AN ABSTRACT OF THE THESIS OF

CHARLES HARRY BALDWIN, JR. for the Doctor of Philosophy
(Name) (Degree)

in PLANT PATHOLOGY presented on August 14, 1969
(Major) (Date)

Title: PECTOLYTIC ENZYME PRODUCTION BY *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI*

Abstract approved: Redacted for Privacy
Dr. Malcolm E. Corden

This investigation was undertaken to determine 1) the effects of various carbon sources in defined media on pectolytic enzyme production, 2) the effect of the incubation period on pectolytic enzyme activity, and 3) the development of improved methods for the purification and characterization of the polygalacturonases (PG) produced by *Fusarium oxysporum* f. sp. *lycopersici*.

Optimum PG and pectinesterase (PE) production on pectin medium occurred in nine days while 11 days were required for optimum pectin methyl trans-eliminase (PMTE) synthesis. PMTE was the predominant enzyme synthesized on polygalacturonic acid (PGA) with trace amounts of PG and PE. Optimum PMTE synthesis occurred in five days, while PG and PE activity decreased after three days of incubation. Only trace amounts of PG and PMTE were detected in cultures in which glucose was the sole carbon source, and PE production was greater on glucose than on PGA. Polygalacturonate

trans-eliminase was not detected in any of the cultures.

The action of these enzymes was determined on purified pectic substrates, using improved viscosity reduction and reducing group assays, as well as thin layer chromatography (TLC) procedures for the detection of hydrolysis products. Only endo-PG and endo-PMTE activity was present in the pectin, PGA, and glucose culture filtrates. The results obtained from the TLC of the PG-reaction-mixtures indicated a preferential release of mono-galacturonic acid from PGA, which suggest exo-PG activity. However, this enzyme probably was not an exo-PG because only a small number of the total α-1, 4 linkages were hydrolyzed at relatively high levels of viscosity reduction.

About 90% of the PG activity in a pectin medium culture filtrate was adsorbed on CM-cellulose resulting in a 24-34 fold increase in specific activity. Most of the PE activity was similarly adsorbed on CM-cellulose, but the endo-PMTE was not adsorbed. All the pectolytic enzyme activity in the PGA culture filtrates was lost following dialysis against 0.01M acetate buffer (pH 4.0). Most of the endo-PG in the filtrates from glucose cultures was not adsorbed on CM-cellulose.

An enzyme isolated from the glucose cultures hydrolyzed sodium polypectate reducing its viscosity but yielding an unidentified aldohexose as the major hydrolytic product. Thus, suggesting that this enzyme may have been misclassified in the past as a PG, but it is

probably more closely related to the hemicellulases.

The chromatographic patterns obtained by gel filtration and ion-exchange chromatography of the PG produced on pectin, PGA, and glucose were correlated with the amount of carbohydrate present in the enzyme fractions. The size of the enzyme units and the number of peaks produced on Sephadex G-75 and CM- and DEAE-cellulose columns increased with increasing carbohydrate concentration. Dialyzed culture filtrate and the fraction not adsorbed on CM-cellulose produced more peaks on the Sephadex G-75 and ion-exchange columns than did the fraction adsorbed on CM-cellulose.

Pectolytic Enzyme Production by *Fusarium oxysporum* f. sp. *lycopersici*

by

Charles Harry Baldwin, Jr.

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1970

APPROVED:

Redacted for Privacy

Professor of Botany and Plant Pathology
in charge of major

Redacted for Privacy

Head of Department of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented August 14, 1969

Typed by Donna L. Olson for Charles Harry Baldwin, Jr.

ACKNOWLEDGEMENT

The author would like to express his gratitude to Dr. Malcolm E. Corden for this opportunity to continue my education and for his guidance and constructive criticism throughout the course of this investigation. I would also like to extend my sincerest appreciation to Dr. Norman I Bishop for his assistance in the preparation of this manuscript. My gratitude is also extended to Dr. Thomas C. Allen, Dr. Donald L. MacDonald , and Dr. Roy O. Morris for serving on my committee. My sincerest thanks are extended to Mrs. Helen Gehring, Mrs. Cynthia Dort, Miss Judith Gonzolves, and Miss Heather Evans for their invaluable assistance. The financial assistance of a Public Health Service traineeship is gratefully acknowledged.

I would also like to express my sincerest thanks to my wife, Mary Katherine, and my family for without their encouragement, patience, and understanding this work could not have been completed.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	3
Pectic Enzymes	3
Role of Pectolytic Enzymes in Plant Diseases	5
Pectic Enzyme Production by *Fusarium oxysporum* f. sp. *lycopersici*	7
Enzyme Purification	10
Methods of Characterization	12
MATERIALS AND METHODS	17
Fungal Growth	17
Partial Purification and Concentration of Pectolytic Enzymes	18
Ion Exchange Column Chromatography	19
Sephadex G-75 Filtration	20
Development of the Reducing Group Assay	20
Pectic Substances and Assay Preparations	20
Procedure for the Alkaline-Copper Reducing Group Assay	21
Procedure for the DNS Reducing Group Assay	22
The Effects of Deproteinization	24
The DNS vs. the Modified Alkaline-Copper Reducing Group Assays	26
Enzyme Assays	29
Preparation of Specific Reaction-Mixtures	29
Cup Plate Assays	30
Viscosity Reduction	30
Reducing Group Release	31
TLC of Reaction-Mixtures	31
Carbohydrate Determination	32
Protein Determination	32
RESULTS	33
Production of Pectolytic Enzymes	33
Growth and pH	33

Page

Polygalacturonase	35
Pectin Methyl Trans-Eliminase	37
Pectin Esterase	38
Protein and Carbohydrate	38
Partial Purification	39
Characterization of the Pectolytic Enzymes	42
Mode of Action of the Major Fractions	42
TLC of Enzyme-Reaction-Mixtures	47
Column Chromatography of PG	49
DISCUSSION	59
SUMMARY	67
BIBLIOGRAPHY	70

LIST OF FIGURES

Figure		Page
1.	The effects of $Ba(OH)_2$ on galacturonic acid, pectic substances, and enzyme reaction-mixtures.	25
2.	The effectiveness of the DNS method in measuring the reducing groups of pectic substances.	27
3.	The growth and culture pH of *Fusarium oxysporum* f. sp. *lycopersici* growing on shake culture medium.	34
4.	The effects of partial purification on the pectolytic enzymes synthesized by *F. oxysporum* f. sp. *lycopersici* during the pectin, PGA, and glucose time course experiments.	36
5.	Characterization of the polygalacturonases obtained from the pectin and glucose time course experiments by reverse flow Sephadex G-75 column chromatograhy.	50
6.	Characterization of the polygalacturonases obtained from the pectin and glucose time course experiments by gradient elution ion-exchange column chromatography	55

LIST OF TABLES

Table		Page
1.	Effect of dialysis against water or buffer on polygalacturonase activity in the culture filtrates of F. oxysporum f. sp. lycopersici.	40
2.	The mode of action of polygalacturonase from cultures of F. oxysporum f. sp. lycopersici growing on a pectin medium.	43
3.	The mode of action of PMTE in the culture filtrates of F. oxysporum f. sp. lycopersici growing on PGA and pectin media.	44
4.	The mode of action of the pectolytic enzymes produced by F. oxysporum f. sp. lycopersici growing on a glucose medium.	46
5.	Differential viscosity reduction of pectic substrates by pectolytic enzymes produced in pectin and glucose cultures by F. oxysporum f. sp. lycopersici.	46

PECTOLYTIC ENZYME PRODUCTION BY *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI*

INTRODUCTION

The role of pectolytic enzymes in plant pathogenesis and, particularly the vascular wilts, has been the subject of intensive investigation over the past decade. One of these vascular diseases is *Fusarium* wilt of tomato induced by *Fusarium oxysporum* f. sp. *lycopersici*. In recent reviews, Bateman and Millar (13), Dimond (29), and Wood (98, p. 122-187 and 326-354) have elucidated our present knowledge concerning the physiology of the vascular wilts and implied a role for the pectic enzymes in disease development. Of particular interest are those pectic enzymes responsible for tissue degradation which appear to be polygalacturonases (Poly-α-1, 4 galacturonide glycanohydrolase E. C. 3.2.1.15) (PG).

To fully understand the role of these pectolytic enzymes in wilt diseases, we must first isolate and characterize the various types of pectic enzymes the pathogen produces *in vitro* and *in vivo*. There are a number of reports (58, 68, 70, 74, 79, 92, 101) on some of the factors affecting the synthesis and characterization of PG enzymes produced by this fungus *in vitro*, but there appeared to be considerable variability in the amount of enzyme produced, the

effects of various carbohydrates on synthesis and/or repression of PG, and the methods used for the characterization of the secreted enzyme.

This investigation was undertaken with the expressed purpose of 1) determining the effects of various pectic substances on pectolytic enzyme production, 2) elucidating the types of pectolytic enzymes produced over a period of 17 days on various carbon sources, and 3) developing methods for the characterization and purification of PG produced by Fusarium oxysporum f. sp. lycopersici.

LITERATURE REVIEW

Pectic Enzymes

The literature dealing with pectic enzymes in plant pathology was reviewed recently by Bateman and Millar (13), Dimond (29), and Wood (98, p. 122-187 and 326-354). There are many reports of various types of pectolytic enzymes and Bateman and Millar (13) have included the following enzymes under this general heading: pectinesterase (PE), formerly referred to as pectin methylesterase (PME); polygalacturonase (PG); polymethylgalacturonase (PMG); pectin methyl trans-eliminase (PMTE); and polygalacturonate trans-eliminase (PATE). This classification is based in part on the one presented by Demain and Phaff (28).

