AN ABSTRACT OF THE THESIS OF

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Title PRODUCTION OF PECTIC ENZYMES BY VERTICILLIUM ALBO-ATRUM

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The main objectives of this study were to determine the effect of certain environmental conditions on the quantity of polygalacturonase (PG) produced by V. albo-atrum, and to partially purify and characterize the PGs produced by this fungus in culture and in infected tomato plants.

V. albo-atrum produced twenty times as much PG when sodium polypectate was the carbon source as when grown on pectin. Optimum PG production occurred 6-8 days after inoculation of the medium. Less pectinmethyesterase (PME) was produced when V. albo-atrum was grown on polypectate as the carbon source than when grown on pectin. PME production was observed early in the growth of the fungus. No PME activity could be detected from

culture filtrates after eight days when either pectin or sodium polypectate was the carbon source.

Three elution schedules were used to purify PGs from a DEAE-cellulose column. Step-wise elution produced residual peaks of PG activity, and gradient elution gave poor separation of the PGs. Flushing the DEAE-cellulose column with distilled water and NaCl gave the best separation of the two endo-PGs. PME was associated with all fractions containing PG activity.

Assays of PG activity in fractions separated by column chromatography suggested that V. albo-atrum produced at least two endo-PGs in culture. One endo-PG preferentially hydrolyzed sodium polypectate to large fragments; however, the fragments were eventually hydrolyzed to monogalacturonic acid. The second endo-PG preferentially hydrolyzed small fragments from the substrate. Fractions containing the endo-PG that preferentially hydrolyzed the substrate to large fragments gave ratios of viscosity reduction to reducing group liberation at least five times as high as those obtained for fractions containing the endo-PG that preferentially hydrolyzed the substrate to small fragments.

Culture filtrates from cultures incubated at room temperature but not on a shaker produced less PG than cultures grown on a shaker at room temperature. When culture filtrates of the still

cultures were eluted from the DEAE-cellulose column, fractions from the first PG peak eluted from the column hydrolyzed tri-galacturonic acid slowly to monogalacturonic acid.

Cultures grown at 30°C produced only trace amounts of PG activity. Growth of the fungus at 30°C was similar to cultures grown at lower temperatures. However, the cultures grown at lower temperatures produced relatively high PG titers.

The crude enzyme preparation of V. albo-atrum from infected tomato plants yielded pectic fragments as well as monogalacturonic acid. This indicated the presence of an endo-PG that hydrolyzed small fragments from the substrate. Column chromatography of this enzyme on DEAE-cellulose using a step-wise elution yielded a PG fraction that preferentially hydrolyzed the substrate into small fragments similar to fractions obtained by a step-wise elution of culture filtrates.

Evidence was obtained that V. albo-atrum produced trace amounts of pectintranseliminase in culture and in infected plants. Additional studies will be required to understand the significance of this enzyme in the Verticillium wilt syndrome.

PRODUCTION OF PECTIC ENZYMES BY *VERTICILLIUM ALBO-ATRUM*

by

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PRODUCTION OF PECTIC ENZYMES BY *VERTICILLIUM ALBO-ATRUM*

INTRODUCTION

Verticillium albo-atrum Reinke and Berth. is a soil-borne plant pathogenic fungus that infects a wide variety of hosts. In tomato plants, the fungus enters the host through the roots, rapidly penetrates to the xylem, and then grows upward in the xylem to the stem and leaves. Infected tomato plants develop a combination of symptoms including epinasty, wilting, yellowing, and stunting. In late stages of the disease the pathogen proliferates throughout the host, and sporulates on the surface of dead stems and leaves.

Pectic substances of the host are a potential source of food for the pathogen. Thus, pectic enzymes produced by *V. albo-atrum* could play a vital role in this disease by making pectic substances of the host available to the pathogen.

Gothoskar *et al.* (17) suggest that in *Fusarium* wilt of tomato, a disease similar to *Verticillium* wilt, pectic enzymes produced by the pathogen partially hydrolyze pectic substances of the host. Pectic fragments then increase the viscosity of the vascular sap or form gels in the vessels, either of which could reduce water flow up the stem and thus contribute to wilting of the foliage. Dimond (14) suggests that many wilt pathogens act upon their host

by a limited number of biochemical processes. Thus, the hypothesis of Gothoskar et al. (17) may apply to the role of pectic enzymes in the Verticillium wilt syndrome.

Wood (47) suggests that before the exact role of pectic enzymes in the wilt syndromes can be determined, additional basic information on pectic enzymes will be required, and that these enzymes should be isolated from diseased plants and compared with corresponding enzymes in healthy plants.

To better understand the role of pectic enzymes in Verticillium wilt of tomato, experimental procedures were designed with the following objectives in mind:

A. To isolate and purify polygalacturonase (PG) produced by V. albo-atrum in culture.

B. To determine the effect of various environmental conditions on the production of PG in culture.

C. To isolate PG from tomato plants inoculated with V. albo-atrum, and compare it with PG produced in culture.

The fulfillment of these objectives should broaden our knowledge of the Verticillium wilt syndrome, and aid in understanding wilt diseases caused by other pathogens in a wide range of hosts.

LITERATURE REVIEW

Pectic substances. -- A lack of knowledge of the complex structure of pectic substances has severely limited the characterization of pectic enzymes. Pectic substances are naturally occurring constituents of plants found primarily in the middle lamella and primary cell walls. The nature of pectic substances has been reviewed several times in recent years (12, 13, 22). All pectic substances consist of D-galacturonic acid units linked by α-1, 4 glycosidic bonds. The properties of pectic substances are due primarily to the chain length of the macromolecules, and the chemical configuration of the No. 6 carbon of the galacturonic acid units. If all the No. 6 carbons are free carboxyls, the pectic substance is termed "pectic acid" or, in the salt form, "pectate". If the No. 6 carbons are esterified with methyl groups, the term "pectinic acid" or "pectinate" is used to describe the pectic substance. All pectinates with a methyl ester content exceeding seven percent on a wt/wt basis are termed "pectin". In theory, the methyl ester content of pectin may reach 16.3 percent on a wt/wt basis but in actuality no pectin has been isolated with an ester content greater than 14.0 percent.

"Protopectin" is the term used to describe the water-insoluble pectic substances found mainly in the matrix of primary

cell walls (22). They are soluble in dilute acids and upon hydrolysis yield pectinates with a lower methoxyl content and properties that vary with the type and age of plant tissue and species. The water insolubility of protopectin is not clearly understood, but it may be due to: A) very high molecular weight of individual chains, B) cross-linkages between adjacent chains by polyvalent cations, C) association with cellulose or hemicelluloses, D) formation of anhydride bridges between chains, or E) a combination of the above (47).

Pectic enzymes. -- Pectic enzymes can be divided into two basic groups depending on their site of action. Pectinmethylesterase (PME) demethylates pectinates, and polygalacturonase (PG) hydrolyzes the α-1, 4 glycosidic linkages of the pectic substances. Many plant pathogenic bacteria and fungi produce both types of pectic enzymes extracellularly, and these enzymes have been implicated in plant disease syndromes of several types (47).

Pectinmethylesterase. -- PME produces pectinates of a lower methoxyl content and methanol. Hydrolysis is rarely complete. About ten percent of the methyl ester groups resist deesterification (47). PME from different organisms has characteristic properties, e.g. pH for optimum activity,

behavior towards inhibitors, and activation by salts (47). Certain divalent cations (e.g. Ca^{++}, Mg^{++}) stimulate PME activity probably by forming a complex with deesterified carboxyls, thus preventing inactivation of the enzyme by the free carboxyl groups (23). PME activity can be determined by measuring the increase in carboxyl groups by titration with NaOH, or by analyzing for the methanol produced during deesterification.

PME is found extensively in the roots, stems, and leaves of higher plants. This enzyme is strongly adsorbed to the cell wall materials, and must be eluted by high pH or salt concentrations to be isolated. Consequently, expressed plant juices contain only a small fraction of the total PME present in the tissues (22).

Production of PME by microorganisms is usually by an adaptive mechanism (35, 39), but Fusarium oxysporum f. lycopersici (44) and Penicillium chrysogenum (35) produce PME on nonesterified carbon sources such as mucic acid, glucose, and galacturonic acid.

Scheffer et al. (39) detected PME in culture filtrates of V. albo-atrum grown on moist bran or bran plus dextrose and pectin. Their results were not conclusive because PME was not always detected and, when present, PME activity was extremely low. Kamal and Wood (21) obtained similar results with V. dahliae.

Wood (48) obtained greater PME production from V. albo-atrum grown on a pectin medium in still culture for 14 days. However, Wood's PME preparations hydrolyzed only about ten percent of the available ester linkages of the pectin substrate. PME from cultures grown on sodium polypectate, glucose, and carboxymethylcellulose hydrolyzed even fewer ester linkages. Deese and Stahmann (11) reported low PME activity in five-day-old cultures in bran inoculated with V. albo-atrum, and no activity at the end of the seventh day.

Wood (48) presented the only evidence for increased PME in plants infected with V. albo-atrum. Increased PME was considered a response of the host to the pathogen, rather than production of PME by the pathogen in the host.

Polygalacturonase. -- Several types of PG are produced by microorganisms. Deuel and Stutz (13) have classified PGs as: Type I (the liquefying PGs) that randomly hydrolyzes low methoxyl pectinic acids to smaller fragments; Type II (a-pectin glycosidase) that randomly hydrolyzes chains of high methoxyl pectinic acids; and Type III (the saccharifying PGs) that attacks end group linkages yielding only monogalacturonic acid.

Demain and Phaff (12) have suggested a different classification system for the PGs. They distinguished between PGs that

hydrolyze pectic substances randomly (endo-) and the PGs that hydrolyze terminal groups of pectic substances (exo-). Endo- and exo-enzymes are further classified depending on their substrate preference. If the enzymes hydrolyze pectin more easily than pectic acid, they are termed endo- or exopolymethylgalacturonases. If pectic acid is attacked in preference to pectin, the enzymes are termed endo- or exopolygalacturonases. The endopolymethylgalacturonases are subdivided further according to the optimum pH for hydrolysis into Type I (pH 5.0-6.0) and Type II (pH 8.0-9.0).

Albersheim et al. (1) recently discovered a new pectic enzyme that hydrolyzes pectic substances by a transelimination process. Discovery of this new enzyme has diminished the value of the classification systems of Demain and Phaff (12) and of Deuel and Stutz (13).

Three different assays may be used to evaluate PG activity. When a glycosidic linkage of a pectic substance is hydrolyzed by PG, a reducing group is exposed and the polygalacturonic chain is shortened. Shortening of the chains reduces the viscosity of the pectic substances. Reduction in viscosity can be measured as an index of PG activity, and the smaller pectic fragments can be detected by paper chromatography. Determination of the number

of reducing groups exposed is a third method for measuring PG activity.

Three methods are commonly used to measure reducing groups in PG assays: the modified Willstätter-Schudel hypoiodite test (19), the copper-reduction test of Somogyi (42), and the dinitrosalicylic acid test of Deese and Stahmann (10). Reducing group determinations are a more accurate measure of PG activity than viscosity reduction methods, particularly in the later stages of hydrolysis (46). However, viscosity changes give a rapid indication of the early stages of hydrolysis of pectic substances. Problems associated with the viscosity reduction technique have been reviewed by Wood (46).

Presence of PG in higher plant tissues was a point of speculation for many years, but in 1955 McCready et al. (27) isolated PG from tomato and avocado fruits. They concluded that plant PG was similar to "mold polygalacturonase" in that it hydrolyzed pectic acid to monogalacturonic acid by a random mechanism. PGs have been found in other plant tissues, but their titer is extremely low (47).

Many fungi and bacteria produce PGs only when grown on pectic substances (47) while others produce PGs in a wide variety of media (21, 48). Often PGs are produced under conditions

unfavorable for growth of the organism (47).

Little has been done to characterize PGs produced by plant pathogenic organisms, particularly the enzymes produced in diseased plants (47). Young (49) characterized the PGs produced by two races of F. oxysporum f. lycopersici in culture and in infected tomato plants. He concluded that the enzymes from the two sources were similar. However, Bateman (3) has noted a difference between the PGs produced by Rhizoctonia in culture and in infected bean plants.

Although Bewley (6) first demonstrated a hydrolytic enzyme produced by V. albo-atrum, Scheffer et al. (39) were the first to discover PG in culture filtrates of V. albo-atrum. This PG hydrolyzed pectic substances to monogalacturonic acid. Their results have since been confirmed by Wood (48) and by Deese and Stahmann (9, 11).

Wood (48) found a three-fold increase in PG production when V. albo-atrum was grown on sodium polypectate instead of pectin, but he did not find this enzyme in infected tomato plants. Deese and Stahmann (9, 11) obtained evidence for a PG that produced monogalacturonic acid in tomato-stem tissue inoculated with V. albo-atrum. They (9) isolated from the sap of potato plants inoculated with V. albo-atrum a PG that produced monogalacturonic

acid and large pectic fragments. Blackhurst and Wood (7) obtained a preparation with weak PG activity from susceptible and resistant tomato varieties inoculated with V. albo-atrum, but did not characterize these enzymes.

