.5 units/ml, but only about 0.1 as much lyase was formed as in the original broth (0.015 unit/ml). Since one of the objects of the purification procedure was to obtain pectinesterase free of lyase activity,

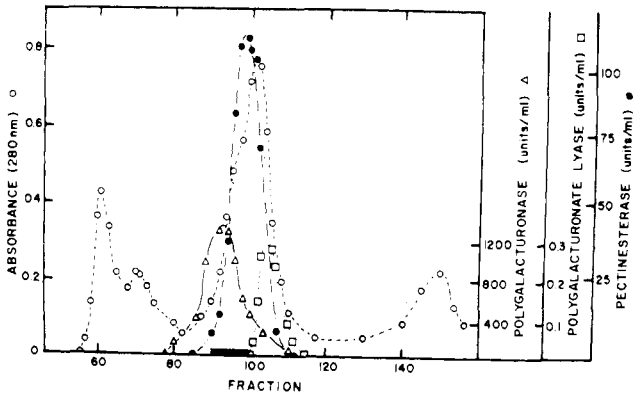

FIGURE 1: Elution profile from ascending chromatography on Sephadex G-75 superfine. The sample (20 ml) contained a total of 10,800 units of pectinesterase. The column was eluted with 0.05 M sodium acetate buffer, pH 5.0, and 10-ml fractions were collected with a flow rate of 30 ml/hr. The void volume was 610 ml: ○, protein absorbance at 280 nm; ●, pectinesterase; □, polygalacturonate lyase; △, polygalacturonase. Bar represents pooled fractions.

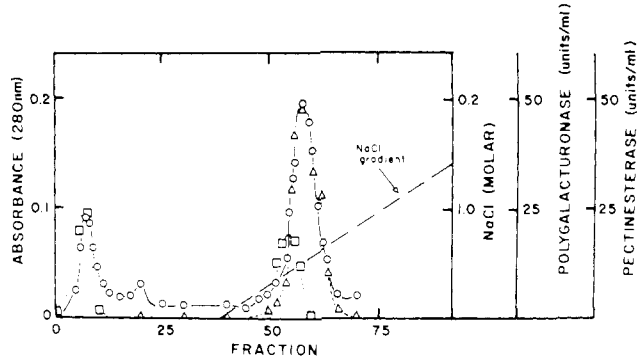

FIGURE 3: Elution profile from ion-exchange chromatography on CM-cellulose. Concentrated eluate (7 ml) from CM-Sephadex containing a total of 308 units of pectinesterase was applied to the column. The column was eluted first with 160 ml of 0.05 M sodium acetate buffer, pH 5.0, and then with a linear gradient of sodium chloride (0–0.2 M) in the same buffer. Fractions (4 ml) were collected with a flow rate of 75 ml/hr: ○, protein absorbance at 280 nm; △, pectinesterase; □, polygalacturonase.

only the second batch of medium was employed as the source of crude pectinesterase. Fermentation broth was clarified by adding 2% Celite (Johns Manville) and then filtering through Whatman No. 1 paper. The broth was then dialyzed against 10 volumes of distilled water for 16 hr at 5° and lyophilized to dryness. The powder was dissolved in a volume of distilled water equivalent to 5% of the original volume. The enzyme solution was centrifuged to remove insoluble particulate matter and adjusted to pH 6.0 for maximum stability. This crude enzyme solution was used in the following purification studies.

Results

Crude fungal pectinesterase also contained polygalacturonate lyase and polygalacturonase which would interfere in studies on the action pattern of the esterase. Therefore, purification was directed primarily to removal of these contaminating enzymes. In a preliminary experiment it was found that all three enzymes adsorbed readily on the cationic exchanger CM-Sephadex (C-50) at pH 5 and 8, but not on the anionic exchangers, DEAE-Sephadex A-50 and QAE-Sephadex A-50, at either pH value. Although pectinesterase did not adsorb, the specific activity increased about threefold by passage through a column of DEAE-Sephadex equilibrated in 0.1 M sodium acetate buffer (pH 5.0). Passage through the column also resulted in a considerable decrease in viscosity of enzyme preparations. The following procedure was adopted as a first step in purification of the esterase. The concentrated crude enzyme solution (300 ml) was passed through a 5 × 60 cm column of DEAE-Sephadex A-25 previously equilibrated in 0.1 M sodium acetate buffer (pH 5.0). The column was eluted with the same buffer and 50-ml fractions were collected. Fractions 8–15 were relatively colorless and contained about 60% of the pectinesterase activity. Since later fractions contained most of the pigment and viscous material characteristic of the crude concentrate, fractions 8–15 were combined and used in the subsequent purification steps. The specific activity increased from 35 to 110 by this treatment.