PE is characterized by its ability to hydrolyze the methoxyl group on carbon atom No. 6 of the galacturonic acid unit in the pectin or pectinic acid polymer. This enzyme may be isolated from a wide variety of higher plants and is produced by many microorganisms. The pH requirements for PE from higher plants varies from pH 4.0 to 8.0 with an optimum of approximately pH 6.0 (48, p. 360-374), while the pH optimum for PE from microbial origin is generally between 4.5 and 5.0. Activity of PE isolated from higher plants is stimulated by divalent cations while the enzyme from fungi is much less affected (48, p. 360-374). Demethylation of pectins by PE

makes them susceptible to the action of PG and PATE.

The remainder of the pectolytic enzymes degrade pectic substances by cleaving the α-1, 4 linkages between the galacturonic acid units by either a hydrolytic or trans-eliminative mechanism. PMG preferentially hydrolyzes pectin and pectinic acids by either a random (endo) or terminal (exo) mode of action. The PMG requires the presence of both methoxyl and carboxyl groups on the No. 6 carbon of adjacent galacturonic acid units (28). PMG enzymes can be classified further on the basis of their pH optima and the degree to which they hydrolyze the substrate.

The PMTE enzymes degrade pectin by a trans-eliminative mechanism whereby the proton on the No. 5 carbon atom of the methoxylated galacturonic acid unit is removed and a double bond inserted between the No. 4 and No. 5 carbon atoms (3). PMTE enzymes normally have pH optima between 8.0 and 9.0 and are usually stimulated by the addition of low levels of Ca^{++} (13). The action of these enzymes may be terminal or random. These enzymes are produced by phytopathogenic and saprophytic fungi, as well as higher plants (2, 13).

The PATE enzymes preferentially hydrolyze demethylated pectic substances by trans-eliminative action producing unsaturated products (86). They have pH optima between 8.0 to 9.0 and are generally stimulated by relatively low levels of Ca^{++}. PATE action

may be by an endo or exo mechanism. These enzymes have been isolated from a wide variety of phytopathogenic fungi and bacteria, as well as, saprophytic and symbiotic bacteria (13).

The most important pectolytic enzymes in plant diseases are the polygalacturonases. These enzymes are characterized by the preferential hydrolysis of the α-1, 4 linkages of demethylated pectic substances (pectic and polygalacturonic acids) and generally exhibit pH optimum between 3.5 and 4.5. The action of these enzymes may be either by an endo or exo mechanism. PG enzymes are distinguishable from the PATE enzymes on the basis of their pH optima and cation stimulation. PG enzymes are inhibited by Ca^{++} and other divalent cations by action on the substrate (23) or by reaction with the enzyme (63, 83). However, Mg^{+} stimulates the hydrolytic degradation of sodium polypectate by these enzymes (23). PG enzymes have been isolated from higher plants, as well as being produced *in vitro* and *in vivo* by saprophytic and phytopathogenic fungi and bacteria (13, 98, p. 164-167).

The Role of Pectolytic Enzymes in Plant Diseases

Although the PE enzymes are not capable of degrading the α-1, 4 linkages of the pectic materials present in the middle lamella and primary cell walls of plants, they may play a role in plant diseases by demethylating native plant pectins which then may be further degraded by PG and PATE enzymes. At one time PE

enzymes were believed to induce vascular browning which occurs in the vascular wilt diseases (29), but this phenomenon has since been associated with the action of polyphenol oxidases (29). The source of the PE isolated from diseased plants has been a subject of study since both the host and pathogen are capable of synthesizing this enzyme, and it increases at the infection site in plant diseases (13). Increased PE activity is accomplished by accumulation of cations which may release PE attached to the plant cell wall (13). However, this elution of enzyme is insufficient to explain fully the increased activity in diseased tissue and some of the increase must be due to the presence of PE produced by the pathogen (13, 29).

Based on our present knowledge, PG, PMG, PMTE, and PATE enzymes appear to function in penetration of plants by various pathogens (13), in formation of vascular pectic plugs in the wilt diseases (13, 29, 98, p. 326-354), in the intercellular-ramification of pathogens (13, 98, p. 122-153), and as a toxic substance causing cell death (13, 29). The importance of these enzymes in soft and dry rots of plant storage organs is well documented, but there is still some doubt as to the extent to which the individual pectolytic enzymes are involved in tissue maceration since little work has been reported with purified enzymes (13, 29, 98, p. 140-148).

Our understanding of the role of pectolytic enzymes in vascular wilt diseases is similarly handicapped by the lack of studies with

purified enzyme preparations. Although these enzymes, primarily PG, have been implicated in the production of pectic plugs in the xylem vessels, there is little evidence to document their relation to disease development and attempts to isolate these chain splitting pectolytic enzymes from the xylem sap of diseased plants have been unsuccessful (29). However, Young (101, p. 61-80) successfully isolated a PG from stem tissue of Fusarium infected tomato plants. The enzyme had properties similar to PG produced by *F. oxysporum* f. sp. *lycopersici in vitro*. The exact role of PG, PMG, PMTE, and PATE enzymes in the wilt disease syndrome will not be determined until the individual enzymes have been purified, the current enzyme assay procedures improved, and the substrates used in enzyme assays sufficiently purified.

Pectic Enzyme Production by *Fusarium oxysporum* f. sp. *lycopersici*

Waggoner and Dimond (92) reported the first extensive study on the production of pectic enzymes by *F. oxysporum* f. sp. *lycopersici*. They found that PG and PE were produced when *F. oxysporum* f. sp. *lycopersici* was grown on a defined medium containing pectin and NH_4NO_3 as the carbon and nitrogen sources,

respectively. However, when glucose was the carbon source, PE was the only pectic enzyme produced. The data of Paquin and Columbe (68) corroborated these results, and they pointed out that PE production on glucose was dependent on the temperature and pH of the growth medium. Later, Mussell and Green (58) found no PG in glucose cultures of F. oxysporum f. sp. lycopersici and Patil and Dimond (70, 74) reported maximum PG production occurred when this fungus was cultured on a defined medium containing pectin, but PG production was repressed by the addition of glucose, galacturonic acid, and 2-deoxy-D-glucose to this medium, while sorbitol and galactitol inhibited PG production. However, in Patil and Dimond's reports (70, 74), it was noted that on a glucose medium PG production was greater than that recorded for the other sugar and sugar alcohol media.

These discrepancies may have been the result of the enzyme assay and purification procedures used by these workers. There are several factors which could result in the loss of PG activity. Gupta and Rautela (37) demonstrated that dialysis against distilled water resulted in a 85% loss of PG activity and the majority of the PG purification procedures reported have included a water dialysis step (4, 7, 44, 46, 47, 56, 74, 87-89, 90, 92, 101, p. 21-22). This could limit the detection of PG activity if the initial concentration of

the enzyme was low. Another factor which could have prevented the detection of PG would be the use of only a single assay procedure such as viscosity reduction or reducing group liberations (43, 66, 70-74, 81, 87) that favor endo and exo activity, respectively. There have also been reports (4, 43, 44, 46, 47, 56, 66, 70-74, 81, 97) where substrates such as sodium polypectate (NaPP) containing some nonuronide material, were used in the enzyme-reaction-mixtures for the detection of specific pectolytic enzymes. Gremli and Neukom (34) have recently isolated an α-L-arabinofuranosidase which hydrolyzes pectin and NaPP. Therefore, it is possible that the enzyme produced on glucose (4, 8, 58, 72), with activity measured on NaPP, may not be a PG. This would also apply to the reports by Bateman (10) and Winstead and McCombs (97) where glucose was released during the enzymatic hydrolysis of NaPP.

Similar discrepancies as those noted for production of PG on glucose exist with other carbon sources. Sherwood (79) reported that _F. oxysporum_ f. sp. _lycopersici_ produced more PMTE on pectin than on NaPP while the reverse was found for PG production. However, Young (101, p. 78-80) failed to detect any appreciable amount of PMTE when the same fungus was grown on a synthetic medium containing pectin and NH_4NO_3 as the sole carbon and nitrogen sources respectively, but PG production was favored under these conditions. In

addition. Mussell and Green (58) found that optimum PG production by *F. oxysporum* f. sp. *lycopersici* occurred on PGA. Although Mussell and Green (58) gave no information on their assay procedures for detecting pectolytic enzyme activity, it would appear that their PG may have been a PATE or PMTE.

Enzyme Purification

There have been relatively few attempts to purify the pectolytic enzymes produced by *F. oxysporum* f. sp. *lycopersici*. Waggoner and Dimond (92) dialyzed their culture filtrates against running tap and distilled water for four and 12 hours, respectively. Following dialysis, they adsorbed and then eluted the pectolytic enzymes from celite. Patil and Dimond (74) dialyzed their culture filtrate against deionized water overnight at 3-4°. The purification procedure employed by Swinburne and Corden (89) involved dialysis against running tap water for 18 hours and running distilled water for eight hours, concentration by flash evaporation at 30°, and freezing. In addition, Young (101, p. 21-22) attempted to purify the pectolytic enzymes produced by *F. oxysporum* f. sp. *lycopersici* by dialyzing the culture filtrate against running tap and distilled water for 16 and four hours, respectively, at room temperature. He then concentrated

the enzyme by flash evaporation at 30° and either precipitated the enzyme with ethanol or chromatographed the concentrated fraction on DEAE-cellulose. However, all of these procedures failed to effectively separate PG from the PE enzymes. In addition these researchers failed to use purified substrates in their enzyme reaction-mixtures, and this could have resulted in the detection of enzymes other than PG but which were designated as PG.