Protopectinase. -- Protopectinases are thought to hydrolyze protopectin to soluble pectinic acids, but because the nature of the protopectin substrate is poorly understood there is some question as to the existence of this enzyme. Elarosi (16) suggested that protopectinase is a system of related PG enzymes, and that the activity of protopectinase depends on the types and amounts of the enzymes present. Bateman (4) and others (12, 22, 47), similarly suggested that protopectinase is not a single enzyme but rather a complex enzyme system.

Pectintranseliminase. -- Pectintranseliminase (PTE) hydrolyzes the α-1, 4 linkages of pectin to give smaller pectic fragments containing a double bond between the number 4 and 5 carbon atoms of the galacturonic acids. Since this enzyme was first discovered in a commercial pectinase preparation (1), Nagel and Vaughn (30) have found a PTE produced by Bacillus polymyxa that hydrolyzes trigalacturonic acid to monogalacturonic acid and an altered dimer that was characterized as an α-β-unsaturated

digalacturonic acid. PTE from several sources requires calcium for optimum activity (30, 43). PTE activity can be measured by the thiobarbituric acid test of Neukon (30).

The proposed role of pectic enzymes in wilt disease syndromes. -- Two hypotheses have been suggested regarding the role of pectolytic enzymes in the Fusarium wilt syndrome. Because of the similarity of the two syndromes, these hypotheses can be extended to the Verticillium wilts.

Gothoskar et al. (17) suggested that F. oxysporum f. lycopersici produces a depolymerase PG (i.e. an endo-type PG) in vessels of tomato plants. The enzyme diffuses out of the vessels and hydrolyzes the pectic substances of the middle lamella and primary cell walls of xylem parenchyma. Partially hydrolyzed pectic fragments diffuse into the vessels, increase the viscosity of the vascular sap, and eventually form gels which slow the translocation of water. Impaired upward flow of water in vessels then causes the diseased plants to wilt.

Beckman et al. (5) presented an hypothesis concerning the role of pectic enzymes in banana wilt caused by F. oxysporum f. cubense. They suggested that formation of gels in vessels of the roots could block the openings in the vessel end-walls and thus prevent the upward movement of the pathogen. In this wilt disease,

the pathogen spreads through its host as microconidia. Before the pathogen can advance from one vessel element to the next, the microconidia must germinate and penetrate the pectic gel at the vessel end-wall. This could be done mechanically or by dissolving the plug through the action of pectic enzymes. This hypothesis finds less application to a host such as tomato in which the mature vessel elements lack end-walls.

Acceptance of these hypotheses depends on proof that PGs produced by the pathogen do hydrolyze pectic substances to large fragments that could form gels. Deese and Stahmann (9) have found a PG of this type in potato stems infected with V. albo-atrum, but all other studies (11, 39, 48) have indicated that V. albo-atrum produces a PG that carries the hydrolysis to monogalacturonic acid. However, none of these studies were exhaustive enough to be conclusive.

Purification of pectic enzymes. -- Within the past 15 years the complex nature of the pectic enzymes has been revealed. Prior to this time, all pectic enzymes were considered either PME or PG. The complex nature of these enzymes makes their separation an important prerequisite to understanding their action in plant disease syndromes. Studies on the pectolytic enzymes of V. albo-atrum have been made with crude enzyme preparations

derived from culture filtrates (7, 11, 39, 48), sap from inoculated tomato stems (7, 11), or infected tomato plants (7). These studies would be more meaningful if the enzyme preparations had been more clearly defined. Many methods are available for separation and purification of pectolytic enzymes. However, early attempts at purification of microbial pectic enzymes were only partially successful because PME was not completely separated from PG (24, 28, 36, 38, 40, 44), or most of the PG was inactivated during the separation (20, 25, 49).

Recently, Patel and Phaff (34) obtained a soluble PG from tomato fruit by column chromatography. The crude enzyme was adsorbed on columns of pectic acid or calcium phosphate gels. The columns were eluted with pH 5.0 acetate buffer and a water-soluble PG preparation was obtained in the eluate.

Diethylaminoethane substituted cellulose (DEAE-cellulose) has been used in column chromatography to successfully separate enzymes and other proteins (41). Nagel and Vaughn (29) identified two active PG fractions from culture filtrates of Bacterium polymyxa using this technique. Albersheim and Kilías (2) purified PTE from pectinol R-10 by gel filtration on Sephadex and DEAE-Sephadex column chromatography, and Young (49) obtained at least two partially purified PGs from culture filtrates of F. oxysporum

f. lycopersici using DEAE-cellulose columns. These recently developed methods for separation of pectic enzymes are superior to previous methods, and were used in these investigations for the separation of pectic enzymes produced by V. albo-atrum.

GENERAL METHODS AND MATERIALS

Culture of Verticillium. -- The isolate of Verticillium albo-atrum used in this study was supplied by Dr. W. B. Raymer of the Agricultural Research Service, U.S.D.A., Beltsville, Maryland, and bore the U.S.D.A. designation No. 12. This fungus was isolated from Katahdin variety of potato in Presque Isle, Maine, in 1953, and was identified by Dr. R. E. Webb of the U.S.D.A. as a strongly carbonaceous dauermycelium isolate of V. albo-atrum pathogenic to potato, and Bonny Best, Loran Blood, and V. R. Moscow varieties of tomato.

Potato-dextrose broth was inoculated aseptically with this fungus, and the culture was incubated on a Gyrotory Shaker for five days. One ml of bud cells was removed aseptically from the flask and a dilution series was made in one percent potato-dextrose agar (PDA). Petri plates were poured from this dilution series and incubated at room temperature for five days. A single, isolated colony was cut aseptically from one of the plates and inoculated on slants of PDA. These slants were the stock cultures used throughout this investigation. They were grown at room temperature and transferred periodically to maintain an actively growing mycelium.

Inoculum for experiments was obtained by transferring the

fungus aseptically to Petri plates containing PDA where it was allowed to grow for five days at 20°C. Disks were then cut aseptically from the margin of the culture with a No. 3 cork borer. The visible margin of the fungus extended to the center of each disk. Each flask in each experiment was inoculated with a single disk.

Verticillium was grown in Erlenmeyer flasks in a modified Richard's medium containing the following salts: 5.0 g NH_4NO_3, 2.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.01 g $ZnSO_4$, and 0.06 g $FeCl_3$ per liter. The carbon source was either one percent (wt/v) Sunkist Growers sodium polypectate (Product No. 6024) or one percent (wt/v) Sunkist Growers pectin N.F. (Product No. 3442). Ten grams of pectin or pectate were added to 500 ml of mineral solution containing all the salts except the KH_2PO_4 and mixed for one minute in a Waring Blendor. One hundred ml of KH_2PO_4 were placed in 125 ml Erlenmeyer flasks, and 100 ml of the mineral solution containing the pectin or sodium polypectate were placed in 500 ml DeLong culture flasks fitted with Morton closures. The flasks containing the solutions were autoclaved 20 minutes at 15 psi. After the solutions had cooled to room temperature, sufficient 1.0 N NaOH was added to the KH_2PO_4 solutions to give a final pH of 5.0 when the KH_2PO_4 solution was added aseptically to

the pectin- or pectate-salts mixture.

The medium was inoculated with the disks previously described and the cultures were incubated under various conditions for six days. At the end of this period the medium was combined aseptically into one flask. Aliquots were removed from the combined culture filtrate and plated in PDA to check for contamination. The remainder of the culture filtrate was filtered through cheesecloth and centrifuged for 20 minutes at 6000 rpm. The supernatant was collected after centrifugation and passed through a presterilized Millipore (HA 0.45) filter.

Toluene (1 ml/l) was added to the culture filtrate to prevent contamination. The filtrate was placed in dialysis tubing and dialyzed against running tap water for approximately six hours. The filtrate was then checked for contamination by plating in PDA. Toluene was again added (1 ml/l) to the filtrate. The filtrate was reduced to one-tenth of its original volume in a flash evaporator at 30°C under reduced pressure. The concentrate was again checked for contamination by plating in PDA, and toluene was added. The concentrate was frozen until used for column chromatography.

Column chromatography. -- DEAE-cellulose (Lot No. 502888, cellex D anion exchange cellulose, California Biochemical Corporation) was used in all column chromatography. The DEAE-

cellulose columns were prepared by a slight modification of the method of Albersheim and Killias (2). Fifteen grams of DEAE-cellulose were weighed and suspended in 200 ml of 1 N NaOH. The suspension was filtered through Whatman No. 1 filter paper (12.5 cm) in a Buchner funnel. The DEAE-cellulose was washed by allowing one liter of distilled water to pass through the DEAE-cellulose on the filter paper. Then the DEAE-cellulose was scraped from the filter paper and resuspended in 200 ml of distilled water. This suspension was adjusted to pH 5.0 with 2 N acetic acid and again filtered on Whatman No. 1 filter paper. The DEAE-cellulose was washed with one liter of 0.01 M acetate buffer, scraped from the filter paper into a beaker, and suspended in 200 ml of 0.01 M acetate buffer (pH 5.0). The DEAE-cellulose was allowed to stand at 4°C for 15 minutes before the cloudy supernatant was poured off and discarded. This last process was repeated three times. The DEAE-cellulose was poured into a column and allowed to settle by gravity, and finally packed under 2 psi air pressure until the height of the column was 190 mm. This gave a column width to height ratio of 1:9.5. Next, 500 ml of 0.01 M acetate buffer were allowed to drip through the column overnight. Culture filtrate containing 750 mg of protein (5-12 ml) was placed on the column and it was capped with a filter paper

disk. Pectic enzymes were displaced from the columns by various elution methods.

Protein determination. -- Protein concentration of various enzyme preparations was determined by reading the optical density of the enzyme fractions at 260 and 280 mμ on a Beckman Model DB Spectrophotometer and then converting these optical densities to milligrams of protein per ml using the nomograph prepared by E. Adams of California Corporation for Biochemical Research. This nomograph is based on the extinction coefficient for enolase and nucleic acids given by Warburg and Christian (45).

Determination of polygalacturonase activity by viscosity reduction. -- One percent wt/v sodium polypectate that had previously been reprecipitated from ethanol (8) was added to 0.1 M acetate buffer (pH 5.0), and mixed for one minute in a Waring Blendor. Toluene was added during the mixing (1 ml/l). Ten ml of this substrate were then pipetted into an Ostwald-Fenskie No. 300 viscosimeter which was suspended in a waterbath at 30°C ± 0.1°. Twenty minutes later one ml of enzyme was added to the substrate and the mixture was shaken for 20 seconds. The first viscosity reading was taken one minute after the addition of the enzyme to the substrate. Additional readings were made

periodically, depending on the activity of the enzyme. The time necessary to reduce the viscosity of the substrate by 50 percent was designated as the n-R_{50} and the activity of the enzyme was expressed as the reciprocal of this value (46). This technique gave excellent replication of duplicate samples.

Determination of polygalacturonase by reducing group determination. -- PG activity was measured by determining the rate of liberation of reducing groups from pectic substances. Reducing groups were determined by the method of Somogyi (42). One percent (wt/v) sodium polypectate that had been reprecipitated from ethanol (8) was mixed with 0.1 M acetate buffer (pH 5.0) in a Waring Blendor for one minute. Toluene was added to the mixture (1 ml/l). Two ml aliquots were pipetted into plastic centrifuge tubes (16 x 120 mm). One ml of enzyme was added to each centrifuge tube and the mixture was agitated for approximately 20 seconds by a Vortex Junior Mixer. The enzyme-substrate mixture was then incubated at 30°C for various lengths of time.

After the desired incubation, 0.1 ml was withdrawn from the mixture and plated in PDA to check for contamination. Enzyme action in the remainder of the mixture was stopped by adding 1 ml of 0.3 M $Ba(OH)_2$. Five minutes later, one ml of five percent $ZnSO_4$ was added and a heavy white precipitate formed. The

mixture was agitated for 20 seconds on a Vortex Mixer, and then centrifuged for 20 minutes at 6,000 rpm. The supernatant was poured into a test tube and a 2 ml aliquot was withdrawn and placed in a Folin-Wu blood sugar tube graduated at 25 and 50 ml. Two ml of Somogyi copper reagent (42) were then added to the Folin-Wu tube. The tubes were capped with aluminum foil and placed in a boiling water bath for 20 minutes. The tubes were removed from the water bath, and cooled to room temperature. Two ml of arsenomolybdate color reagent were then added to each tube (30). A blue color that varied in intensity with the number of reducing groups developed in the mixture. The samples were brought to a specific volume with distilled water, and the optical densities (O.D.) were determined. Optical densities below 0.100 were disregarded and samples with an O.D. above 0.700 were diluted into the range 0.100-0.700 so that more accurate readings could be made. A standard curve was prepared by plotting optical density against concentration of monogalacturonic acid. A linear relationship was obtained between 50 and 400 μg/ml. The activity of the PG was expressed as μg of monogalacturonic acid per ml of enzyme per minute.

Determination of polygalacturonase by the cup-plate method. -- For a rapid determination of PG activity, a modification of the

cup-plate assay of Reid (37) was used. One percent sodium polypectate was mixed in a Waring Blendor with one percent agar in 0.1 M acetate buffer (pH 5.0). The mixture was autoclaved for 20 minutes at 15 psi. Forty-five ml aliquots were placed in bottles and autoclaved at 15 psi for 20 minutes. The bottles were cooled and stored at 4°C until needed. When an assay was to be run, the medium was melted and 15 ml aliquots were poured into Petri dishes. Five 12.7 mm filter paper disks were placed on the medium in each plate and three drops of enzyme preparation were placed on each disk. The medium was then incubated at 30°C for 24 hours. The disks were removed from the surface of the medium with forceps, and the plates were flooded with 4 N HCl. PG activity was evident as cleared zones in the agar medium where the disks had been.