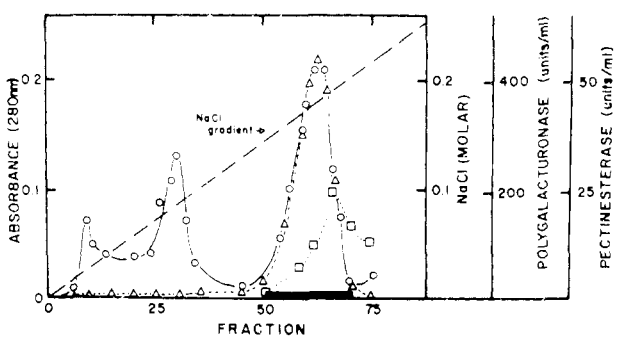

FIGURE 2: Elution profile from ion-exchange chromatography on CM-Sephadex. A concentrated eluate (5 ml) from Sephadex G-75 containing a total of 3250 units of pectinesterase was added to the column and eluted with a linear gradient of sodium chloride (0–0.2 M) in 0.05 M sodium acetate buffer, pH 5.0. Fractions (5 ml) were collected with a flow rate of 60 ml/hr: ○, protein absorbance at 280 nm; △, pectinesterase; □, polygalacturonase. Bar represents pooled fractions.

The combined fractions (400 ml) were lyophilized and reconstituted to 40 ml and a 20-ml sample was applied to the bottom of a 5 × 82 cm column of Sephadex G-75, superfine, previously equilibrated with 0.05 M sodium acetate buffer (pH 5.0). The elution profile (Figure 1) shows that pectinesterase activity was present in a single symmetrical peak which corresponded approximately to the largest protein peak as measured by absorbance at 280 nm. The peaks for polygalacturonase and polygalacturonate lyase overlapped with the one found for pectinesterase. The remaining 20 ml of the concentrated preparation from DEAE-Sephadex was passed through the same column of G-75 and fractions 90–100 of both columns were combined, dialyzed overnight against distilled water, lyophilized to dryness, and reconstituted in 20 ml of distilled water. This preparation was free of polygalacturonate lyase activity. Insoluble material was removed by centrifugation and 5 ml of the supernatant was placed on the top of a 2.5 × 25 cm column of CM-Sephadex C-50. The column was eluted with a linear gradient of sodium chloride in 0.05 M sodium acetate buffer (pH 5.0). The elution profile (Figure 2) shows pectinesterase activity coinciding with the major protein peak; however, all of the fractions were contaminated with polygalacturonase. The remainder of the preparation from Sephadex G-75 was chromatographed on the same column of CM-Sephadex C-50 and fractions 50–70 from all runs were combined, lyophilized to dryness, and reconstituted in 40 ml

TABLE I: Purification of Pectinesterase from *Fusarium oxysporum*.

Fraction	Vol (ml)	Total Protein (mg)	Pectinesterase			Polygalacturonate Lyase[a]	Polygalacturonase[b]
			Total (units)	Sp Act. (Units/mg of Protein)	Yield (%)		
Dialyzed culture broth	6000	1500	39,000	26	100	+	+
Concentrate[c]	300	780	27,300	35	70	+	+
DEAE-Sephadex eluate	400	144	15,800	110	41	+	+
Concentrate[c]	40	144	21,600	150	55	+	+
Sephadex G-75 eluate	220	110	13,300	121	34	−	+
Concentrate[c]	20	74	13,000	176	33	−	+
CM-Sephadex eluate	420	63	12,300	196	32	−	+
Concentrate[c]	40	44	9,000	205	23	−	+
CM-cellulose eluate	155	14	2,850	203	7	−	−

[a] + indicates that the lyase reaction mixture containing 10% v/v enzyme increased in absorbance at 235 nm; − indicates no detectable increase in absorbance occurred during a 30-min reaction period. [b] + indicates a positive test for polygalacturonase activity with a 0.04-ml sample by the cup-plate method; − indicates no detectable activity. [c] Concentrates (usually tenfold) were prepared by lyophilizing fractions to dryness and dissolving the residues in distilled water.