Improved purification procedures of pectolytic enzymes isolated from higher plants and produced *in vitro* by other fungi and bacteria have been reported. The methods which have been utilized are: precipitation of the enzymes in the culture filtrates or dialyzed culture filtrates with acetone (37, 57, 60, 88), methanol (99, 100), ethanol (37, 84), and $(NH_4)_2SO_4$ (14, 31, 47, 82). Another technique employed a combination of gel filtration (Sephadex G-75 and G-200) and CM-Sephadex or CM-cellulose column chromatography (8, 18, respectively). It seems that the most satisfactory purification scheme was recently reported by Rexova-Benkova and Slezarik (76-78). Their procedure was as follows: 1) precipitation with $(NH_4)_2SO_4$, 2) reprecipitation with cold ethanol, 3) precipitate solubilized and lyophilized, 4) Sephadex G-25 column chromatography and lyophilization of the protein fractions, and 5) column

chromatography on DEAE-cellulose at pH 7.0 in $NaHPO_4$ buffer by gradual elution with buffer containing an increasing concentration of salt. Five peaks of activity were recovered from the column. Peak C contained the PG, peak D the PME, and peak E the PMG. This purification sequence enabled these workers to determine the molecular weight and amino acid content of the extracellular endo-PG produced by *Aspergillus niger*.

Methods of Characterization

There have been various assays developed to measure pectolytic enzyme activity. The cup plate assay of Dingle *et al.* (30) is based on the ability of the enzyme to hydrolyze NaPP as the enzyme diffuses through an agar medium. The activity is detected by an opaque zone formed by the precipitation of the hydrolyzed substrate molecules with 2N HCl. A similar cup plate assay was developed by Mann (54) to detect PE activity. For the PE assay, pectin is used as the substrate and a pH indicator is added to the medium; thus, enabling a visual estimation of enzymatic activity since the hydrolysis results in the formation of a carboxyl group and a concomittant lowering of the pH and color change of the surrounding medium. However, the data obtained by these assays are not quantitative unless standard incubation time and temperature conditions are maintained at all times.

Two additional assay procedures have been used for measurement of activity and characterization of pectolytic enzymes. These assays depend on the ability of the enzyme to break the α-1, 4 clycosidic linkages of pectic substances with a resultant reduction in viscosity and the release of reducing groups. The substrates generally used in the viscosity reduction assay has been NaPP for PG (8, 9, 44, 46, 47, 51, 56, 66-68, 75-79, 81, 88, 89, 92, 101, p. 22-24) and pectin for PMG (85). However, Rexova-Benkova and Slezarik (76-78) used pectic acid for PG and pectinic acid for PMG in their viscosity reduction and reducing group assays. The viscosity reduction of a reaction-mixture has been determined by measuring the time required for a measured amount of the reaction-mixture to flow through a capillary tube (92) or by directly reading the viscosity change in centipose from the dial of a rotating spindle apparatus (80).

Three assays have been used to determine the reducing groups released from pectic substances by PG, PMG, PATE, and PMTE, and these are the hypoiodite or iodometric titration procedure (45), the dinitrosalicylic acid (DNS) assay (55), and Somogyi's alkaline-copper colorimetric assay (83). The DNS (55) assay procedure was used by several workers (8, 24-27, 38-41, 44, 46, 47, 56, 75-78, 82, 88) while Somogyi's (83) assay has been used in this laboratory (53, 101, p. 22-24) and in several others (39, 59, 69). In this

connection, Whistler and Wolfrom (95) pointed out that Somogyi's (83) assay was the most sensitive of the available reducing group assays.

The thiobarbituric acid (TBA) colorimetric assay developed by Neukom (64) and later modified by Ayers *et al.* (7) has been used to measure PATE, PMTE, and PG activity by measuring the absorbancies of the samples at wavelengths of 540 mμ (PATE and PMTE) and 515 mμ (PG), respectively. Another technique which was developed by Albersheim *et al.* (2, 3) measures PMTE and PATE activity by determining the increase in absorbancy of the unsaturated 4-5 bonds in an enzyme-reaction-mixture at wavelengths of 230 and 235 mμ, respectively.

Various combinations of solvent systems and chromagenic reagents have been used for determining the hydrolytic products in the reaction-mixture by paper and thin layer chromatography (TLC). The sodium formate-formic acid-water system of Page (65) appeared to be the best system of separation of the uronic acids while the pyridine-ethylacetate-acetic acid-water system of Block *et al.* (16, p. 204-205) resulted in the maximum separation of other compounds from the uronic acids. Furthermore, some of the chromogenic reagents used by previous workers, such as ammonical $AgNO_3$ (8, 9, 57), aniline (12, 76, 82), and brom phenol blue (6, 12, 53, 57, 88, 101, p. 24-28), are not quantitative since they do not

differentiate the uronic acids from other possible products in the reaction-mixture. However, the CD-1 (2-aminobiphenyl) chromogenic reagent developed by Gordon et al. (33) differentiated between the aldopentoses, aldohexoses, uronic acids, aminosugars, and other carbohydrates.

Two additional techniques used to characterize pectolytic enzymes involves their behavior during gel filtration and ion-exchange column chromatography. The gel filtration technique has been used as a procedure for purification (8, 17-19, 42, 76-78, 82, 99), molecular weight estimations (1, 5, 8, 78, 88, 89), and homogeneity determinations (88, 89).

Anion (DEAE-cellulose) and cation (CM-cellulose, Duolite CS-101, CM-Sephadex, Cellulose-phosphate, and Ecteola-cellulose) exchangers have been used for the purification and characterization of pectolytic enzymes. Young (101, p. 34-61) isolated five peaks containing pectolytic enzyme activity from a DEAE column of the enzymes produced by F. oxysporum f. lycopersici. The majority of the enzymatic activity (PG) was eluted in the void volume, but Young failed to separate PE from PG by this method. Swinburne and Corden (89) obtained similar results with the Fusarium enzyme on DEAE-cellulose but when this enzyme was chromatographed stepwise on Duolite CS-101, three peaks resulted with only 0.7% of the enzyme coming off at salt concentrations of 0.01M and 0.1M, while the

majority of the PG was eluted with 1M sodium acetate. On re-chromatography, the major Duolite peak resulted in the production of a single positive peak indicating that it was homogenous. However, chromatography of the second Duolite peak on DEAE resulted in the production of two peaks (98%, +, 2%, -) which were similar to the peaks produced by the original sample. Swinburne and Corden suggested that the formation of isoenzymes or complexing of the enzyme with other molecules (substrate) was responsible for this anomalous behavior during ion exchange chromatography.

MATERIALS AND METHODS

Fungal Growth

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) Snyder and Hans. strain R 5-6 was grown in a liquid medium on a rotary shaker at 25° with 260 ft-c of artificial light on a 16-hour day. One liter of the liquid medium contained: 5.0g NH_4NO_3, 2.5g KH_2PO_4, 1.0g $MgSO_4 \cdot 7HOH$, 40mg $FeCl_3 \cdot 6HOH$, 20mg $ZnSO_4 \cdot 7HOH$, and 10g of either glucose, pectin N.F. (Sunkist product no. 3442), or polygalacturonic acid (Sunkist product no. 3491). The pH of the medium was adjusted to 5.0 with HCl, autoclaved and then 200 ml of the medium in 500 ml Erlenmeyer flasks were inoculated with 7.0 mm discs cut from the margin of actively growing *Fusarium* colonies on potatoe-dextrose-agar. After 3, 5, 7, 9, 11, and 17 days of incubation, 800 ml of culture fluid was collected, the pH was measured, and fungal growth was estimated by determining the spore concentration using a standard dilution plate assay.

A sterile culture filtrate for enzyme assays was obtained by centrifuging the liquid culture at 7,500 x g to remove the fungal cells, then filtering the supernatant fluid through a Millipore HA filter (0.45 μ). Sodium azide was added to a final concentration of 0.02% (w/v) to preserve the culture fluid; at this concentration the azide had no effect on pectolytic enzyme activity.

Partial Purification and Concentration of Pectolytic Enzymes

Step 1. The culture filtrate in 1.0 l batches was dialyzed for 48 hours against three changes of 10 l of 0.01M acetate buffer (pH 4.0). The dialyzate was passed through a Millipore filter (0.45 μ) and sodium azide was added.

Step 2. The filtrate from Step 1 was added in 400-500 ml batches to 40g of CM-cellulose (Cellex CM, Bio Rad Laboratories) in 500 ml of 0.01M acetate buffer (pH 4.0). The resulting slurry was filtered, the CM-cellulose was washed with two batches of the acetate buffer totaling 750 ml, and the filtrates were combined. The pectolytic activity in this fraction was termed "Enz^-" on the basis of its behavior on the cellulose ion exchanger.

Step 3. The CM-cellulose from Step 2 was eluted with three batches totaling 1750 ml of 0.01M acetate buffer (pH 4.0) containing 1.0M KCl/liter. The enzymatic activity in this fraction was designated "Enz^+".

Step 4. The Enz^- and Enz^+ fractions were passed through a Millipore filter (0.45 μ) and the Enz^+ fraction was dialyzed for 48 hours against three changes of 10 l of 0.01M acetate buffer (pH 4.0) to remove the KCl.

Step 5. The Enz^- and Enz^+ fractions from Step 4 were concentrated 10-fold in a Diaflo ultrafiltration cell under 100 psi of He using

a UM-2 membrane with a molecular weight cutoff of 1000. The concentrated fractions then were passed through a Millipore filter (0.45 μ) and sodium azide was added.