Polygalacturonase assay by paper chromatography. -- The chromatographic method of Young and Corden (50) was used to determine the presence of breakdown products of pectic substrates. Fifty µl aliquots were withdrawn from enzyme-substrate mixtures at predetermined intervals, and spotted on Whatman No. 4 paper (18 cm x 50 cm). The chromatograms were developed at 23°C using an ascending solvent system containing 60 mg of sodium formate and 50 mg of brom phenol blue dissolved in a mixture of

15 ml of 88 percent formic acid and 85 ml of 77 percent ethanol (32). The paper chromatograms were removed from the solvent system after the solvent front had traveled approximately 30 cm past the point where the enzyme-substrate mixture had been applied to the paper. The wet, yellow chromatographs were then hung in a fume hood until dry.

The developed chromatographs showed bright yellow spots of mono-, di-, tri-, and tetragalacturonic acids on a blue background. Rf's of approximately 0.62, 0.41, 0.31, 0.21 for mono-, di-, tri-, and tetragalacturonic acids respectively were obtained for active enzyme preparations. With weak enzyme preparations the Rf's were apt to be lowered (50).

Determination of PME activity. -- The method of Kertesz (21) was used with slight modifications to measure the activity of PME in crude culture filtrates of Verticillium. Twenty-five ml of a 0.5 percent solution of pectin N. F. were placed in a 50 ml beaker containing a stirring bar. The electrodes of a Beckman Expanded Scale pH meter were inserted, and the mixture was adjusted to pH 7.5 with dilute NaOH. Then 0.5 ml of merthiolate (1:1000 dilution) and 0.75 ml of 1.0 M $CaCl_2$ were added to the mixture. When three drops of phenol red were added, the mixture turned a pink color. Two ml of a culture filtrate containing PME

were added to the mixture, and the pH of the enzyme-substrate mixture was readjusted to 7.5 with 0.005 N NaOH. The enzyme-substrate mixture was incubated in a 30°C water bath and whenever the reaction mixture began to lose its pink color, sufficient 0.005 N NaOH was added to readjust the pH to 7.5. This assay was carried on for 24 hours, and the total ml of NaOH used was recorded. After the assay the reaction mixture was plated in PDA to check for contamination. PME activity was expressed as microequivalents of carboxyl groups released per ml of enzyme per minute.

Determination of PME activity by cup-plate assay. -- The cup-plate method of McComb and McCready (26) was used for a rapid assay of PME activity. One gram of pectin and 2 gm of agar were added to 100 ml of pH 6.3 acetate buffer (0.1 M). The mixture was dissolved by stirring in a boiling water bath. Forty-five ml aliquots were withdrawn and placed in bottles, and they were autoclaved for 15 minutes under 15 psi, cooled to room temperature, and stored at 4°C until needed.

When an assay was to be run, the medium was melted, and approximately 15 ml aliquots were poured into Petri dishes. After the pectin-agar substrate had solidified, five 12.7 mm filter paper disks were placed on the surface of the medium and three drops of

an enzyme preparation were placed on each disk. The plates were then incubated for 24 hours at 30°C. The disks were removed and 3 ml of a solution of hydroxylamine hydrochloride (3.75 gm dissolved in 100 ml of water) were added. While agitating the plate, 3 ml of a solution containing 9.4 gm NaOH dissolved in 100 ml of water were added. The plate was allowed to stand for 10 to 15 minutes before 3 ml of 4 N HCl were added followed by 3 ml of a fresh solution of 10.0 gm ferric chloride hexahydrate dissolved in 100 ml of 0.1 N HCl was added. After a few minutes PME activity could be detected as clear areas in the medium where the disks had been. The rest of the medium turned a red-brown color.

RESULTS

SEPARATION OF PECTIC ENZYMES

Pectic enzyme production on pectin and pectate. -- The initial objective of this study was to measure the production of PME and PG by V. albo-atrum growing on pectate or pectin as the sole carbon source in a synthetic medium. The time required for optimum enzyme production was determined.

Flasks containing 200 ml of either pectin or pectate medium were inoculated with Verticillium and incubated on a shaker. The culture filtrates from five flasks of both pectin and pectate medium were collected 2, 4, 6, 8, and 10 days after the initiation of the experiments. Dialyzed culture filtrates were tested for PG activity by the viscosity reduction assay and for PME activity by titration with NaOH.

Two days after inoculation, culture filtrates of the fungus grown on pectin had twice the PME of filtrates from the pectate medium (Fig. 1). At four days, PME activity of the culture filtrate from the fungus grown on pectate was only slightly less than that of the pectin culture filtrate. The PME from the culture filtrate of the fungus grown on pectin was only slightly less active at four days than at two days. After six days no PME activity was

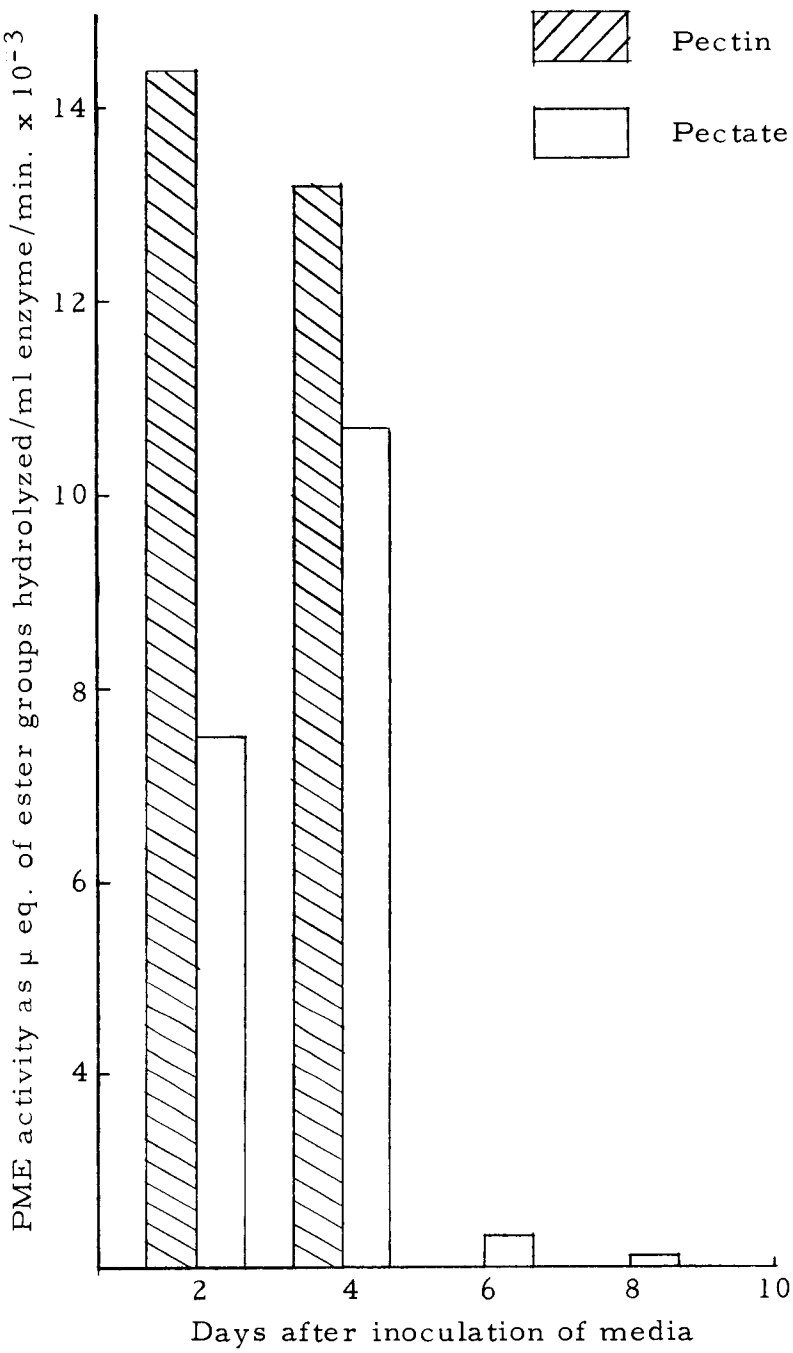

Figure 1. Production of PME by V. albo-atrum grown in a nutrient medium on pectin or pectate as the carbon source.

obtained in the culture filtrates from the fungus grown on pectin medium. However, slight PME activity was obtained from culture filtrates when the fungus was grown on pectate for six and eight days. No activity was observed for either culture filtrate harvested ten days after inoculation.

Verticillium produced almost twenty times as much PG on the pectate medium as on the pectin medium (Fig. 2). This increase in PG activity might be explained if the fungus required hydrolysis products of pectic substances to induce production of PG. PG usually hydrolyzes demethylated pectic substances more readily than the highly methylated type (48). Low PME activity of the culture filtrates would limit demethylation of pectin, and thus, PG activity which is necessary for production of hydrolysis products. Lack of pectic fragments could then limit the production of additional PG.

The twenty-fold increase of PG in culture filtrates of Verticillium grown on pectate versus pectin is significantly higher than the three-fold increase that Wood (48) obtained. This difference may be due to variation in ability to produce PG by the two strains of V. albo-atrum, or it may be due to use of culture filtrates from still cultures by Wood as compared to shake cultures in this study. Shaking causes the fungus to grow by budding, and a high

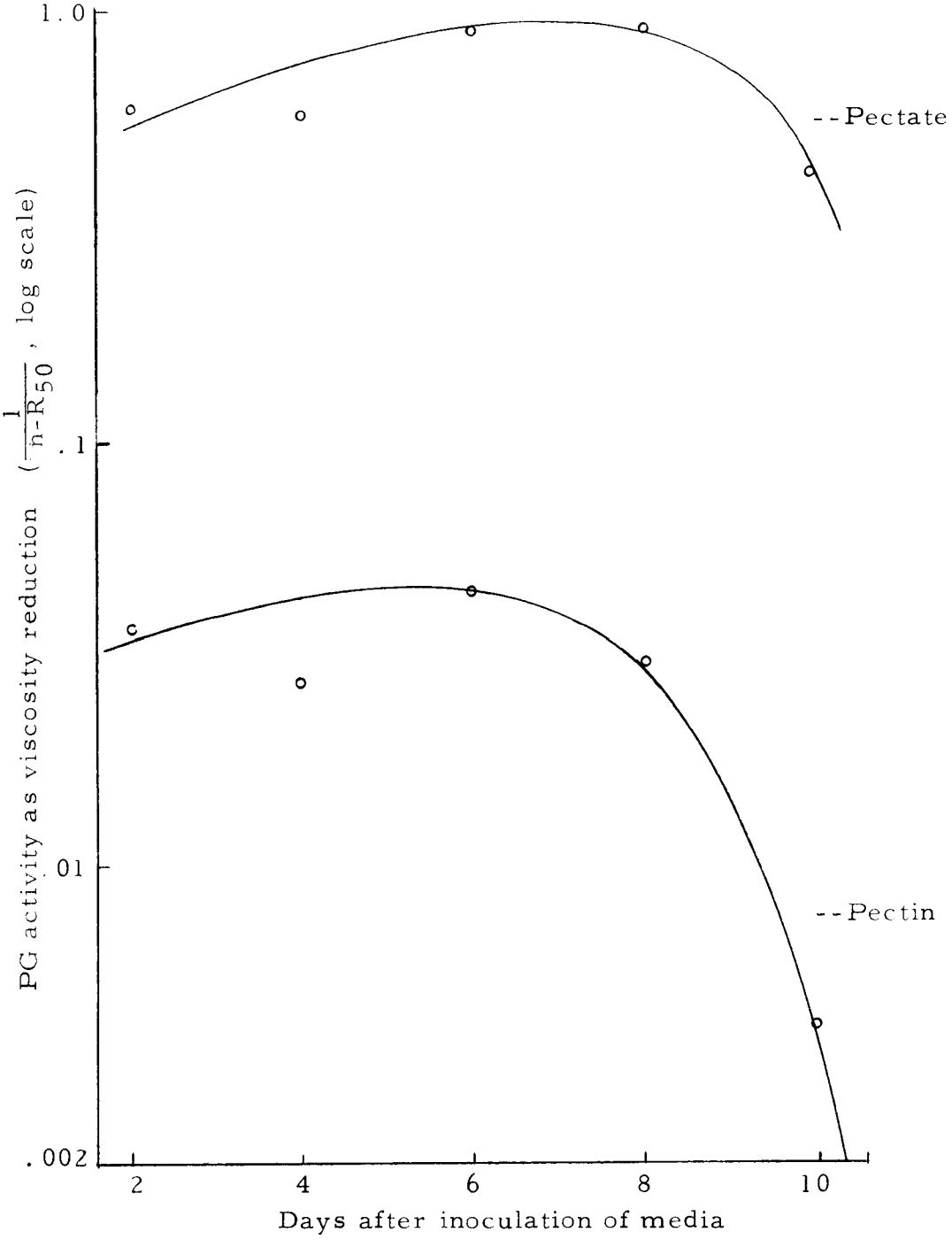

Figure 2. Production of PG by V. albo-atrum grown in a nutrient medium on pectin or pectate as the carbon source.

percentage of these cells are actively metabolizing and producing PME. In still culture, Verticillium produces a mycelial mat in which only the hyphal tips are actively growing and producing PME. These hyphal tips represent only a small percentage of the total fungal mass in these cultures.