of distilled water. The specific activity of pectinesterase increased from 176 to 205 units per mg by this procedure. This preparation (7 ml) was applied to the top of a 1.7 × 9 cm column of CM-cellulose and the column was eluted with a linear gradient of NaCl in 0.05 M sodium acetate buffer (pH 5.0). Once again the pectinesterase activity coincided with the major protein peak (Figure 3). Polygalacturonase activity was eluted in two peaks. No polygalacturonase activity was detected in tubes 60–66 by the cup-plate method, and these fractions when combined represented the final purified esterase preparation. A summary of the complete purification procedure is shown in Table I.

Analysis of Purified Esterase. Since the cup-plate method is not as sensitive as the viscosimetric method for measuring polygalacturonase activity, a sample of the purified esterase was subjected to the latter analysis as an additional indication that the contaminating enzyme had been removed. The sample (0.5 ml) containing 25 units of pectinesterase was mixed with 9.5 ml of the reaction mixture and flow times were determined periodically in a viscosimeter as described under Materials and Methods. No change in flow time occurred during the first hour of the reaction. After 24 hr, however, an 8% decrease in relative viscosity was detected. This indicated the possibility that a trace of polygalacturonase activity remained in the purified esterase preparation.

A 5-ml reaction mixture containing 0.1% polygalacturonic acid, 0.02 M sodium acetate buffer (pH 5.0), and 50 units of purified pectinesterase was incubated for 24 hr at 28°. Merthiolate (1 mg) was added as a preservative. A sample equivalent to 100 μg of polygalacturonic acid was analyzed by paper chromatography. Faint spots corresponding to monogalacturonic acid and the lower oligogalacturonides up to tetramer were detected. Another reaction mixture was prepared exactly as above except that 0.02 M imidazole hydrochloride (pH 7.0) was substituted for the acetate buffer. No oligogalacturonides were detected in this reaction mixture after incubation for 24 hr.

Crude and purified enzyme preparations were analyzed by disc gel electrophoresis and typical results are shown in Figure 4. At least 7 bands were detected in the crude preparation on gels run at pH 4.3. The final purified enzyme gave 2 bands which stained with approximately equal intensity on gels at pH 4.3. No bands were detected in the purified preparation on gels run at pH 9.5.

Apparent Molecular Weights of Various Pectic Enzymes. The apparent molecular weights of pectinesterase and polygalacturonate lyase from *F. oxysporum* f. sp. *vasinfectum* were determined by gel filtration chromatography on Sephadex G-75 as described under Materials and Methods. A purified sample of the esterase and a crude sample of the lyase were employed and for comparative purposes the molecular weight of tomato pectinesterase was also determined on the same column. Data in Figure 5 show that the apparent molecular weight of the fungal esterase (35,000) is somewhat larger than fungal polygalacturonate lyase (24,000) and tomato pectinesterase (27,500). In addition, the molecular weight of the apparent complex of pectinesterase and exopolygalacturonate lyase from *C. multifermentans* was estimated by gel filtration chromatography on Sephadex G-200 (Figure 6). The value found (400,000) is considerably higher than the total combined weight of the fungal esterase and lyase (59,000).

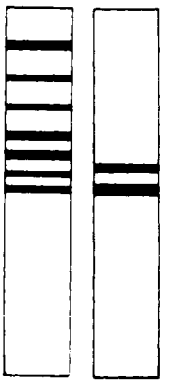

FIGURE 4: Disc gel electrophoresis of pectinesterase at pH 4.3: left, crude enzyme (260 μg of protein); right, purified enzyme (30 μg of protein).