Ion Exchange Column Chromatography

Enzyme samples (10 ml) were applied to DEAE-cellulose (0.84 meq/g, coarse, Sigma Chemical Co.) and CM-cellulose columns (1.5 cm x 30 cm) equilibrated with 0.01M acetate buffer (pH 5.6 and 4.0) respectively. A linear gradient from 0 to 0.62M NaCl in 0.01M acetate buffer (pH 5.6) was applied to the DEAE-cellulose, and the CM-cellulose was eluted with a linear gradient from 0 to 1.25M KCl in 0.01M acetate buffer (pH 4.0). The flow rates of the DEAE- and CM-cellulose columns were 2.5 ml and 4.8 ml/min respectively. Ten ml fractions were collected from both columns and the pectolytic enzyme activity was measured by the cup-plate method.

The DEAE-cellulose was regenerated with 1.0 N NaOH, washed with distilled water, pH adjusted to 5.6 with 2.0 N Acetic acid, and washed repeatedly with 0.01M acetate buffer (pH 5.6). CM-cellulose was regenerated with 1.0 N HCl, washed with distilled water, pH adjusted to 4.0 with 1.0 N NaOH, and washed repeatedly with 0.01M acetate buffer (pH 4.0).

Sephadex G-75 Filtration

Sephadex G-75 was packed into columns (2.5 cm x 30 cm) adapted for reverse flow and equilibrated with 0.05M acetate buffer (pH 5.0) containing 0.02% sodium azide. Enzyme samples (5 ml) were placed on the columns and the buffer was allowed to flow at a rate of 1.0 ml/min. Five ml fractions were collected and the pectolytic enzyme activity was measured by the cup-plate method.

Development of the Reducing Group Assay

Pectic Substances and Assay Preparations

The pectic substances used in these experiments were the same as described before with the exception of pectic acid-PA (Nutritional Biochemical Co., Cont. No. 1962). A series of dilutions with proportional amounts of the pectic compounds were prepared from 1% or 2% (w/v) stock solutions for the polyuronides and a 350 μg/2 ml galacturonic acid monohydrate stock solution.

In experiments with polygalacturonase, the substrates were prepared in 0.1 M acetate buffer (pH 5.0). The dilution of the reaction-mixture by the addition of 1 ml of crude fungal PG or other solutions [$Ba(OH)_2$ and $ZnSO_4$] was accounted for by the deletion of buffer, and thus the substrate concentration remained the same in all experiments.

Procedure for the Alkaline-Copper Reducing Group Assay

The reagents used in the modified alkaline-copper technique for reducing group determination were prepared according to the method of Somogyi (83). The alkaline-copper reagent was prepared before each experiment by mixing four parts of solution I with one part of solution II. Two ml of this alkaline-copper reagent were pipetted into 16 X 125 mm screw cap test tubes containing 2 ml of the pectic substance being assayed. The tubes were capped, solutions mixed, and then placed in a constant level boiling water bath and heated for five minutes. A 2000 watt electric hot plate was used as the heat source for the water bath because any hot plate smaller than this did not supply enough heat to maintain continuous boiling when the tubes were placed in the bath or while the bath was refilling after the sample tubes had been removed. Following boiling, the tubes were cooled under running tap water.

After cooling, 2 ml of Nelson's (63) arsenomolybdate chromogenic reagent was added to each tube and the tubes shaken until effervescence ceased. The samples were poured into large test tubes and diluted to 25 ml with distilled water. These diluted samples were then thoroughly mixed on a vibra-mix and filtered through Whatman No. 4 filter paper into a clean set of large test tubes. The filtered solutions were again thoroughly mixed on a vibra-mix and

their absorbancies determined at 540 mμ.

Since the original alkaline-copper method was designed for blood sugar determinations, 0.3 N $Ba(OH)_2$ solution was added as a deproteinizing agent (63, 83) as protein interferes with the reducing group measurements. To prevent any interference in color development by the presence of excess Ba^{++}, $ZnSO_4$ (5%) was added to precipitate the excess Ba^{++}. The proportion of $Ba(OH)_2$ and $ZnSO_4$ used in these experiments was 12.5% of the total volume of the test samples or 2.5 ml of each solution per tube. In those experiments which involved the deproteinization of pectolytic enzyme-reaction-mixture, 1 ml of the enzyme (PG) was added to the appropriate substrate, mixed for 20 seconds on a vibra-mix, and then the $Ba(OH)_2$ was added to each of the samples. The samples were again thoroughly mixed, the $ZnSO_4$ (2.5 ml) solution was added and mixed, and this mixture filtered through Whatman No. 4 filter paper into clean large test tubes. The 20 second mixing period was deleted for those samples which contained no enzyme. The reducing groups present in triplicate samples of each one of the filtrates were then determined as before.

Procedure for the DNS Reducing Group Assay

The DNS reagent (55) was prepared as follows: 10g, dinitrosalicyclic acid; 2g, phenol; and 10g, NaOH were placed in a one

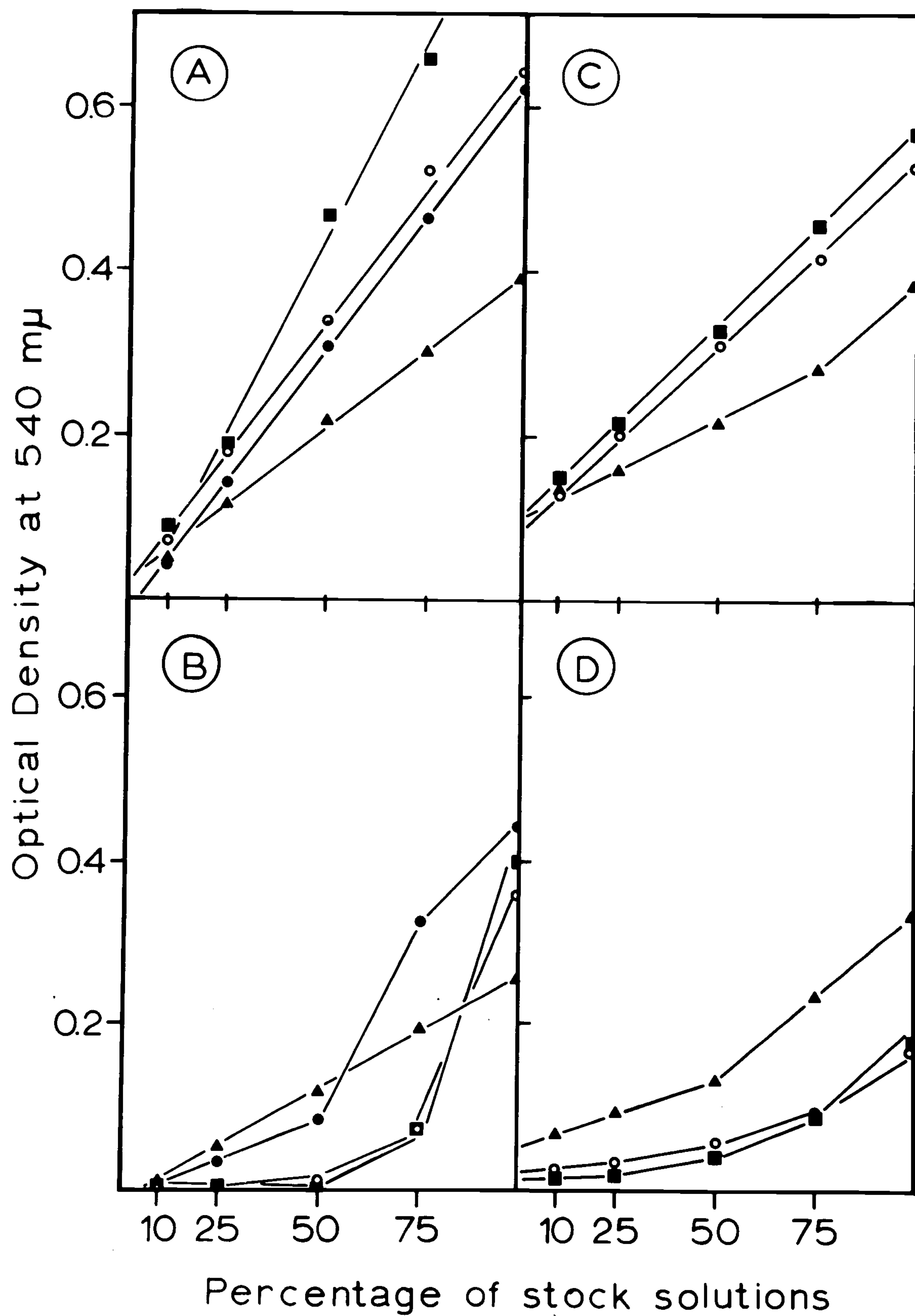
liter volumetric flask and brought up to volume with distilled water. This solution comprised the stock reagent. At the time of use, 0.05% $NaSO_3$ was added to the amount of stock reagent that was expected to be used for that day's experiment. In addition, a 40% solution of Rochelle salt ($NaKC_4H_4O_6 \cdot 4H_2O$) was prepared and placed in a bottle fitted with a repipet device. The DNS determinations on the pectic substances and galacturonic acid were conducted in 24 mm x 20.5 cm and/or 16 x 150 mm test tubes. Three ml of the test material was pipetted into test tubes containing 3 ml of the reduced DNS reagent. The tubes were boiled in a water bath for five minutes, removed, 1 ml of the Rochelle salt solution immediately added to each tube with thorough mixing, and the tubes cooled. In all instances, with the exception of galacturonic acid, heavy precipitates formed in the tubes. Therefore it was necessary to centrifuge the cooled assay mixtures at 7000 x g for five minutes because filtration resulted in a drastic loss of color by the samples. Following centrifugation, the supernatant fluid was poured into clean test tubes and their absorbancies determined at 575 mμ. Triplicate samples of each substrate and galacturonic acid monohydrate were tested at all concentration levels. Identical amounts of substrate were used in both the DNS and alkaline-copper experiments.