Enzyme separation by step-wise elution of chromatographic columns. -- The next step in this study was to purify by column chromatography the PGs produced by Verticillium in culture. Concentrated culture filtrate (750 mg protein) was placed on a DEAE-cellulose column. The column was eluted in a step-wise manner with 200 ml of distilled water followed by consecutive additions of 200 ml of 0.01 M, 0.07 M, 0.17 M, and 0.4 M NaCl (49). Two psi air pressure was used to obtain a flow rate through the column of 2 ml per minute. One hundred and twenty consecutive ten ml fractions were collected from the column with a fraction collector. Each fraction was assayed for PME by the cup-plate assay, and for PG by the viscosity reduction assay.

Six peaks of PG activity were obtained from the fractions collected from the column (Fig. 3). PG in the fractions of peak A caused such a rapid reduction in the viscosity of sodium polypectate

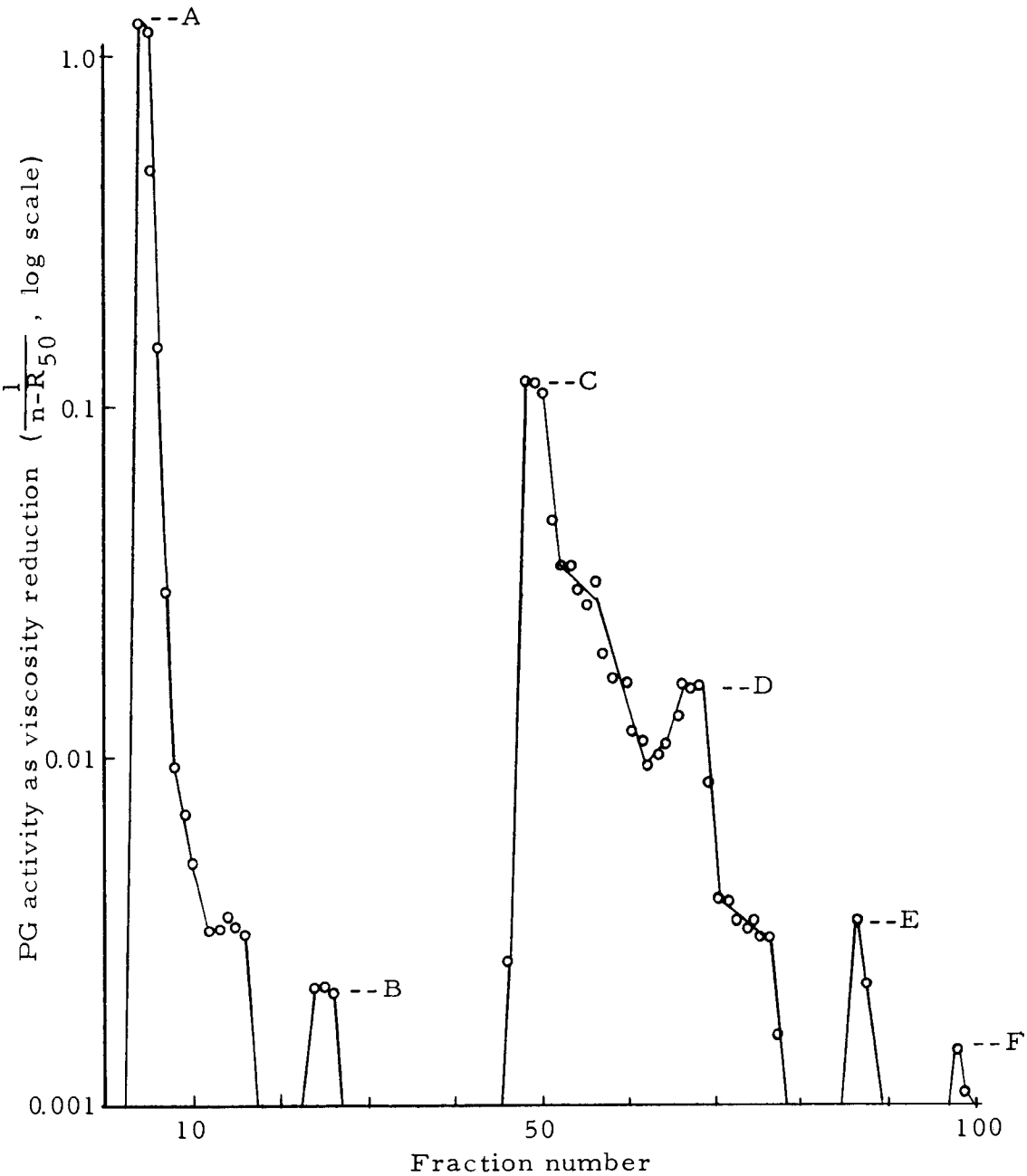

Figure 3. PG activity of enzyme fractions produced by V. albo-atrum and separated by column chromatography on DEAE-cellulose using a step-wise elution schedule.

that they were diluted. Consequently, when the dilution factor was multiplied by the reciprocal of the n-R_{50}, the reciprocal was greater than one.

Peaks A and C contain the most distinctive fractions. Peak B is probably a residual fraction of A separated by the change from water elution to NaCl at fraction number 20. Similarly, peaks D, E, and F are probably residuals of fraction C separated by the step-wise changes in NaCl concentration during elution.

PME was found in all fractions containing PG activity, and thus these enzymes were not separated by the step-wise elution of DEAE-cellulose columns.

The fractions in each peak were combined into six separate grouped fractions (Table 1). The letters designating the grouped fractions corresponded to the letters for the original peaks (Fig. 3) from which they came. Each grouped fraction was tested for PG activity by viscosity reduction and reducing group assays. Samples (50 µl) from the reaction mixtures used for the reducing group assay were taken 4, 8, 24, 48, 72, 96, 120, and 168 hours after initiation of the experiment and analyzed for the presence of pectic hydrolysis products by paper chromatography.

Viscosity reduction by the grouped fractions (Table 2) followed the pattern that would be expected from the activity of their

Table 1. Grouping of fractions from a DEAE-cellulose column used to separate PG enzymes of V. albo-atrum by three elution schedules.

Grouped fraction	Fraction Numbers		
	Step-wise elution	Gradient elution	Distilled water elution
A	3-5	3-5	3-5
B	24-26	29-32	85-87
C	48-51	46-50	93-98
D	66-68	-	-
E	86-88	-	-
F	97-98	-	-

Table 2. PG activity of grouped fractions obtained by the stepwise elution of a DEAE-cellulose column.

Group	Viscosity reduction[1]	Reducing groups released[2]	Ratio[3]
Crude culture filtrate	11.0	1.9	5.77
A	0.89	0.0383	23.4
B	0.0034	0.00082	4.15
C	0.02174	0.011	1.97
D	0.0128	0.0052	3.76
E	0.00084	0.00073	1.03
F	0.0008	0.00028	2.93

1 Viscosity reduction expressed as $\frac{1}{n\text{-}R_{50}}$.

2 Reducing groups expressed as μM galacturonic acid/ml enzyme/minute.

3 $\frac{1}{n\text{-}R_{50}}$ divided by μM galacturonic acid/ml enzyme/minute.

component fractions (Fig. 3). The ability of these grouped fractions to liberate reducing groups was similar to the viscosity reduction pattern. For example, grouped fraction A was the most effective in reducing viscosity, and liberated reducing groups more rapidly than grouped fractions C or D. To gain a better understanding of the mode of PG action, the ratios of viscosity reduction to reducing group liberation were computed for these grouped fractions.

The ratios formed a series from high viscosity reducing power relative to liberation of reducing groups (e.g. A) to groups with low viscosity reduction relative to reducing group liberation (e.g. C and E). These ratios indicate that the fractions contain varying proportions of at least two enzymes: an endo-type PG that cleaves pectic chains into relatively large fragments by a random mechanism of hydrolysis (i.e. high viscosity reduction relative to reducing group production); and either an exo-type PG that cleaves pectic chains primarily by hydrolysis of the terminal galacturonic acid groups or an endo-PG that preferentially cleaves relatively small fragments from the pectic chains (i.e. high reducing group activity relative to viscosity reduction). A large amount of hydrolysis by an exo-PG or an endo-PG cleaving small units from the pectic chains would eventually lead to a reduction in

viscosity. If only one enzyme were present, the ratios for the grouped fractions should be similar. However, these assays are interrelated in that endo-PG action which rapidly reduces viscosity slowly releases reducing groups, and terminal hydrolysis by an exo-PG eventually reduces the viscosity of the substrate. These assays by themselves cannot be used to separate endo- from exo-type activity.

Additional evidence on the mode of action of PGs can be obtained by analyzing the sequence of hydrolysis products released. This was accomplished by paper chromatographic analysis as described earlier.

Grouped fraction A released pectic fragments, tetra-, tri-, and digalacturonic acid during the first four hours of incubation (Table 3). No monogalacturonic acid was detected at this time. These results are consistent with the classification of this fraction as an endo-PG based on the relatively high ratio of viscosity reduction action to reducing groups released. An endo-type PG would initially produce pectic fragments before monogalacturonic acid. Through continued action, however, the fragments should be hydrolyzed to monogalacturonic acid, and this occurred within 120 hours.

The chromatographic data obtained for grouped fractions C

Table 3. Chromatographic analysis of hydrolysis products of sodium polypectate incubated with PGs obtained by column chromatography of enzymes produced by V. albo-atrum.

Source of enzyme and products of hydrolysis	Products of enzymatic hydrolysis produced after varying periods of incubation (hrs.)[1]							
	4	8	24	48	72	96	120	168
A. Monogalacturonic Acid	0	1	3	4	4	4	4	4
Digalacturonic Acid	1	1	2	2	1	3	0	0
Trigalacturonic Acid	2	2	3	2	2	0	0	0
Tetragalacturonic Acid	1	0	1	0	0	0	0	0
Pectic Fragments	3	0	0	0	0	0	0	0
C. Monogalacturonic Acid	0	0	0	1	2	3	3	3
Digalacturonic Acid	0	0	0	0	0	2	1	1
Trigalacturonic Acid	0	0	0	0	1	2	3	2
Tetragalacturonic Acid	0	0	0	0	1	0	1	0
Pectic Fragments	0	0	1	1	1	3	3	0
D. Monogalacturonic Acid	0	0	0	0	2	2	2	3
Digalacturonic Acid	0	0	0	0	1	1	1	1
Trigalacturonic Acid	0	0	0	0	1	1	2	1
Tetragalacturonic Acid	0	0	0	0	0	0	0	0
Pectic Fragments	0	0	1	1	1	3	0	0

[1] The amount of each hydrolysis product was rated on the paper chromatogram according to the following scale:

0 - none
1 - trace
2 - slight amount
3 - moderate amount
4 - heavy

and D (Table 3) are consistent with the low ratios of viscosity reducing activity to reducing group liberation (Table 2) obtained for these fractions. The presence of pectic fragments and small polymers of galacturonic acid indicates that these fractions contain an endo-type PG and not an exo-type PG. Exo-PG would produce only monogalacturonic acid in the reaction mixtures. Monogalacturonic acid was present in the reaction mixture for grouped fractions C and D when only trace amounts of pectic fragments were produced. In grouped fraction A, however, monogalacturonic acid did not appear in the reaction mixture even when moderate amounts of pectic fragments were present. This suggests that grouped fractions C and D preferentially cleave small fragments from the pectic substrate while fraction A preferentially cleaves large fragments from pectic chains. This could account for the lower ratio of viscosity reduction to reducing group liberation for fractions C and D in comparison to grouped fraction A. Early production of monogalacturonic acid in relation to production of pectic fragments by fractions C and D would account for the relatively high activity in the reducing group assay.

The three grouped fractions (B, E, and F) with weak viscosity reduction and reducing group liberation activity produced no breakdown products detectable by paper chromatography during

168 hours incubation with the substrate.

Reliability of viscosity reduction and reducing group assays. -- The purpose of this phase of the study was to determine the limits of enzyme concentration and length of assay periods in the viscosity reduction and reducing group assays that would give a linear relationship with enzyme activity. Only when a linear relation is obtained can viscosity reduction be compared validly with release of reducing groups through calculation of ratios. At high enzyme concentrations the log of viscosity reduction and reducing groups released per unit time becomes non-linear because of substrate saturation. At low enzyme concentrations incubation times become too long to be practical.

A dilution series of concentrated culture filtrate was made and tested for viscosity reduction and release of reducing groups at 30°C. Reducing group determinations were made 4, 8, 24, 48, 72, and 96 hours after the enzyme was added to the substrate.

Enzyme concentration, and activity as judged by viscosity reduction were linearly related up to a reciprocal of the n-R_{50} of 0.1 (Fig. 4). Therefore, assays in which the reciprocal of the n-R_{50} exceeds 0.1 are not valid measurements of viscosity reducing ability and were not used to compute ratios with reducing group values.