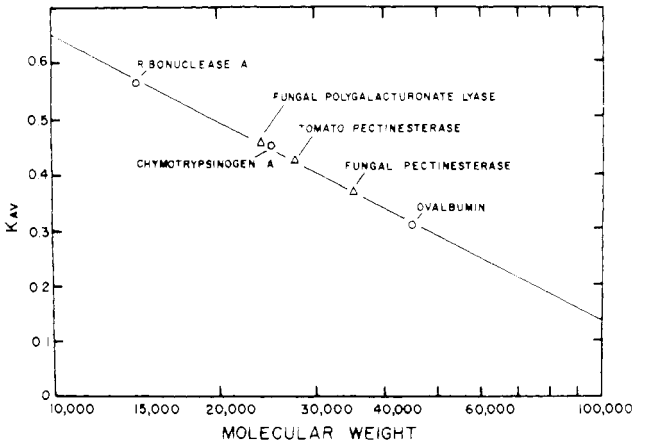

FIGURE 5: Estimation of molecular weight by ascending gel filtration chromatography on Sephadex G-75, superfine. The column was eluted with 0.05 M sodium acetate buffer, pH 5.0, and 2-ml fractions were collected with a flow rate of 12 ml/hr. $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e = the elution volume; V_0 = void volume (154 ml); V_t = bed volume (417 ml).

Action of Fungal Pectinesterase on Highly Esterified Pectin. The activities of fungal pectinesterase (0.7 unit) and esterase-free clostridial exopolygalacturonate lyase (1.2 units) were monitored simultaneously in the same reaction mixture by the procedure described under Material and Methods. The results of the assays were plotted (Figure 7) so that the scale for esterase activity was exactly twice that for lyase. This facilitated comparison of the two enzymes since one molecule of unsaturated digalacturonic acid, the product of the exolyase, contains two carboxyl groups, products of the esterase. Exopolygalacturonate lyase cannot catalyze the degradation of highly esterified pectin in the absence of pectinesterase (Macmillan *et al.*, 1964). When both enzymes are present the esterase creates deesterified substrate (polygalacturonate) for the lyase. Comparison of the two rates (Figure 7) shows that the deesterified portion of the substrated produced by the action of the esterase was degraded by the lyase at about 57% of the rate at which this deesterified substrate was produced. Approximately the same percentage difference in the two rates was found in a second

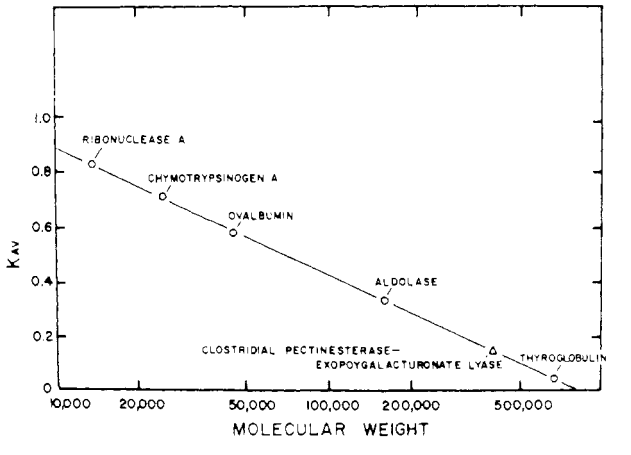

FIGURE 6: Estimation of molecular weight by ascending gel filtration chromatography on Sephadex G-200. The column was eluted with 0.02 M potassium phosphate buffer, pH 6.0, and 2-ml fractions were collected with a flow rate of 10 ml/hr. V_0 = 138 ml; V_t = 382 ml (see Figure 5).

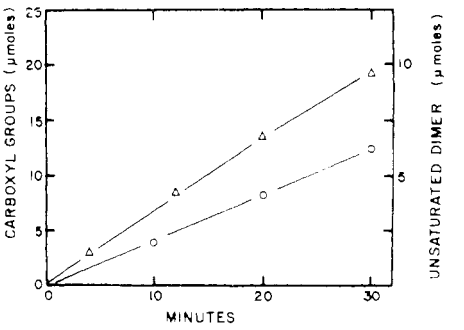

FIGURE 7: Comparison of fungal pectinesterase and clostridial exopolygalacturonate lyase activities. The reaction mixture contained 0.125% polymethyl polygalacturonate methyl glycoside, 0.002 M $CaCl_2$, 0.7 unit of pectinesterase, and 1.2 units of polygalacturonate lyase in a final volume of 10 ml. Enzyme activities were monitored simultaneously by the method described in the text: ○, exopolygalacturonate lyase (unsaturated dimer); △, pectinesterase (carboxyl groups).