The Effects of Deproteinization

The development of the modified alkaline-copper method of reducing group determination as reported here was gradually developed over several years. This method was primarily derived from the colorimetric methods described by Nelson (63) and Somogyi (83). Addition of $Ba(OH)_2$ and $ZnSO_4$ to the pectic substances drastically reduced the absorbancies obtained for the samples (Figure 1B). At low PA and PG concentrations, few reducing groups were detected. The data given in Figure 1A-B clearly shows this effect of reduction in reducing ends. Not only does it occur with long chain polyuronides, but this phenomenon causes a drastic reduction in available reducing groups in galacturonic acid solutions (Figure 1B). The effect of the $Ba(OH)_2$ decreases with increasing polyuronide or galacturonic acid concentration. From the data given in Figure 1A and B, the percentage of lost reducing power for each of the pectic materials and galacturonic acid were as follows: PGA, 97 to 43%; PA, 98 to 57%; NaPP, 55 to 34%; and galacturonic acid, 74 to 25%.

The addition of $Ba(OH)_2$ to the enzyme-reaction-mixture similarly resulted in the reduction of reducing groups (Figure 1C and D).

Figure 1. The effects of $Ba(OH)_2$ on galacturonic acid, pectic substances, and enzyme reaction-mixtures with: A) pectic substances, B) pectic materials + BA^{++}, C) pectic materials + enzyme, and D) pectic substances + enzyme + $Ba(OH)_2$. The pectic materials are represented by the following symbols: galacturonic acid, ●——●; PGA, ○——○; PA, □——□; and NaPP, △——△. The concentration of the pectic materials used were 0.1 to 1.0 g/100 ml while the concentration of galacturonic acid was 35 to 350 μg/2 ml.

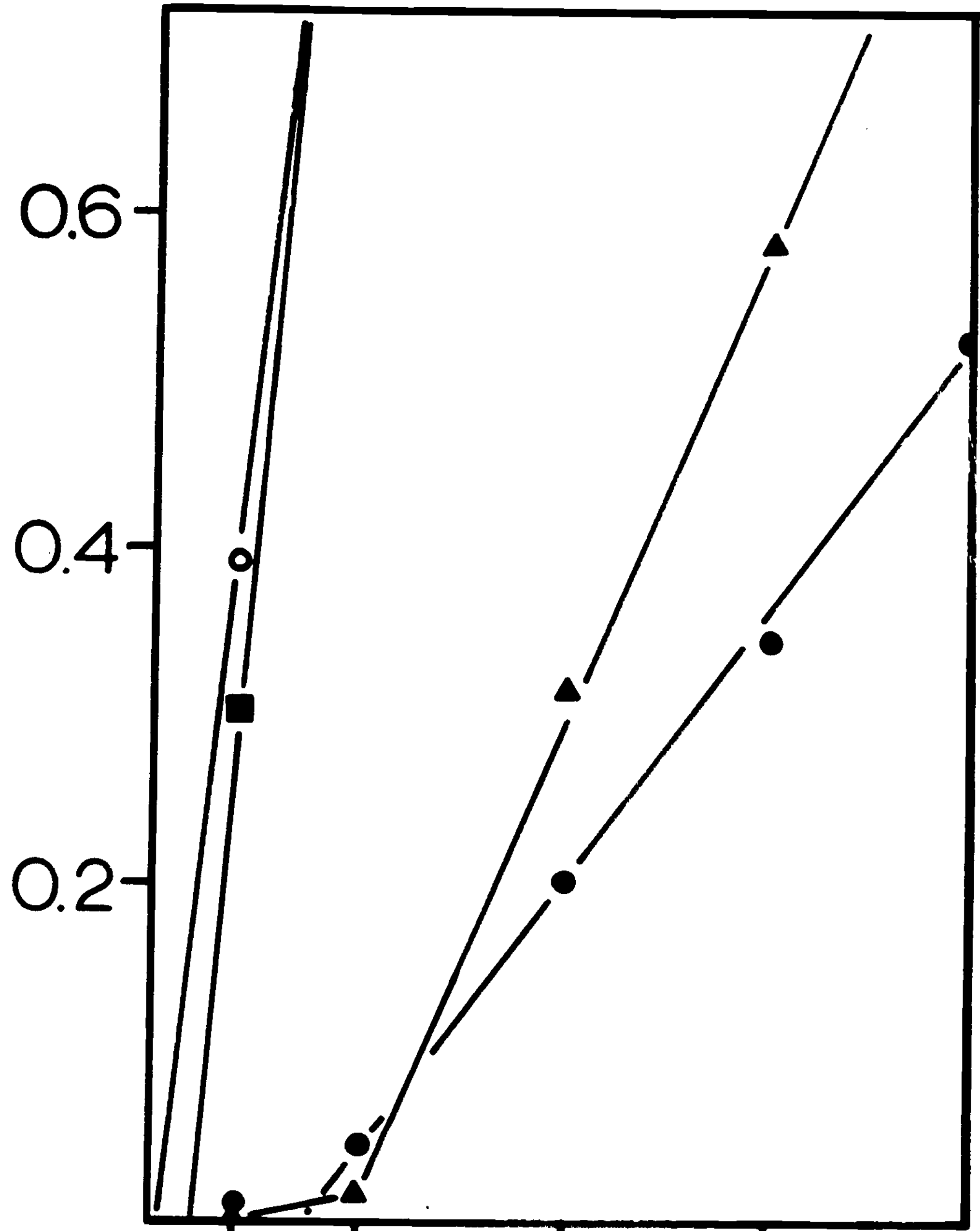

A
C
B
D
0.6
0.4
0.2
Optical Density at 540 mμ
10 25 50 75
Percentage of stock solutions

Here again, the greatest loss in reducing ends occurred with the more purified substrates PA and PGA rather than with NaPP. The effect of the $Ba(OH)_2$ on the loss of the available reducing groups in the enzyme-reaction-mixtures were found to range from 84 to 68% for PGA; 92 to 68% for PA; and 40 to 14% for NaPP from low to high levels of substrate concentration (0.1 to 1.0 g/100 ml), respectively. The objective in adding the $Ba(OH)_2$ was to precipitate the protein for blood sugar determination and to inactivate the enzyme in the enzyme-reaction-mixture by precipitation. However, this was achieved by the copper present in the reagent, and any other method of inhibiting enzyme activity was unnecessary. Corden _et al_. (23) showed that copper was very effective in inhibiting hydrolysis of NaPP by pectolytic enzymes. Furthermore, the concentration of Cu^{++} present in the alkaline-copper reagent was considerably higher than the level which Corden _et al_. (23) reported to be inhibitory. It is therefore suggested, that the Cu^{++} was just as effective as the Ba^{++} in preventing the enzymatic hydrolysis of NaPP or any of the other substrates.

The DNS vs. the Modified Alkaline-Copper Reducing Group Assays

A comparison of these two methods of reducing group determinations are shown in Figures 1A and 2. The data presented here

Figure 2. The effectiveness of the DNS method for measuring the reducing groups of pectic substances with: galacturonic acid, ●——●; PGA, o——o; PA, ■——■; and NaPP, ▲——▲. The concentration of the pectic substances was 0.1 to 1.0 g/100 ml while the concentration of galacturonic acid was 52.5 to 525 μg/3 ml.


Optical Density at 575mμ
0.6
0.4
0.2
10
25
50
75
% of stock solution

indicates that all of the polyuronide compounds used in this study were degraded by the DNS reagent upon boiling in a water bath (Figure 2), thereby, resulting in absorbancies that were much higher than would normally be expected. Furthermore, pectic substances are known to be polymers of galacturonic acid, and should, therefore, generate concentration response curves which would parallel the galacturnoic acid standard curve at equivalent concentrations. As noted in Figure 2 this was not the case, and the degree of divergence from the galacturonic acid curve increased with chain length and degree of purity of the pectic material in this case PGA and PA. Sodium polypectate is also decomposed by the DNS reagents with increasing concentration of the substrate, but to a lesser extent than occurred with PA and PGA. This reduced rate of breakdown must undoubtedly be related to the presence of several nonuronide substances which are known to be associated with NaPP (48, p. 78-89), such as mannans, glucans, arabinose, and xylose.

The results of the modified alkaline-copper tests in Figure 1A show that PGA generated a concentration response curve which was parallel to the galacturonic acid standard curve. Although the PGA curve was parallel to the galacturonic acid standard curve, pectic acid was more susceptible to heat degradation due to its increased chain length (3) as indicated by its deviation from the galacturonic acid standard curve. However, NaPP always failed to generate a

concentration response curve which paralleled the galacturonic acid standard curve. The reduction in slope of the NaPP curve was probably due to the complexing of the copper with this substrate. In experiments where the polyuronide concentration range was from 0.01 to 0.1 g/100 ml the DNS curves had a much steeper slope than did those obtained by the modified alkaline-copper assay as the result of continued substrate decomposition by the DNS reagents. The absorbancies obtained for this low concentration range using the alkaline-copper method varied from 0.0 to 0.66 O.D. units vs. 0.0 to 0.485 for the DNS assay, thus further establishing the direct decomposition of the polyuronide compounds by the DNS reagent.