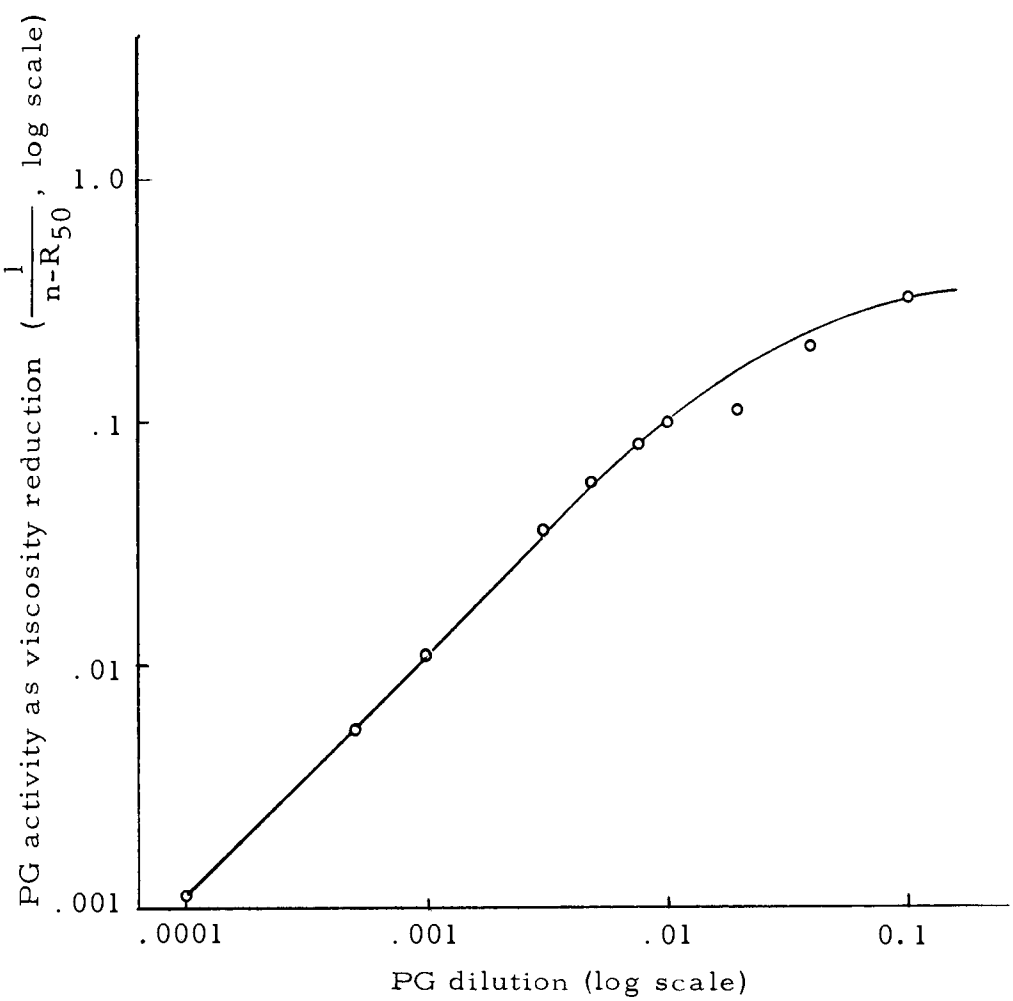

Figure 4. PG activity of various dilutions of crude culture filtrate of V. albo-atrum.

The liberation of reducing groups by the 0.01 dilution (Fig. 5) was so rapid that the reaction rate became non-linear in less than 24 hours. Therefore, although the viscosity reducing activity is a linear function at this enzyme concentration (Fig. 4), the enzyme is too active for a valid estimation of reducing group liberation after 24 hours. To obtain a reducing group value that is a linear function of time, the reaction mixture must be incubated for shorter periods of time or the enzyme diluted. The 0.0005 dilution does not give an accurate reducing group test until after 24 hours incubation. Intermediate enzyme concentrations were linear functions with activity between 8 and 48 hours incubation.

The ratios of viscosity reduction to reducing group liberation were calculated for the various dilutions of the culture filtrate incubated 4, 8, 24, 48, 72, and 96 hours with the substrate (Table 4). The ratios between 3.79 and 7.76 were accepted as valid. All ratios larger than 7.76 were unacceptable because they were calculated from values for reducing group liberation that had been obtained when reducing group liberation was no longer a linear function of time.

Enzyme separation by gradient elution of chromatographic columns. -- Although several PG fractions were obtained by stepwise elution of DEAE-cellulose columns, it was hoped that their

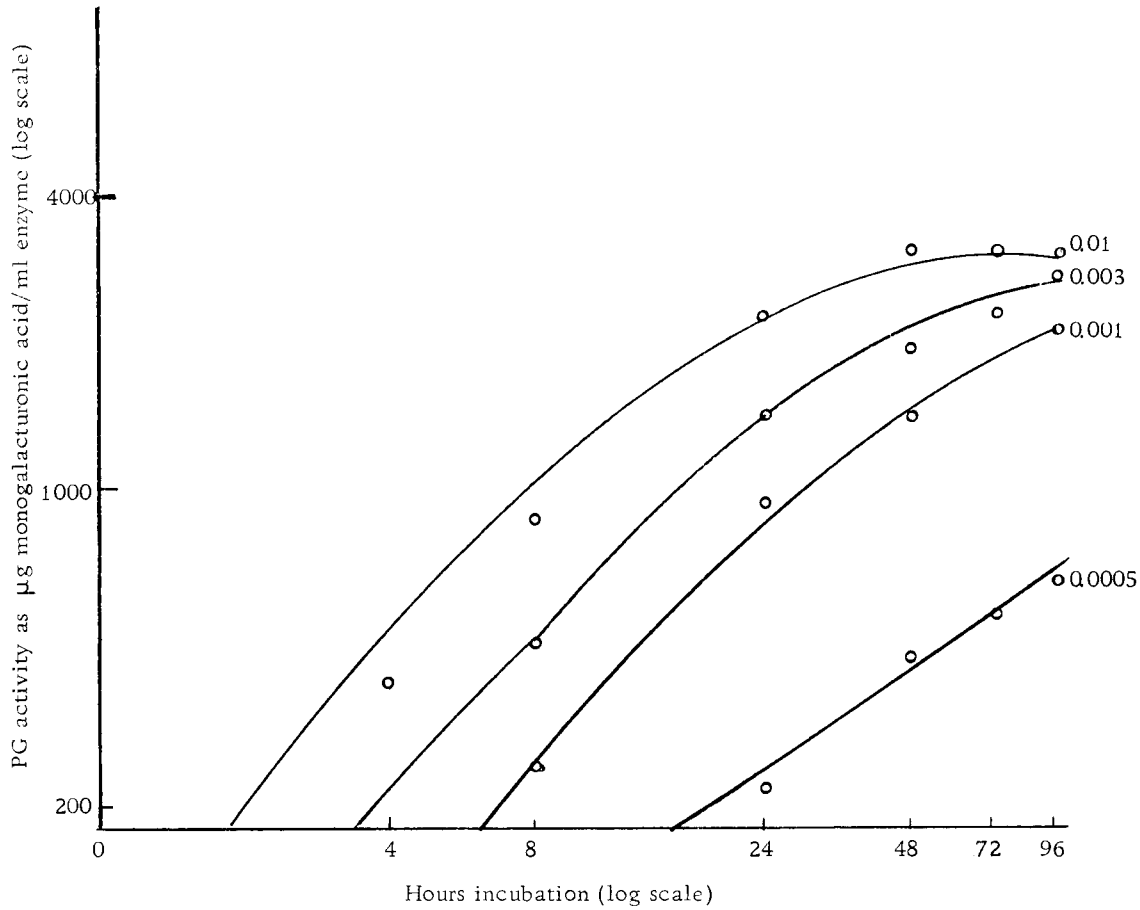

Figure 5. PG activity of various dilutions of crude culture filtrate of V. albo-atrum.

Table 4. PG activity of dilutions of crude culture filtrates of V. albo-atrum.

Dilution	Ratios of viscosity reduction[1] to production of reducing groups[2] at varying periods of incubation (hrs.)					
	4	8	24	48	72	96
0.01	10.58	13.74	17.23	23.65	29.80	39.10
0.003	**[3]	6.39	6.26	9.30	12.07	13.04
0.001	**	3.79	4.20	4.40	--[4]	5.79
0.0005	**	**	5.75	5.75	7.07	7.76

[1] Viscosity reduction expressed as $\frac{1}{n-R_{50}}$.

[2] Reducing groups expressed as μM of galacturonic acid/ml enzyme/minute.

[3] No reducing group data available to compute the ratio.

[4] No determination made.

separation could be improved by a gradient elution. Gradient elution tends to give narrower and higher peaks than the step-wise elution, elutes substances of widely differing adsorption properties in a single operation, and gives more easily interpreted results because there are no residual peaks due to abrupt concentration change (18, p. 67).

The gradient elution mixing chamber and reservoir used in this study were similar to those described by Palmer (33). Distilled water (600 ml) was placed in the mixing chamber and 0.4 M NaCl (600 ml) was placed in the reservoir. The enzyme was eluted from the column using 2 psi air pressure, to obtain a flow rate of 2 ml per minute. One hundred twenty, 10 ml fractions were collected using a fraction collector. The final concentration of NaCl in the elution mixture was 0.38 M. All fractions were tested for PME and PG activity by the respective cup-plate methods. PG activity was also determined in the viscosity reduction assay.

It was hoped that gradient elution might provide a better separation of PG from PME, but just as in the step-wise elution, all fractions containing PG activity contained at least a trace of PME activity. The PG cup-plate method indicated the presence of two PG peaks from this elution.

Three peaks of PG activity were obtained by the viscosity

reduction assay (Fig. 6), whereas step-wise elution had given six peaks (Fig. 3). This suggests that some residual peaks were obtained by the step-wise elution (i.e. some fractions were not completely eluted from the column before the next higher molarity of NaCl was added).

To determine if the fractions obtained by gradient elution were similar to those obtained by step-wise elution, the fractions that made up each of the three peaks were combined into grouped fractions (Table 1). Labeling of the grouped fractions corresponded to the designations of the original peaks.

Grouped fractions were assayed for PG activity by the viscosity reduction and reducing group liberation assays. Ratios of viscosity reduction to liberation of reducing groups were obtained for the grouped fractions. Only values for the reciprocal of the $n\text{-}R_{50}$ and reducing group liberation were used that fell within the previously mentioned acceptable range for these two tests. Chromatographic determinations of hydrolysis products were made from the reaction mixture used for the reducing group tests. Samples were withdrawn and chromatographed 4, 8, 24, 48, 72, 96, and 120 hours after the incubation period was initiated.

Viscosity reduction and release of reducing groups by the grouped fractions (Table 5) followed the pattern that would be

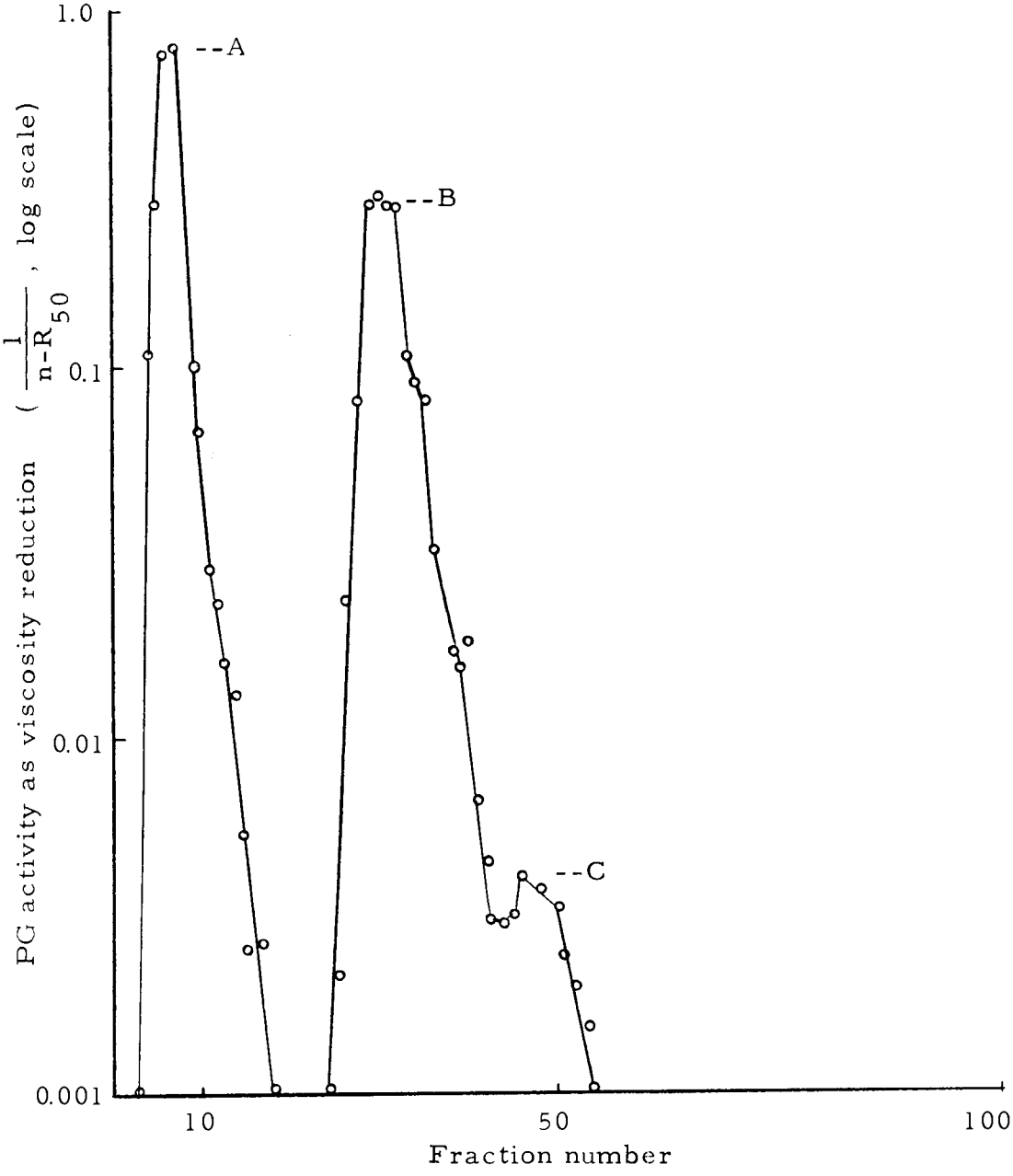

Figure 6. PG activity of enzyme fractions separated by column chromatography on DEAE-cellulose using a gradient elution.