experiment in which 2.4 units of clostridial lyase was assayed simultaneously with 0.7 unit of pectinesterase. The results were about the same as shown in Figure 7 indicating that the amount of lyase present in both experiments was in excess and that the rate for lyase was limited by the amount of substrate formed by fungal esterase. Similar results were found for tomato pectinesterase (Lee *et al.*, 1970), and as before they were interpreted as meaning that about half of the initial pectinesterase activity must have occurred near the reducing ends of the highly esterified pectin chains. The remaining initial activity appears to occur at some other locus on the chain. Other interpretations are untenable in view of the known action pattern of exopolygalacturonate lyase. Presumably the action of the esterase proceeds along the chain in a linear manner ("unzippering"). Additional evidence for this interpretation was obtained in the following experiment in which the action of exopolygalacturonate lyase was monitored on a substrate consisting of highly esterified pectin that was being deesterified nonenzymatically under slightly alkaline conditions.

Action of Fungal Pectinesterase on Randomly Deesterified Pectin. Deesterification was accomplished by maintaining the pH of a sample of highly esterified pectin at pH 9.4 in the pH-Stat. According to Kohn and Furda (1967a,b) under alkaline conditions the ester linkages in pectins are hydrolyzed by a random process. This pH value was selected for two reasons. First, the rate of random deesterification which occurred was appropriate for the experiment and was easily monitored, and second, exopolygalacturonate lyase has about the same activity at pH 9.4 as at 7.0, the pH value used in previous experiments employing enzymatically deesterified substrate for the lyase (the optimal pH for exolyase is 8.5). Once the conditions were established, 10 ml of 0.125% highly esterified pectin was randomly deesterified in the presence of 1.2 units of exopolygalacturonate lyase. The activity of the lyase compared to the rate of nonenzymatic deesterification is shown in Figure 8. The two upper curves are plots of lyase and esterase activity obtained when the two enzymes act together at pH 7.0. The two lower curves show that nonenzymatically deesterified pectin is not a good substrate for the lyase. Presumably this is because the ester groups near the reducing ends of the molecules block its action. After 100 min, 16 μmoles of carboxyl groups was formed and evidently sufficient nonenzymatic deesterification had occurred near the reducing ends to allow the lyase to begin to degrade the polymer. The upper curves

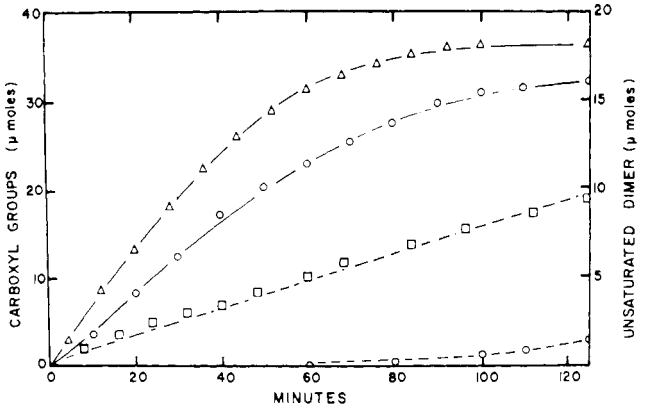

FIGURE 8: Comparison of clostridial exopolygalacturonate lyase activity on substrate deesterified with fungal pectinesterase and on substrate randomly deesterified at pH 9.4. The reaction mixture represented by the solid lines contained 0.125% polymethyl polygalacturonate methyl glycoside, 0.002 M $CaCl_2$, 0.7 unit of pectinesterase, and 1.2 units of polygalacturonate lyase in a final volume of 10 ml. The reaction mixture represented by the broken lines contained the same components except that pectinesterase was omitted and the reaction was carried out at pH 9.4. Activities were monitored simultaneously by the method described in the text: △, carboxyl groups produced by pectinesterase activity at pH 7.0; □, carboxyl groups produced nonenzymatically at pH 9.4; ○, exopolygalacturonate lyase activity (unsaturated dimer).