Enzyme Assays

Preparation of Specific Reaction Mixtures

Polygalacturonase activity was measured on PGA purified by repeated extractions with acidified ethanol (48, p. 116) containing 12.5 mg/l of dithizone to remove metal contaminants. Following extraction the anhydrogalacturonic acid (AGA) content was 98-100%.

Polygalacturonic acid was solubilized and adjusted to pH 5.0 with 1.0 N NaOH, then filtered through Whatman no. 1 paper. Two and one-half ml of enzyme solution was mixed with 22.5 ml of purified

substrate solution (final concentration 2% w/v) and incubated at 30°.

PATE activity was measured on purified PGA as was PG except the final reaction mixture contained 1.0 mmole $CaCl_2$ and 25 mmoles tris-HCl buffer (pH 8.0). The reaction mixture for PMTE was similar to that for PATE except the substrate was pectin at a final concentration of 1.0% (w/v).

Cup-Plate Assays

Polygalacturonase (30) and PE (54) activities were estimated in rapid cup-plate assays on NaPP (Sunkist product no. 6024) and pectin, respectively. The PE assay was modified by substituting 0.75g of bromocresol purple for methyl red and by adding 0.2 mmoles of $CaCl_2$ to 200 ml of the reaction mixture. All cup plates were incubated for 16 hours at 40°. Enzymatic activities were expressed in relative units from standard curves in which zone diameters were plotted against the log of enzyme concentrations (30 mm zone = 1000 units).

Viscosity Reduction

Reductions in the viscosity of pectic substrates by enzymatic action was measured with a Brookfield model LVF viscometer using a U. L. adapter (80) and spindle speeds of 30 and 6 rev/min for the 2% PGA, and 1% pectin substrates, respectively.

Reaction mixtures were incubated at 30°, and six viscosity readings were made during the course of each enzyme assay. Results were expressed as percentage viscosity reduction relative to the boiled enzyme or diluted substrate control. At the end of each experiment, the reaction mixtures were boiled for ten minutes and stored at 5° before being analyzed chromatographically for the products of enzymatic hydrolysis.

Reducing Group Release

Release of reducing groups following hydrolytic and trans-eliminative cleavage of α-1, 4 linkages of pectic substrates was determined in 2.0 ml aliquots of the reaction mixture by the above modification of Somogyi's alkaline-copper method (83). Reaction mixtures were incubated at 30° and periodically assayed for enzymatic activity which was expressed as μmoles of reducing groups [as anhydrogalacturonic acid (AGA)] released per minute.

TLC of Reaction-Mixtures

Uronides through the tetramer arising by the enzymatic cleavage of pectic substrates were determined by thin layer chromatography (TLC) on MN-cellulose Powder 300 (Brinkmann Instruments Inc.) and developed with sodium formate (60 mg) - formic acid (15 ml of 88%, v/v) - ethanol (85 ml of 77% v/v) (65) for 90 minutes. Dried plates

were sprayed with CD-1 chromogenic reagent (33) to detect mono-galacturonic acid and the oligouronides.

Carbohydrate Determination

The uronide content of pectic substances used as enzyme substrates was determined by the carbazole method (52) which was however, unsatisfactory for detecting uronides in enzyme fractions because of the interference from relatively high levels of salts and proteins. The total carbohydrate content of these fractions was determined by the phenol-sulfuric acid method (95, p. 388-389).

Protein Determination

Protein was estimated by the method of Lowry *et al*. (49) using bovine serum albumin (Sigma Chemical Co.) as a standard.

RESULTS

Production of Pectolytic Enzymes

Growth and pH

F. oxysporum f. sp. *lycopersici* was grown on pectin, PGA, or glucose in shake culture medium to determine the range of pectolytic enzymes produced by this fungus. On all three substrates, near maximum growth was obtained on the second or third day following inoculation when the first measurements were made (Figure 3). The fungal population remained relatively constant at slightly above 10^8 cells per ml in the pectin and glucose cultures, but on PGA the concentration of viable spores declined significantly after the ninth day (Figure 3B).

Results with several fungal species have demonstrated an influence of culture pH on the type of pectolytic activity obtained (13). On the three substrates used here, distinctly different pH curves were obtained in the culture media (Figure 3). On pectin, after an initial drop from 4.6 to 3.7, the pH gradually increased to 6.1 by the 17th day (Figure 3A). On PGA, the pH increased rapidly from 4.9 to 7.6 after inoculation then gradually increased to 8.4 by the 17th day (Figure 3B). The pH of the glucose cultures increased throughout the 17 days but never became basic (Figure 3C). The fact that

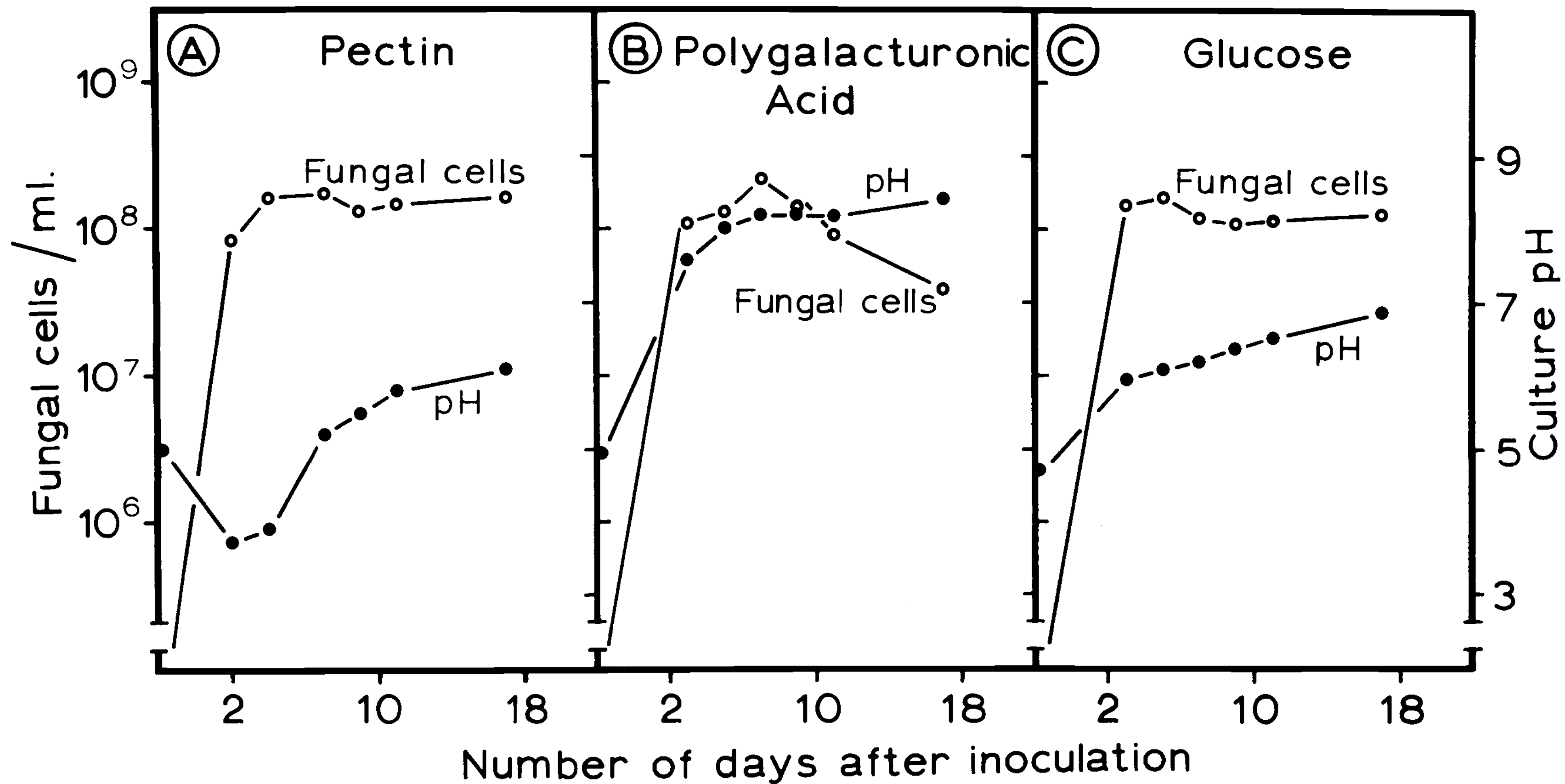


Figure 3. The growth and culture pH of *Fusarium oxysporum* f. sp. *lycopersici* growing on shake culture medium with: A) pectin, B) polygalacturonic acid, and C) glucose as the sole carbon sources. The fungal cells/ml are represented by o--o and the pH by ●--● .

culture filtrates of _F. oxysporum_ f. sp. _lycopersici_ maintained an acid reaction in the pectin cultures was probably due to release of acid groups from pectin through the action of PE.