Table 5. PG activity of grouped enzyme fractions obtained by gradient elution of a DEAE-cellulose column.

Group	Viscosity reduction[1]	Reducing groups released[2]	Ratio[3]
Crude culture filtrate	11.0	1.9	5.77
A	0.6088	0.033	18.3
B	0.091	0.017	5.35
C	0.00098	---[4]	---

[1] PG activity expressed as viscosity reduction $\left[\frac{1}{n\text{-}R_{50}}\right]$.

[2] PG activity expressed as μM galacturonic acid/ml enzyme/minute.

[3] $\frac{1}{n\text{-}R_{50}}$ divided by μM galacturonic acid/ml enzyme/minute.

[4] No activity.

expected from the action of their component fractions. Ratios of viscosity reduction to reducing group liberation were computed from these values.

Group A had a relatively high ratio of viscosity reduction to reducing group liberation while the ratio for fraction B was relatively low. No ratio could be obtained for grouped fraction C because no reducing group liberation could be detected. These ratios suggest that group A is predominantly an endo-PG that cleaves the substrate into large fragments. The relatively low ratio for grouped fraction B suggests that this fraction contains an endo-PG that initially cleaves pectic substances into small fragments. Grouped fraction B is probably similar to grouped fractions C and D of the step-wise elution.

The chromatography results with fraction A further suggests that this fraction contains an endo-PG that initially cleaves the substrate into large fragments (Table 6). The pectic fragments are soon hydrolyzed to di- and monogalacturonic acid. The hydrolytic products produced by fraction B are not consistent with the classification of this fraction by the ratio of viscosity reduction to reducing group liberation. Production of large quantities of pectic fragments suggested that the grouped fraction hydrolyzed the substrate into large fragments similar to grouped fraction A.

Table 6. Chromatographic analysis of hydrolysis products of sodium polypectate incubated with PGs obtained by column chromatography of enzymes produced by V. albo-atrum.

Source of enzyme and products of hydrolysis	Products of enzymatic hydrolysis produced after varying periods of incubation (hrs.)[1]						
	4	8	24	48	72	96	120
A. Monogalacturonic Acid	1	2	2	3	3	4	4
Digalacturonic Acid	1	1	1	2	1	2	2
Trigalacturonic Acid	1	2	2	2	2	2	0
Tetragalacturonic Acid	0	1	0	0	0	0	0
Pectic Fragments	1	1	0	0	0	0	0
B. Monogalacturonic Acid	0	0	2	2	2	3	4
Digalacturonic Acid	0	0	0	1	1	1	1
Trigalacturonic Acid	0	0	2	2	2	2	2
Tetragalacturonic Acid	0	0	0	1	0	0	0
Pectic Fragments	1	4	1	0	0	0	0

[1] The amount of each hydrolysis product was rated on the paper chromatogram according to the following scale:

0 - none
1 - trace
2 - slight amount
3 - moderate amount
4 - heavy

Grouped fraction A of the step-wise elution (Table 2) was similar to grouped fraction A of the gradient elution. However, the step-wise elution separated fractions that contained an endo-PG that produced small pectic fragments (fractions C and D). This type of PG was not isolated in the gradient elution. In both elutions the endo-PG that cleaved the substrate to large fragments was weakly adsorbed on the DEAE-cellulose columns. It was eluted from the column primarily by water. The endo-PG that cleaved the substrate to small fragments was adsorbed more strongly and required NaCl to elute it from the column.

A DEAE-cellulose column was exhaustively washed with distilled water to determine if all the endo-type PG that produced large pectic fragments could be removed from the column, and thus, separated from the other PG. Water elution (800 ml) was followed by elution with 0.4 M NaCl (200 ml) to elute the endo-PG that cleaves small pectic fragments from the substrate. One hundred and ten, 10 ml fractions were collected using a fraction collector. The fractions were tested for PME and PG by the cup-plate methods, and PG was also tested by the viscosity reduction assay.

PME was in all fractions containing PG as determined by the cup-plate assays.

Three peaks of PG activity were obtained in the viscosity reduction assay (Fig. 7). Peak A, containing high PG activity, was obtained immediately after the void volume of the column was released. This peak corresponded to the A peaks in the step-wise and gradient elutions. Two additional peaks of PG activity were obtained when the NaCl solution was added to the column. The first of these (Peak B), reduced the viscosity of the substrate very rapidly while the other (Peak C) had very weak PG activity.

The fractions from these peaks were grouped (Table 1), and labeled to correspond to the designations of the original peaks. The grouped fractions were then tested for PG activity by viscosity reduction and reducing group liberation assays. Hydrolysis products of sodium polypectate were analyzed by paper chromatography.

Viscosity reduction by the grouped fractions (Table 7) followed the pattern that would be expected from the activity of their component fractions. Reducing group liberation by the grouped fractions followed a similar pattern.

Ratios of viscosity reduction to reducing group liberation were computed for these grouped fractions, and they were compared with the results of paper chromatographic analysis of the hydrolytic products produced by these enzyme fractions.

Hydrolysis products obtained from the reaction mixture of

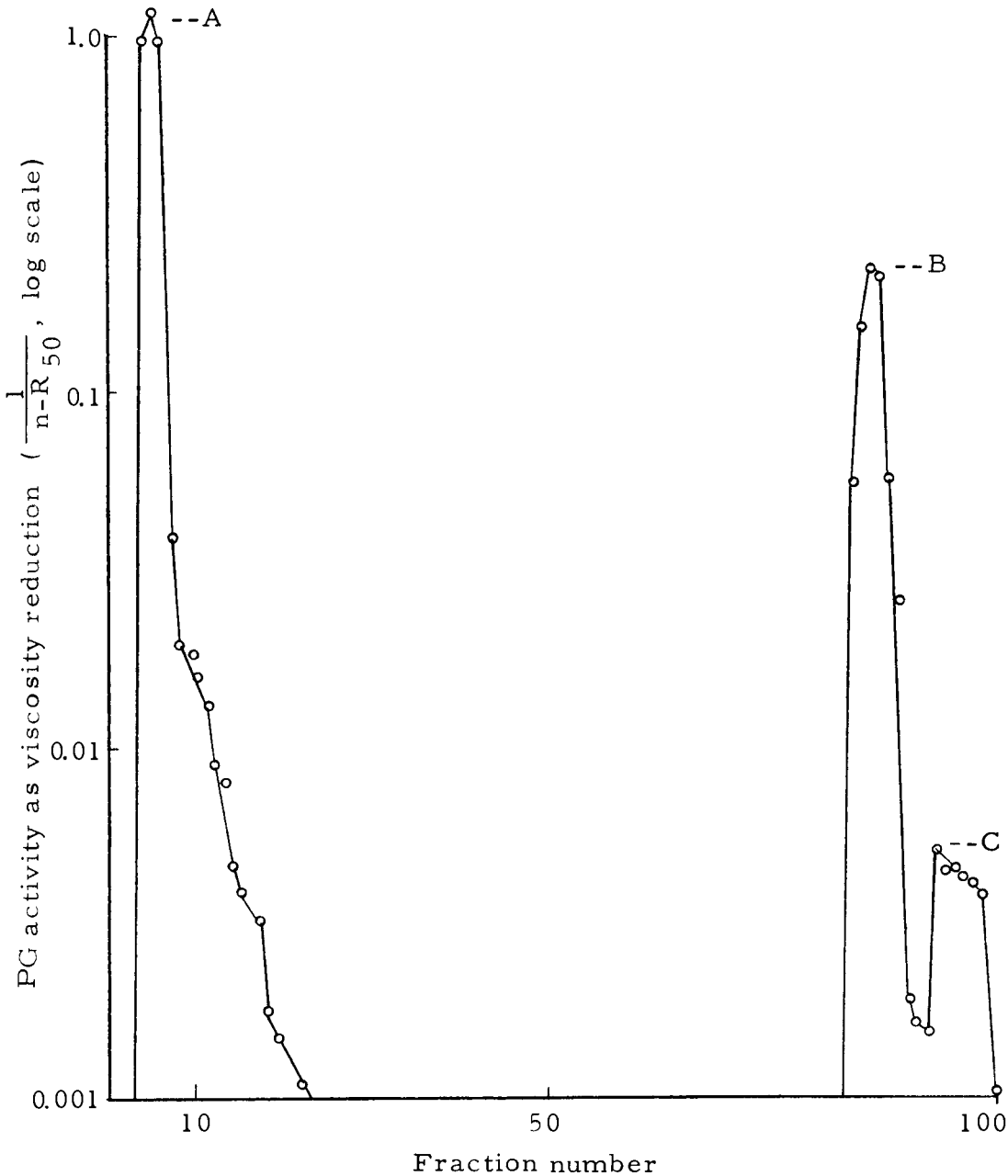

Figure 7. PG activity of enzyme fractions separate by column chromatography on DEAE-cellulose using a water and NaCl elution.

Table 7. PG activity of grouped enzyme fractions obtained by column chromatography on DEAE-cellulose eluted with distilled water and NaCl.

Group	Viscosity reduction[1]	Reducing groups released[2]	Ratio[3]
Crude culture filtrate	11.0	1.9	5.77
A	1.15	0.059	19.62
B	0.13	0.038	3.42
C	0.0036	0.002	1.8

1 PG activity expressed as viscosity reduction $\left[\frac{1}{n\text{-}R_{50}}\right]$.

2 PG activity expressed as μM galacturonic acid/ml enzyme/minute.

3 $\frac{1}{n\text{-}R_{50}}$ divided by μM galacturonic acid/ml enzyme/minute.

grouped fraction A (Table 8) where similar to those obtained from the "A" peaks in the step-wise and gradient elutions. The relatively high ratio of viscosity reduction to reducing group release for the A fraction (Table 7) was also similar to the values obtained for the A fractions in the other elution schedules. Elution of this enzyme with water gives a satisfactory separation of it from the other PG.

The low ratio of viscosity reduction to reducing group liberation (Table 7) and the preferential cleavage of the substrate into small fragments (Table 8) suggest that grouped fraction B corresponds to fractions C and D of the step-wise elution. There is little relation between this grouped fraction and those obtained in the gradient elution.

Grouped fraction C had a low ratio of viscosity reduction to reducing group liberation (Table 7) and was similar to grouped fractions E and F of the step-wise elution. However, as with grouped fractions E and F of the step-wise elution, no hydrolysis products could be detected by paper chromatography of this fraction due to its low activity.

Table 8. Chromatographic analysis of hydrolysis products of sodium polypectate incubated with PGs obtained by column chromatography of enzymes produced by V. albo-atrum.

Source of enzyme and products of hydrolysis	Products of enzymatic hydrolysis produced after varying periods of incubation (hrs.)[1]						
	4	8	24	48	72	96	120
A. Monogalacturonic Acid	1	3	3	4	4	4	4
Digalacturonic Acid	2	2	2	3	3	2	2
Trigalacturonic Acid	2	3	3	4	3	1	1
Tetragalacturonic Acid	1	1	0	0	0	0	0
Pectic Fragments	1	1	0	0	0	0	0
B. Monogalacturonic Acid	1	1	2	3	4	4	4
Digalacturonic Acid	0	2	2	2	2	1	0
Trigalacturonic Acid	0	1	2	2	1	1	0
Tetragalacturonic Acid	0	1	1	0	0	0	0
Pectic Fragments	4	3	0	0	0	0	0

[1] The amounts of each hydrolysis product was rated on the paper chromatogram according to the following scale:

0 - none
1 - trace
2 - slight amount
3 - moderate amount
4 - heavy

PRODUCTION OF POLYGALACTURONASES UNDER VARYING ENVIRONMENTAL CONDITIONS

Studies were undertaken to determine the influence of varying environmental conditions on the production of PGs by Verticillium. Cultures that were usually grown at room temperature, on a shaker with 12 hours of diffuse light (standard conditions) were grown under various environmental conditions (Table 9). Six days after inoculation, the cultures were harvested in the usual manner. A ten ml sample was withdrawn aseptically from each culture and pipetted into a pre-weighed beaker. The beakers containing the bud-cells were placed in an oven at 100°C for 24 hours, and dry weights of the bud-cells were subsequently determined.