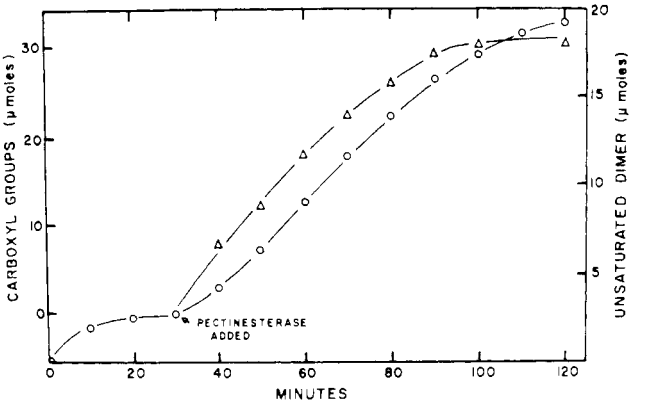

FIGURE 9: Comparison of fungal pectinesterase and clostridial exopolygalacturonate lyase activities on pectin N.F. The reaction mixture contained 0.125% pectin N.F., 0.002 M $CaCl_2$ in a final volume of 10 ml. The lyase (1.2 units) reacted for 30 min before pectinesterase (0.7 unit) was added to the reaction mixture. Enzyme activities were monitored simultaneously by the methods described in the text: ○, exopolygalacturonate lyase (unsaturated dimer); △, pectinesterase (carboxyl groups).

show that the lyase begins acting almost immediately in the presence of esterase and by the time 16 μmoles of carboxyl groups was produced (25 min) the lyase had already formed 5 μmoles of unsaturated dimer.

Conceivably, nonenzymatic degradation of glycosidic bonds could have occurred in the reaction mixture at pH 9.4 since the elimination reaction is known to be catalyzed by alkali. This was not the case, however, since only a slight increase in absorbance at 235 nm occurred in a control reaction mixture containing no enzyme.

Action of Fungal Pectinesterase on Pectin N.F. In another experiment the combined action of fungal esterase and clostridial exopolygalacturonase was monitored simultaneously on 10 ml of 0.125% pectin N.F. (about 68% esterified with methanol compared to 96% esterification for highly esterified pectin). Prior to the addition of esterase, however, lyase was incubated with the substrate for 30 min in order to split off any unesterified galacturonide residues at the reducing end of the pectin molecules. At the end of 30 min approximately 2.6 μmoles of unsaturated digalacturonate was produced (Figure 9). This corresponded to about 8% degradation of the substrate which was the same as the value previously reported (Macmillan *et al.*, 1964). At the end of 30 min there was no lyase activity, but immediately upon addition of pectinesterase the lyase activity began to increase and the rates for the two enzymes began to parallel one another. With pectin N.F. as substrate the rate for clostridial lyase was considerably closer to that for esterase than was previously found with the highly esterified pectin as substrate (Figure 8). These results were probably caused by differences in the degree of esterification between the two substrates. With highly esterified pectin the esterase hydrolyzes ester linkages at the reducing end of the molecule and at some other locus further along the molecule. Unesterified substrate produced at loci other than the reducing end of the molecule would not immediately be available for degradation by the lyase. With pectin N.F. as substrate (assuming a random distribution of ester linkages) it would be expected that each ester linkage hydrolyzed near the reducing end of the molecule would effectively release more substrate for the lyase than would be released with a single hydrolysis at the reducing end of a highly esterified pectin molecule. Thus even if the esterase is also acting further along the pectin N.F. chain, more substrate is available at the reducing end and the rates for lyase and esterase would be closer to one another.

Action Pattern of Clostridial Pectinesterase. A preparation of clostridial exopolygalacturonate lyase containing clostridial pectinesterase was assayed on highly esterified pectin as previously reported (Lee *et al.*, 1970). This experiment reconfirmed our original observation that all of the clostridial esterase activity occurs at the reducing ends of pectin chains and that the clostridial esterase and lyase have very similar action patterns.

Discussion

Previously we found (L. Miller and J. D. Macmillan, unpublished data) that crude fungal pectinesterase could be resolved into two peaks by gel filtration chromatography on Sephadex G-75; one peak was retained on columns of the gel and the other was excluded in the void volume. In the present work only one pectinesterase peak was found. It is possible that the activity which had been previously excluded from the gel was adsorbed on DEAE-Sephadex during the first step of the purification.

The elution peaks corresponding to pectinesterase, polygalacturonate lyase, and polygalacturonase were only partially separated from one another by either gel filtration chromatography on Sephadex G-75 or ion-exchange chromatography on CM-Sephadex. Because of these similarities in chromatographic properties only a few fractions from pectinesterase elution peaks were selected in order to eliminate the contaminating pectic activities. Since large amounts of pectinesterase activity were discarded in the contaminated fractions, the final yield in the purified preparation, 7%, was low.