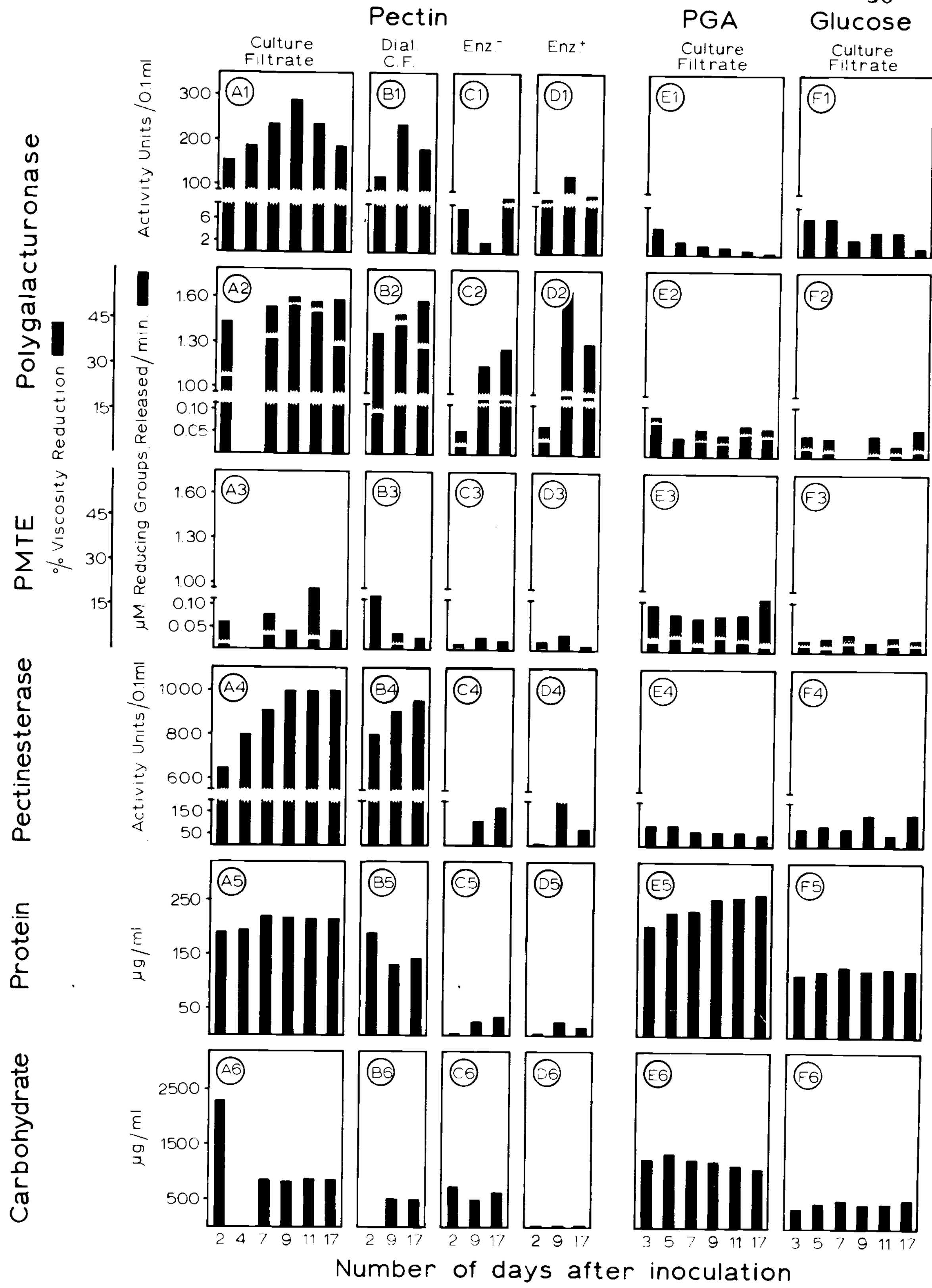
Polygalacturonase

PG was the major pectolytic enzyme detected in culture filtrates of _F. oxysporum_ f. sp. _lycopersici_ growing on pectin as the sole carbon source (Figure 4). Enzymatic activity, as estimated by the cup plate assay (Figure 4A1) followed closely the release of reducing groups from a purified PGA substrate (Figure 4A2). In both assays, maximum activity was obtained on the 9th day following inoculation after which it declined. The ability of the enzymes in the culture filtrates of various ages to reduce the viscosity of PGA, however, was relatively constant from the 7th through the 17th day (Figure 4A2).

Considerably less PG was produced on PGA than on pectin. As estimated by the cup plate (Figure 4E1) and reducing group (Figure 4E2) assays, maximum activity was obtained in the youngest cultures analyzed after which activity decreased through the 17th day. As with pectin cultures, viscosity reduction assays indicated a disproportionately high level of activity in the older (17 day) PGA cultures (Figure 4E2).

The generally lower activity in PGA cultures may be due to

Figure 4. The effects of partial purification of the pectolytic enzymes synthesized by *F. oxysporum* f. sp. *lycopersici.* A) pectin culture filtrates, B) dialyzed pectin culture filtrates, C) concentrated Enz$^-$ fractions, D) concentrated Enz$^+$ fractions, E) PGA culture filtrates, and F) glucose culture filtrates. Cup plate activity expressed in PGU's/0.1 ml; PG activity in μmoles of AGA/min and % V-R at 25 min; PMTE activity in μmoles of AGA/min and % V-R at 25 min; PE activity in PEu's/0.1 ml; protein concentration in μg/ml; and carbohydrate concentration in μg/ml.


Pectin
PGA
Glucose
Culture Filtrate
Dial. C.F.
Enz⁻
Enz⁺
Culture Filtrate
Culture Filtrate
Polygalacturonase
PMTE
Pectinesterase
Protein
Carbohydrate
Activity Units/0.1ml
% Viscosity Reduction
μM Reducing Groups Released/min.
Activity Units/0.1ml
μg/ml
μg/ml
300
200
100
6
2
1.60
1.30
1.00
0.10
0.05
45
30
15
1000
800
600
150
50
250
150
50
2500
1500
500
A1 B1 C1 D1 E1 F1
A2 B2 C2 D2 E2 F2
A3 B3 C3 D3 E3 F3
A4 B4 C4 D4 E4 F4
A5 B5 C5 D5 E5 F5
A6 B6 C6 D6 E6 F6
2 4 7 9 11 17
2 9 17
2 9 17
2 9 17
3 5 7 9 11 17
3 5 7 9 11 17
Number of days after inoculation

the instability of PG at the relatively high pH's attained in these cultures. Similar results have been obtained by others (68, 101, p. 61-80).

In glucose cultures, relatively weak PG activity was obtained, and again production with time, as determined by cup plate assays and release of reducing groups, was similar but viscosity reduction indicated unusually high activity in the older culture filtrates (Figure 4F1 and F2).

Waggoner and Dimond (92) and others (7, 58, 67, 68) failed to detect PG from F. oxysporum f. sp. lycopersici growing on glucose medium, but more recently it has been noted on this substrate (70-74).

Pectin Methyl Trans-Eliminase

On pectin much less PMTE was produced than PG (Figure 4A3), and PMTE activity measured by viscosity reduction and reducing group release was quite variable. In the PGA cultures (Figure 4E3), PMTE activity was generally higher than that found in the pectin or glucose (Figure 4F3) cultures. PMTE production was probably favored by the relatively high pH in the PGA culture filtrates. Mussell and Green (58) reported high PG production by F. oxysporum f. sp. lycopersici growing on PGA, but this could have been PATE or PMTE which they were measuring.

Pectin Esterase

PE production on pectin medium increased until the 9th day then remained constant through the 17th day (Figure 4A4). On the PGA (Figure 4E4) and glucose (Figure 4F4) media relatively little PE was produced.

Protein and Carbohydrate

Protein in the culture filtrates was significantly higher in cultures grown on pectin and PGA (Figure 4A5 and E5) than in glucose cultures (Figure 4F5). The higher protein levels in the PGA cultures were not due to increased pectolytic enzyme production because these cultures demonstrated only limited pectic enzyme activity.

Excluding the two day culture on pectin (Figure 4A6), the carbohydrate levels in the culture filtrates from each substrate were relatively constant during the experiment suggesting that the residual carbohydrate was unavailable for fungal growth. The relatively high carbohydrate level in the two day cultures from pectin is probably unmetabolized pectin that was utilized by the 5th day when maximum growth was achieved.

Partial Purification and Concentration of Pectolytic Enzymes Produced by F. oxysporum f. sp. lycopersici

Culture filtrates of F. oxysporum f. sp. lycopersici growing on pectin, PGA, and glucose media were partially purified by dialysis against 0.01M acetate buffer (pH 4.0) and separated on CM-cellulose into Enz^- and Enz^+ fractions.

PG activity in the pectin culture filtrate was little affected by dialysis against the buffer (Figure 4B1 vs 4A1 and Figure 4B2 vs 4A2). As in the undialyzed filtrate, enzyme activity estimated by the cup plate and reducing group assays was similar but the viscosity reduction assay indicated disproportionately high activity in the 17 day sample (Figure 4B2).

Dialysis at low temperature (5°C) gave results similar to those at room temperature, but when dialysis was carried out against tap water a significant loss of PG activity occurred (Table 1).

Dialysis against water has been used repeatedly in studies on the pectolytic enzymes produced by F. oxysporum f. sp. lycopersici (73, 88, 92, 101, p. 34-61). The results obtained here suggest, however, that dialysis may have provided a highly selective treatment of the mixture of enzymes present in the culture filtrate, and undoubtedly confounded studies on enzyme production.

The carbohydrate content of the culture filtrate was reduced only slightly by dialysis (Figure 4B6), suggesting that the

carbohydrate was either of large molecular weight or small molecules bound to protein.

Table 1. Effect of dialysis against water or buffer on polygalacturonase activity in the culture filtrate of _F. oxysporum_ f. sp. _lycopersici_.