Culture filtrates were concentrated to one-tenth their original volume in the usual manner, and toluene was added (1 ml/l). Concentrated filtrates were stored at - 4°C until used.

For separation of the PGs, a step-wise elution of the enzymes from DEAE-cellulose columns was carried out using increasing concentrations of NaCl, as previously described. One hundred-twenty, 10 ml fractions were collected with a fraction collector. All fractions were analyzed for PG activity by the viscosity reduction assay.

The peaks of PG activity for the culture filtrates produced

Table 9. Growth of V. albo-atrum under various environmental conditions.

Environmental conditions	pH of culture filtrate at harvest	Dry weight of bud-cells[1]
On shaker at 22°C under 16 hours of fluorescent light (925 foot candles) alternated with 8 hours dark.	5.25	147.0
On shaker at 22°C in total darkness	7.5	122.6
Still culture, at 22°C under diffuse light	5.0	125.0
On shaker at 20°C under diffuse light	4.8	117.8
On shaker at 30°C under diffuse light	4.4	133.3
On shaker at 22°C under diffuse light, (standard conditions)	8.0	135.0

[1] Dry weight is expressed as mg/10 ml of culture filtrate.

under the various environmental conditions were similar to those obtained by growth under the standard conditions (Fig. 3), except at 30°C only a trace of PG was produced. The fractions in peaks corresponding to A, C, and D were combined as described earlier, so that three fractions were obtained for the enzymes produced under each of the environmental conditions. The grouped fractions were analyzed for PG activity by viscosity reduction and reducing group assays. Hydrolysis products of pectate decomposition were analyzed by paper chromatography.

Fungal growth was used as the basis for comparing PG production by Verticillium grown under the various environmental conditions. Verticillium grew well under all environmental conditions, but significantly less growth was obtained at 20°C (Table 9). The pH of the medium prior to inoculation ranged from 4.6 to 5.0, and when the cells were collected the pH was measured again to determine its influence on fungal growth and PG production.

No correlation was found between final pH of the medium and the growth of the fungus (Table 9). For example, when Verticillium was grown under standard conditions, the medium had a final pH of 8.0. Verticillium grew as well in the standard culture as it did at 30°C where the pH of the medium was 4.4 when the cells were collected.

The grouped fractions from culture filtrates of Verticillium grown at 30°C had no detectable PG. However, the fungus must have been able to utilize the pectate because it grew well at this temperature. Concentrated culture filtrates from Verticillium grown at 20°C and 30°C were compared for PG activity (Table 10). Production of PG was supressed at 30°C. This suggests that very little PG is required to hydrolyze sufficient pectate to units that can be used by Verticillium as a carbon source. Acid hydrolysis of the substrate at pH 4.4 was ruled out as a possible source of pectic hydrolytic products (21, p. 136).

Table 10. PG activity of concentrated culture filtrates from cultures grown at 20°C and 30°C.

Temperature	PG activity	
	Viscosity reduction [1]	Reducing groups released [2]
20°C	6.1	0.032
30°C	0.001	---[3]

[1] PG activity expressed as viscosity reduction $\left[\frac{1}{n\text{-}R_{50}}\right]$.

[2] PG activity expressed as μM of galacturonic acid/ml enzyme/minute.

[3] No activity after 48 hours incubation.

Grouped fractions A, C, and D from culture filtrates of the fungus grown at 20°C reduced viscosity and liberated reducing groups from the substrate at approximately the same rate as grouped fractions A, C, and D from culture filtrates of Verticillium grown under standard conditions.

Grouped fraction A of the cultures grown in continuous dark had the most active PG when analyzed by viscosity reduction and reducing group liberation assays (Table 11). PG preparations from cultures subjected to 16 hours light (925 foot candles) alternated with eight hours of dark, and the cultures grown under standard conditions were similar in ability to reduce viscosity of a pectic substrate. The A fraction from cultures subjected to continuous dark was about 55 percent more active than the corresponding fraction from the cultures grown under standard conditions.

In ability to liberate reducing groups, fraction A of the enzyme produced in the dark was about three times as effective as the corresponding fraction produced under standard conditions or under 16 hours light alternated with eight hours dark. Grouped fraction C from the cultures grown under standard conditions produced significantly more reducing groups than the C groups from any of the other conditions. No significant differences were found

Table 11. Effect of various environmental conditions on the PG activity of grouped enzyme fractions.

Environmental conditions	Fraction designation[1]	Viscosity reduction[2]	Reducing groups released[3]	Ratio[4]
On shaker at 22°C under 16 hours of flourescent light (925 foot candles) alternated with 8 hours dark	A	0.605	0.0313	19.3
	C	0.014	0.0074	1.94
	D	0.008	0.0023	3.76
On shaker at 22°C in total darkness	A	1.06	0.046	23.04
	C	0.011	0.0051	2.06
	D	0.034	0.0073	4.72
Still culture, at 22°C under diffuse light	A	0.034	0.0027	12.64
	C	0.0019	0.0018	0.975
	D	0.0018	0.00054	3.43
On shaker at 22°C under diffuse light (standard conditions)	A	0.65	0.033	20.0
	C	0.017	0.017	1.0
	D	0.0088	0.0023	3.96

[1] Fraction designations are the same as the letters used in Table 1 for the step-wise elution.

[2] PG activity expressed as viscosity reduction $\left[\frac{1}{n-R_{50}}\right]$.

[3] PG activity expressed as μM of monogalacturonic acid/100 mg dry wt/minute.

[4] Ratio is expressed as $\frac{1}{n-R_{50}}$ divided by the reducing groups released expressed as micromoles of monogalacturonic acid/100 mg dry wt/minute.

in the reduction of viscosity by the C groups. Grouped fraction D obtained from cultures grown in continuous dark released reducing groups and reduced the viscosity of a pectic substrate more readily than the corresponding fractions from the cultures grown under standard conditions or in 16 hours light alternated with eight hours dark. The group D fraction from the culture filtrate of Verticillium grown under standard conditions was about the same as the group D of the culture filtrate of the fungus grown under 16 hours light.

These data suggest that when Verticillium is grown in continuous dark more PG is produced of the types in fractions A and D than if the cultures are illuminated.

When the PGs produced by cultures grown under standard conditions are compared with those produced in still cultures (Table 11), it becomes apparent that agitation of the culture causes a significant increase in the production of PG in all fractions.

As previously suggested, the low PG activity of fractions from still cultures can be explained by low metabolic activity of the fungus in still culture compared to the high metabolic activity in the shake cultures.

The ratio of viscosity reduction to reducing group liberation was obtained for grouped fractions A, C, and D from all

environmental conditions. Only grouped fraction A from the culture filtrate of the fungus grown in still culture was considered significantly different from the results previously described for the fractions in A, C, and D of the step-wise elution. All figures used to obtain these ratios fell within the previously described limits for these assays.

Grouped fraction A of the still culture had a significantly lower ratio than grouped fraction A from the cultures grown under standard conditions. This suggests that growing Verticillium as a still culture causes the production of an endo-PG that can not hydrolyze the substrate into fragments that are as large as those obtained from the hydrolysis of the substrate by grouped fraction A of the culture grown under standard conditions.

The grouped fractions for each environmental condition were incubated with sodium polypectate. Samples for paper chromatography were taken after 4, 24, 48, 72, 96, and 120 hours incubation. Pectic decomposition products were identified and quantatively rated as described earlier.

The sequence and pattern of pectic decomposition products in all treatments was similar to those obtained from the enzyme produced under standard conditions (Table 2) with two exceptions. No di-, tri-, or tetragalacturonic acids were found in the reaction

mixture of the grouped fraction D from the cultures grown in continuous dark (Table 12). Monogalacturonic acid was present in the reaction mixture at 24 hours. The absence of di-, tri-, and tetragalacturonic acids suggest that this peak contained an endo-PG that rapidly hydrolyzed the trace amounts of pectic fragments in the reaction mixture to monogalacturonic acid. It also suggests that the activity of this grouped fraction is greater than the activity usually associated with grouped D fractions.

The second exception was the results obtained for groups A, C, and D from the still culture. Although the PG of grouped fraction A was weaker than the PGs of the other A fractions (Table 11), pectic fragments were obtained from the reaction mixture four hours after the enzyme was added to the substrate. The fragments were slowly broken down to monogalacturonic acid. At the end of 120 hours hydrolysis, trigalacturonic acid accumulated. This suggests that trigalacturonic acid is not readily hydrolyzed by this enzyme, and substantiates the previous suggestion that the endo-PG of this grouped fraction preferentially hydrolyzes the substrate to relatively large fragments.

Grouped fraction C from the still culture produced monogalacturonic acid in the reaction mixture at the end of 4 hours. Pectic fragments appeared in the reaction mixture at 24 hours.

Table 12. Chromatographic analysis of hydrolysis products of sodium polypectate incubated with PGs obtained by column chromatography of enzymes produced by V. albo-atrum grown as a still culture.

Source[1] of enzyme and products of hydrolysis	Products of enzymatic hydrolysis produced after varying periods of incubation (hrs.)[2]					
	4	24	48	72	96	120
Still culture						
A. Monogalacturonic Acid	0	0	1	1	1	1
Digalacturonic Acid	0	1	1	1	0	0
Trigalacturonic Acid	0	2	3	4	4	4
Tetragalacturonic Acid	1	2	2	1	0	0
Pectic Fragments	2	1	1	0	0	0
C. Monogalacturonic Acid	1	1	1	1	1	1
Digalacturonic Acid	0	0	0	0	0	0
Trigalacturonic Acid	0	0	0	0	0	0
Tetragalacturonic Acid	0	0	0	0	0	0
Pectic Fragments	0	1	2	2	2	2
D. Monogalacturonic Acid	0	0	0	0	0	0
Digalacturonic Acid	0	0	0	0	0	0
Trigalacturonic Acid	0	0	0	0	0	0
Tetragalacturonic Acid	0	0	0	0	0	0
Pectic Fragments	0	0	0	1	1	2
Continuous dark						
D. Monogalacturonic Acid	0	2	2	3	4	4
Digalacturonic Acid	0	0	0	0	0	0
Trigalacturonic Acid	0	0	0	0	0	0
Tetragalacturonic Acid	0	0	0	0	0	0
Pectic Fragments	0	0	1	0	0	0

[1] The letters used corresponded to the letters in Table 1 for these fractions.

[2] The amount of each hydrolysis product was rated on the paper chromatogram according to the following scale:

0 - none
1 - trace
2 - slight amount
3 - moderate amount
4 - heavy

Di-, tri-, and tetragalacturonic acid were never detected. The accumulation of monogalacturonic acid before pectic fragments were detected is what would be expected from the ratio of viscosity reduction to reducing group liberation (Table 11). The ratio was low indicating an enzyme that hydrolyzes small fragments from the substrate.

The ratio of viscosity reduction to reducing group liberation had previously indicated that group D from the still culture contained an enzyme that hydrolyzes the substrate into small units. However, the activity of the enzymes was low (Table 10). The appearance of only small amounts of pectic fragments in the reaction mixture at 120 hours incubation confirmed the weak activity of the enzyme (Table 12).

Three environmental conditions that are unrelated to the ability of Verticillium to grow in culture can influence the production of PG by V. albo-atrum. Still culture and incubation at 30°C are detrimental to the production of PGs by the fungus, while the absence of light seems to enhance the ability of Verticillium to produce PGs, particularly endo-PGs in peak D.

EXTRACTION AND PURIFICATION OF POLYGALACTURONASES FROM TOMATO STEMS INFECTED WITH VERTICILLIUM ALBO-ATRUM

The objective of this phase of the study was to characterize the PGs produced by V. albo-atrum in tomato plants. Inoculum for these experiments was obtained from cultures grown five days on a rotary shaker in flasks containing 200 ml of potato-dextrose broth. The flasks were removed from the shaker and combined aseptically. The bud-cells were removed from the medium by centrifuging at 6000 rpm for 20 minutes. The supernatant was discarded and the bud-cells were resuspended in distilled water to give a concentration of about 5×10^5 cells/ml.

Seeds of Bonny Best variety tomatoes were planted in 320 cans containing sandy-loam soil and were grown in a greenhouse at about 21°C. After four weeks the seedlings were thinned to five per can. Two weeks later the root systems of the seedlings were severed by cutting into the soil on one side of the stem at an angle of about 45° to the surface with a spatula. A cut was made about one inch from each stem in such a way that the spatula blade intersected the tap root of each seedling. The roots were severed to insure infection since bud-cells of Verticillium are readily taken into the plant through freshly cut roots. Two hundred-fifty ml of the bud-cell suspension were poured over the surface of the soil in

each can immediately after cutting the roots.

Four weeks after inoculation stems of diseased tomato plants were collected. At this time the fungus was confined to the xylem and general necrotic symptoms of the diseased plants had not developed. Stems of uninoculated healthy tomato plants of the same age were collected as controls.