The purification procedure developed here was successful in removing polygalacturonate lyase from preparations of fungal pectinesterase. Polygalacturonase activity was not

detected in the purified preparation using the cup-plate method for assay at pH 5.0. However, traces of oligogalacturonides were detected after 24 hr in a reaction mixture containing a large sample (50 units) of purified pectinesterase and 0.1% polygalacturonate at pH 5.0. This indicated that at pH 5.0 some polygalacturonase activity was still present in the purified preparation. Since the experiments on the action pattern of fungal pectinesterase were run at pH 7.0, it was essential that the esterase contain no significant polygalacturonase activity when acting at that pH value. Oligogalacturonides were not detected in a reaction mixture at pH 7.0 after 24 hr. Furthermore, a sample containing 25 units of pectinesterase was incapable of lowering the viscosity of 0.5% polygalacturonate after 1 hr at pH 7.0. An 8% decrease in relative viscosity, however, occurred after incubation of the viscosimetric reaction mixture for 24 hr. This decrease would represent an extremely small amount of polygalacturonase activity. The products of the reaction of fungal polygalacturonase corresponded to the products produced by endopolygalacturonase from yeast. Assuming that the fungal polygalacturonase is also an endo- or randomly splitting enzyme, then each unit of pectinesterase would be contaminated with an amount of polygalacturonase capable of breaking only about 4×10^{-5} glycosidic bonds/min. Therefore, it seems unlikely that there was enough polygalacturonase present to interfere with experiments on the action pattern of the esterase.

Two bands were detected by disc gel electrophoresis of the purified pectinesterase preparation. The bands were close to one another and stained with about equal intensity with coomassie blue. It is not known whether these two bands represent pectinesterase and a contaminating protein or two isozymes of pectinesterase.

Fungal pectinesterase acts on highly esterified pectin with an action pattern similar to that found previously for tomato pectinesterase. That is, about half of the esterase activity is initiated at the reducing end of the pectin molecule while the rest of the activity occurs at some other locus or loci on the pectin chain. All of the clostridial pectinesterase activity occurred at the reducing ends of highly esterified pectin chains. It is possible, however, that the action pattern of the fungal and the tomato enzymes are not as completely different from that of the clostridial esterase as the data indicate. Previously evidence was presented indicating that the clostridial esterase is complexed with exopolygalacturonate lyase (Miller and Macmillan, 1970). This possibility is supported by the relatively higher molecular weight found for the complex of clostridial enzymes (400,000) compared to the individual fungal enzymes (24,000–35,000). Although the clostridial esterase activity was inactivated with heat, it was possible that the inactivated enzyme was still complexed with the active exopolygalacturonate lyase. Conceivably this inactive esterase could interfere with studies employing exopolygalacturonate lyase to determine action patterns of other esterases.

A comparison of the action of clostridial lyase on randomly deesterified pectin and on pectin deesterified enzymatically with fungal pectinesterase supports the theory that a considerable amount of pectinesterase activity must occur at the reducing ends of the pectin molecules. Clostridial lyase was not able to degrade randomly deesterified pectin during early stages of the reaction, and only after 100 min did the lyase have sufficient deesterified substrate available at the reducing end so that polymer degradation could be detected. Presumably methyl ester groups near the reducing ends of pectin molecules were initially inhibiting the action of the lyase. These groups at the end are evidently removed sequentially by fungal esterase thereby allowing the clostridial lyase to act at a more rapid rate.

Comparison of the data in Figure 8 with the data in Figure 4 of our earlier publication (Lee *et al.*, 1970) shows that while fungal pectinesterase was capable of forming over 35 μmoles of carboxyl groups on 0.125% highly esterified pectin before activity leveled off, the clostridial esterase formed only about 13 μmoles of carboxyl groups before activity leveled off. One possibility for the difference in the final degree of deesterification catalyzed by the two esterases could be related to the relative stability of the two enzymes. Clostridial pectinesterase is easily inactivated at pH 7.0 (Miller and Macmillan, 1970) while the fungal esterase is considerably more stable at that pH value (L. Miller and J. D. Macmillan, unpublished data).

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