The enzyme extraction procedure was initiated by washing the stems in tap-water, blotting, and recording their fresh weights. The fresh weight of the infected stems for a typical experiment was 13,800 g. Stem tissue of the controls weighed 2,470 g. The stems were then placed in plastic bags and quick-frozen. Frozen stems were cut into one-inch sections and homogenized in 0.05 M (pH 6.0) phosphate buffer in a Waring Blendor for three minutes. About two ml of buffer were used per gram fresh weight of stem tissue. The homogenized stem tissue was placed in a flour sack and the liquid was squeezed out of the fiber and discarded. The fiber was scraped into a pan and phosphate buffer (0.01 M) at pH 7.6 was added at the rate of 0.5 ml per gram original fresh weight. Sufficient NaCl was added to the fiber to obtain a final concentration of ten percent NaCl in the extraction mixture. This mixture was then held for two hours at 4-5°C. The mixture was filtered through a flour sack and the filtrate was centrifuged at

6000 rpm for 20 minutes. The supernatant was collected (8000 ml) and sufficient ascorbic acid was added to obtain a final concentration to 0.01 M. Toluene was added at a rate of 1 ml/l. The supernatant was dialyzed for 18 hours against running tap water to remove the NaCl. This was followed by dialysis against running distilled water for six hours. The dialysis tubing containing the crude enzyme preparation was then suspended in front of a fan at 22°C until the volume of the preparation was reduced to one-half by evaporation. The enzyme preparation was then checked for contamination by plating an aliquot in PDA. The remainder of the enzyme preparation was concentrated to one-tenth the original volume (800 ml) by flash evaporation at 30°C under partial vacuum.

Five ml of the enzyme preparation was incubated with 10 ml of sodium polypectate and samples were periodically tested for the presence of hydrolysis products by paper chromatography, and for reducing groups by the appropriate assay. PG activity was also determined by the viscosity reduction assay.

The enzyme preparation from diseased tomato plants had weak PG activity when tested by viscosity reduction and reducing group assays (Table 13). The low ratio of viscosity reduction to reducing group liberation suggests that the preparation contained an enzyme that produced relatively small pectic fragments on

Table 13. PG activity of crude and partially purified enzymes from tomato plants infected by V. albo-atrum.

Source of enzyme	PG activity		
	Reducing group assay[1]	Viscosity reduction assay[2]	Ratio[3]
Crude extract Inoculated Plants	0.0057	0.0008	0.14
Grouped fraction A	0.0021	0.00053	0.25
Grouped fraction D	---[4]	0.00015	---[5]

[1] PG activity expressed as μM of monogalacturonic acid/ml enzyme/minute.

[2] PG activity expressed as viscosity reduction $\left[\frac{1}{n-R_{50}}\right]$.

[3] Expressed as the ratio of $\frac{1}{n-R_{50}}$ to μM of monogalacturonic acid/ml enzyme/minute.

[4] No activity.

[5] Ratio could not be determined because of no activity in the reducing group assay.

hydrolysis. Paper chromatographic analysis (Table 14) demonstrated the presence of these fragments as well as mono-, di-, and trigalacturonic acid. Thus, this preparation probably contained predominantly an endo-type PG similar to those produced by the fungus in culture (grouped fraction C, Fig. 3). No PG activity was obtained from the extract of the controls.

Separation of the PGs produced in tomato plants was carried out on DEAE-cellulose columns eluted in a step-wise manner. Thirty ml of the crude enzyme were placed on the column (100 mg protein/g DEAE-cellulose) and eluted with NaCl as previously described. One hundred 10-ml fractions were collected with a fraction collector. All fractions were analyzed for PG activity by the cup-plate method.

Three fractions with PG activity were obtained, numbers 4, 5, and 68. Fractions 4 and 5 corresponded in position to peak A and fraction 68 corresponded to peak D of the step-wise elution (Fig. 3). Fractions 4 and 5 were combined and designated grouped fraction A. Fraction 68 was designated grouped fraction D. The grouped fractions were tested for PG activity by viscosity reduction and reducing group assays. Grouped fraction D had weak viscosity reducing ability (Table 13), and no reducing groups were detected after 72 hours incubation. Because of the weak

Table 14. Chromatographic analysis of hydrolysis products of sodium polypectate incubated with crude and partially purified pectic enzymes of V. albo-atrum obtained from infected tomato plants.

Source of enzyme and products of hydrolysis	Products of enzymatic hydrolysis produced after varying periods of incubation (hrs.)[1]						
	24	48	72	96	120	144	168
Crude enzyme from infected plants							
Monogalacturonic Acid	0	0	2	2	2	2	-[2]
Digalacturonic Acid	0	0	1	1	1	2	-
Trigalacturonic Acid	0	0	2	2	2	1	-
Tetragalacturonic Acid	0	0	0	0	0	0	-
Pectic Fragments	0	0	1	1	1	2	-
Grouped fraction A of a step-wise elution							
Monogalacturonic Acid	1	1	1	2	2	-	2
Digalacturonic Acid	0	0	0	0	0	-	0
Trigalacturonic Acid	0	0	0	0	0	-	0
Tetragalacturonic Acid	0	0	0	1	0	-	0
Pectic Fragments	1	1	2	2	2	-	3

[1] The amount of each hydrolysis product was rated on the paper chromatogram according to the following scale:

0 - none
1 - trace
2 - slight amount
3 - moderate amount
4 - heavy

[2] No determination made.

activity of grouped fraction D, no more tests were attempted.

The activity of grouped fraction A was extremely low when tested by viscosity reduction and reducing group assays (Table 13). The ratio of the reciprocal of the n-R_{50} to the production of reducing groups was similar to that of the crude plant enzyme, and suggested a grouped fraction that was an endo-PG that cleaved the substrate into small fragments.

A chromatographic analysis of the reaction mixture of fraction A was carried out to gain additional information on the mode of action of this enzyme preparation. Two ml of enzyme preparation were incubated with 2 ml of one percent sodium polypectate. One hundred µl aliquots were chromatogramed after 24, 48, 72, 96, 120, and 168 hours incubation.

The presence of pectic fragments and monogalacturonic acid further suggest that this enzyme fraction is an endo-PG that cleaves the substrate into small fragments.

These results coupled with the low ratio of viscosity reduction to reducing group liberation suggest that grouped fraction A obtained from infected tomato plants contains the same type of endo-PG found in grouped fraction C (Fig. 3) from the culture filtrate.

PRODUCTION OF PECTINTRANSELIMINASE BY VERTICILLIUM ALBO-ATRUM

Experiments were designed to determine if V. albo-atrum produced a transeliminase (PTE) type of pectic enzyme. One ml of enzyme to be assayed was incubated for 48 hours at 30°C with two ml of one percent sodium polypectate buffered at pH 5.0 with acetate buffer (0.1 M). After 48 hours, 0.2 ml of NaOH was added to 2 ml of the enzyme substrate mixture, and the mixture was allowed to stand for 30 minutes at room temperature. Then, 2.5 ml of 1 N HCl and 10 ml of 0.01 M thiobarbituric acid solution were added to the mixture (23). The volume of the mixture was brought to 16 ml with distilled water. The tubes containing the solution were heated for 30 minutes in a boiling water bath. When PTE were present, a deep red color would form, indicating the presence of unsaturated galacturonic acids. The color intensity was determined at 547 mμ and the optical density at this wavelength was an index of PTE activity.

A culture filtrate of Verticillium grown six days at 20°C was tested for PTE activity along with grouped fractions A and D (Table 6) from culture filtrates, and an enzyme preparation from infected Bonny Best tomato stems. Boiled enzyme was used as the control for all preparations tested.

Slight PTE activity was obtained in all enzyme preparations except those that had been boiled. The deepest color was obtained for Fraction A, however, even this intensity was too low for an optical density determination on the spectrophotometer.

Additional studies will be necessary before the significance of this enzyme can be determined in the Verticillium wilt syndrome. However, PTE is produced by V. albo-atrum in culture and in tomato plants, and the possibility exists that PTE may play a minor role in the wilt syndrome.

DISCUSSION

Analyses of culture filtrates obtained by column chromatography suggest that two endo-PGs are produced by V. albo-atrum. One of these preferentially cleaves large fragments from the substrate and slowly cleaves these fragments to monogalacturonic acid. This enzyme fits the classical description of an endopolygalacturonase (12). The second enzyme is an endo-type that preferentially hydrolyzes relatively small fragments from the pectic substrate and more rapidly produces monogalacturonic acid. Further characterization of these enzymes is required. For example, the ability of the enzymes to hydrolyze specific substrates (i.e. di-, tri-, and tetragalacturonic acids) should be determined.

Young (49) obtained two PGs from culture filtrates of F. oxysporum f. lycopersici, and classified them as endo- and exo-PGs. Young suggested that these enzymes were always associated with one another in varying proportions. However, it seems doubtful that they would be obtained as mixtures after adsorption and elution from DEAE-cellulose chromatography columns. Therefore, it would appear that the low ratios of viscosity reduction to reducing group liberation obtained by Young for some fractions, coupled with the presence of traces of polymers of

galacturonic acid in these fractions, are more readily interpreted as the action of an endo-PG that preferentially cleaves the substrate into relatively small fragments. The presence of monogalacturonic acid in these reaction mixtures can be explained by the random mechanism of hydrolysis of this enzyme.

The only other report of an endo-type PG that produces large fragments is from a study of the enzymes extracted from potato stems infected with V. albo-atrum (9). These workers classified this enzyme as a depolymerase. All other reports (7, 11, 38, 48) fail to classify these enzymes except to show that monogalacturonic acid is produced.

Culture filtrates of Verticillium grown at 30°C had extremely low PG activity. However, the fungus grew well at this temperature although sodium polypectate was the only carbon source in the medium. Possibly PG in very low amounts is capable of producing enough galacturonic acid to satisfy the carbon requirements of the fungus. Partially hydrolyzed pectic fragments could have diffused into the cells and there been broken down by an intracellular glycosidase similar to the one described by Nagel and Vaughn (30) for Bacillus polymyxa.

Enzyme extracts obtained from tomato plants infected with V. albo-atrum contained a PG that produced relatively small

fragments from the pectic substrate and rapidly produced monogalacturonic acid. No evidence was obtained for the endo-PG that produces relatively large fragments initially. However, the low titer of enzymes obtained from infected plants suggests that caution should be used in the interpretation of these results. Young (49) compared the PGs produced by F. oxysporum f. lycopersici, in culture and in infected tomato plants, and found they were similar in ability to reduce viscosity of the substrate and to release reducing groups in the pH range 4.5 to 5.5 Young concluded that the enzymes produced in culture and in infected tomato plants were similar, but his interpretations were based on crude enzyme preparations.

Bateman (3) observed differences in the thermal inactivation and reducing group liberation of PG produced by Rhizoctonia in infected beans and in culture. He concluded that the PGs produced by Rhizoctonia in culture and in infected plants were different.

The ability of the endo-PGs from culture filtrates and infected tomato plants to hydrolyze pectic substances to monogalacturonic acid suggests that large pectic fragments are not formed in the vessels. Therefore, the vessels are unobstructed to the passage of water and microconidia. Thus, the hypotheses advanced by Gothoskar et al. (17) and Beckman et al. (5) are not applicable

to Verticillium wilt. If these hypotheses (5, 17) can not be applied to the role of pectic enzymes in the Verticillium wilt syndrome, another explanation may be considered for the role of these enzymes. Young (49) proposed that PGs may act as a means for F. oxysporum f. lycopersici to obtain a carbon source from the host. This explanation is well suited to Verticillium wilt, particularly when one considers the low PG titer in diseased plants. However, if the poor production of PG at 30°C is compared to the decrease in disease symptoms when infected tomato plants are grown at 28°C (15), the production of large amounts of PG by the fungus in the host may be an important factor in the severity of the disease. If only small quantities of PG are produced, the pectic substances may be so slowly hydrolyzed that the severity of the disease is greatly reduced.

SUMMARY

1. More pectinmethylesterase (PME) is produced when V. albo-atrum is grown on pectin as the sole carbon source than when grown on sodium polypectate. PME is produced during the first few days of growth but rapidly diminishes and is absent in culture filtrates of either a pectin or pectate medium harvested eight days after inoculation of the medium.

2. Twenty times as much polygalacturonase (PG) was produced by Verticillium grown on sodium polypectate as the carbon source as when grown on pectin. Optimum PG production occurred 6-8 days after inoculation of the medium.

3. Three elution techniques of DEAE-cellulose columns were used to isolate the pectic enzymes produced by Verticillium. All fractions with PG activity contained PME.

4. Assays for PG activity in fractions separated by column chromatography of crude culture filtrates suggested that Verticillium produced at least two PG enzymes: 1) an endo-PG that preferentially cleaves the substrate into relatively large fragments, and 2) an endo-PG that preferentially hydrolyzes small fragments from the substrate.

5. Fractions that were high in endo-PG that hydrolyzed the substrate to large fragments gave ratios of viscosity reduction to reducing group liberation at least five times as high as the ratios for endo-PGs that cleaved the substrate into small fragments.

6. Filtrates from still cultures contained less PG than cultures grown on a shaker although the fungus grew equally well in each case. The weak endo-PG of the first peak from the step-wise elution of the culture filtrate of a still culture hydrolyzed trigalacturonic acid slowly.

7. Concentrated culture filtrates obtained from cultures grown at 30°C contained very little PG although the fungus grew well at this temperature.

8. PGs produced by V. albo-atrum in culture and infected tomato plants were similar. However, more enzyme that produced small pectic fragments was obtained from infected tomato plants.

9. Evidence was obtained that Verticillium produces trace amounts of pectintranseliminase in culture and in infected tomato stems.

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