Polygalacturonase activity units[a]			Percent Recovery
Culture filtrate	Water dialyzed	Buffer dialyzed[b]	
44	29	--	64
123	19	--	16
36	0	--	0
190	--	190	100
235	--	235	100
290	--	235	81

[a] Polygalacturonase activity expressed in relative units obtained from a standard curve for the cup-plate assay in which zone diameters were plotted against the log of the enzyme concentration, (30 mm zone = 1000 units)

[b] 0.01 M sodium acetate buffer (pH 4.0).

Dialysis of the glucose culture filtrates against buffer caused about a 10% loss of PG activity, and the enzymatic activity obtained in the various assays failed to indicate a change in the mode of action of the enzyme. PG activity in the PGA culture filtrates, however, was eliminated completely by dialysis; thus, further characterization was impossible.

The dialyzed culture filtrates were separated into Enz^+ and Enz^- fractions by a batchwise technique on CM-cellulose. The

fractions were de-salted by dialysis and concentrated by ultrafiltration in a Diaflo apparatus. In general, this treatment increased the specific activity of the PG three fold in the Enz^{+} fractions but 40 to 60% of the total activity was lost. Most of the PG activity was in the Enz^{+} fraction (Figure 4D1, D2), which contained about the same level of protein as the Enz^{-} fraction but had considerably less carbohydrate (Figure 4D5, D6). Fractionation of the dialyzed glucose culture filtrates gave the opposite results to those obtained with the pectin cultures. Most of the enzymatic activity (95%) was in the Enz^{-} fraction along with the majority of the protein retained (93%) and the carbohydrate (95%). In total, 60 to 80% of the PG activity was lost but the specific activity was increased 1.2 fold for the Enz^{-} fraction but decreased for the Enz^{+} fractions.

The PMTE in pectin culture filtrates was generally reduced by dialysis against acetate buffer at pH 4.0 (Figure 4B3), while all the PMTE activity was lost from the PGA cultures. The PMTE activity from glucose and pectin (Figure 4C3, D3) cultures was evenly divided between the Enz^{-} and Enz^{+} fractions but was extremely low.

The PE activity from the pectin cultures was slightly reduced by dialysis against buffer (Figure 4B4). Following fractionation on CM-cellulose, the PE activity was about evenly divided between the

Enz^- and Enz^+ fractions (Figure 4C4, D4). The PE activity in the two day Enz^- fraction was completely lost during the batchwise separation on CM-cellulose.

Characterization of the Pectolytic Enzymes

Mode of Action of the Major Fractions

The PG produced by F. oxysporum f. sp. lycopersici on pectin was classified as an endo enzyme, i.e. only about 2% of the total α-1, 4 linkages were hydrolyzed at 20% reduction in the viscosity of the substrate (Table 2). The mode of action of the endo-PG was not altered during purification. However, the 17 day Enz^+ fraction had to hydrolyze significantly more α-1,4 bonds to reach 20% viscosity reduction.

These results agree with those obtained by Waggoner and Dimond (92) and others (68, 70, 74). However, Young (101, p. 61-80) suggested that F. oxysporum f. sp. lycopersici produced some exo-PG based on results obtained from paper chromatographic separation of hydrolysis products. The initial product of enzymatic hydrolysis was monogalacturonic acid. This conclusion must be questioned because a PG functioning in a truly random manner would be expected to release monogalacturonic acid, as well as other oligmers. Young's (101, p. 61-80) viscosity reduction and reducing

group data failed to confirm the presence of an exo-PG.

Table 2. The mode of action of polygalacturonase from cultures of F. oxysporum f. sp. lycopersici growing on a pectin medium.

Enzyme Source	Age of culture (days)[a]	Polygalacturonase activity: min. required to reduce the viscosity of PGA 20%	Polygalacturonase activity: Percent hydrolysis of PGA at 20% viscosity reduction
Culture filtrate	2	4.5	1.2
	9	2.5	1.7
	17	3.2	1.5
Dialyzed culture filtrate	2	5.2	2.2
	9	3.5	1.9
	17	3.4	2.1
Enz^-	9	12.0	2.7
	17	7.8	1.9
Enz^+	9	2.4	1.2
	17	9.5	3.5

[a]Number of days from inoculation to harvesting of the culture.

A trace of PG was detected in the PGA and glucose cultures and, while the activity was low, the enzyme appeared to function in a random (endo) manner as indicated by the relatively few bonds (0.5 and 0.06%) hydrolyzed at 7.5 and 9.0% viscosity reduction respectively. These results are in opposition to those reported by Mussell and Green (58) in which they found maximum endo-PG production on PGA and failed to detect PG in glucose

cultures. However, Patil and Dimond (74) reported the production of a significantly greater amount of endo-PG by F. oxysporum f. sp. lycopersici growing on glucose than was detected in these experiments (Table 4). Their results must be questioned, however, because activity measurement were based solely on viscosity reduction assays employing NaPP which contains 30-40% nonuronide material (48, p. 78-89). This could result in the detection of enzymes other than PG that reduce the viscosity of a NaPP solution by hydrolyzing the nonuronide moiety.

The PMTE produced by F. oxysporum f. sp. lycopersici grown on pectin and PGA was designated as endo enzymes based on the fact that about 1.0% of the α-1, 4 linkages were hydrolyzed at 20% viscosity reduction (Table 3). Thus, there were no detectable differences in the mode of action for the PMTE produced on pectin and PGA by this fungus.

Table 3. The mode of action of PMTE in the culture filtrate of F. oxysporum f. sp. lycopersici growing on PGA and pectin media.

Enzyme Source	Age of culture (days)[a]	PMTE activity	
		Min. required to reduce the viscosity of pectin at 20%	Percent hydrolysis of pectin at 20% viscosity reduction
Culture filtrate	7	62.5	1.2
PGA medium	11	63.5	1.1
Culture filtrate	7	79	1.2
pectin medium	11	25	0.9

[a] Number of days from inoculation to harvesting of the culture.

The PMTE activity detected in enzyme fractions from the glucose culture medium was characterized as endo-PMTE. However, the characterization was difficult because of the variability experienced in the viscosity reduction and reducing group assays. There appeared to be a shift toward an exo mode of action in the Enz^- fractions from older cultures, but the low level of activity in these fractions prevented their complete characterization.

An enzyme detected in the Enz^- and Enz^+ fractions from glucose cultures hydrolyzed NaPP, but had only limited activity on PGA (Table 4). The action of this "pectolytic enzyme" on NaPP in both the Enz^- and Enz^+ fractions was of a random nature since it hydrolyzed only 0.1% of the available bonds at 20% viscosity reduction. The difference between this enzyme and PG was further documented by comparing their relative abilities to reduce the viscosity of PGA and NaPP solutions (Table 5). The PG from a pectin culture was about seven fold more active on NaPP but 30 fold more active on PGA than the enzyme from the glucose culture. The reduced activity on PGA demonstrated by the Enz^- and Enz^+ fractions obtained from the glucose cultures would coincide with a reduction in PG activity, while the greater activity of these enzyme fractions on NaPP would suggest the hydrolysis of the nonuronide bonds present in the NaPP molecule.

Table 4. The mode of action of the pectolytic enzymes produced by _F_. _oxysporum_ f. sp. _lycopersici_ growing on a glucose medium.

Enzyme Source	Pectolytic enzyme activity			
	min. required to reduce the viscosity of		Percent hydrolysis	
	PGA 5%	NaPP 20%	PGA at 5% vis. red.	NaPP at 20% vis. red. -
Culture filtrate	13	--	0.02	--
Dial. C.F.	50	--	0.16	--
Enz^-	28	2.7	0.16	0.1
Enz^+	--[a]	29.7	--[a]	0.1

[a]Enzyme activity was too weak for accurate determination of viscosity reduction.

Table 5. Differential viscosity reduction of pectic substrates by pectolytic enzymes produced on pectin and glucose cultures by _F_. _oxysporum_ f. sp. _lycopersici_.

Substrate for enzyme production	Pectolytic enzyme activity of Enz^- fraction	
	min. required to reduce the viscosity of NaPP 70%	min. required to reduce the viscosity of PGA 5%
Pectin	3.8	1.2
Glucose	29	30

TLC of Enzyme-Reaction-Mixtures

TLC of reaction-mixtures of the various PG fractions produced by *F. oxysporum* f. sp. *lycopersici* on pectin, PGA, and glucose further indicated the endo action of this enzyme. Mono, di, tri, and tetragalacturonic acids, as well as larger oligmers were present in all reaction-mixtures with enzymes produced on pectin. The enzyme from PGA and glucose cultures produced primarily oligmers larger than the tetramer.

The TLC of the pectin (pH 8.0) enzyme-reaction-mixtures showed that the PMTE activity detected in the pectin, PGA, and glucose cultures was due to endo enzymes as they failed to release oligmers smaller than tetragalacturonic acid. The failure to detect the unsaturated monomer or monogalacturonic acid in the PMTE reaction-mixtures was probably the result of low enzyme activity, as well as the inability to spot more than 10 μl of these reaction-mixtures on the TLC plates.

When the NaPP enzyme-reaction-mixtures for both the glucose Enz^- and Enz^+ fractions were analyzed by TLC, they produced color streaks atypical of galacturonic acid. The presence of a greyish color indicated that aldohexoses had been released. The hydrolytic products larger than the tetramer in the NaPP-reaction-mixtures were precipitated with 95% ethanol. The

ethanol extract was evaporated to dryness, redissolved in distilled water, spotted on TLC plates along with glucose, galactose, mono-, di- and trigalacturonic acid, developed with a solvent system containing pyridine-ethylacetate-acetic acid-water (5:5:1:3) (16, p. 204-205), and sprayed with 2-aminobiphenyl (CD-1) (33) chromogenic reagent.

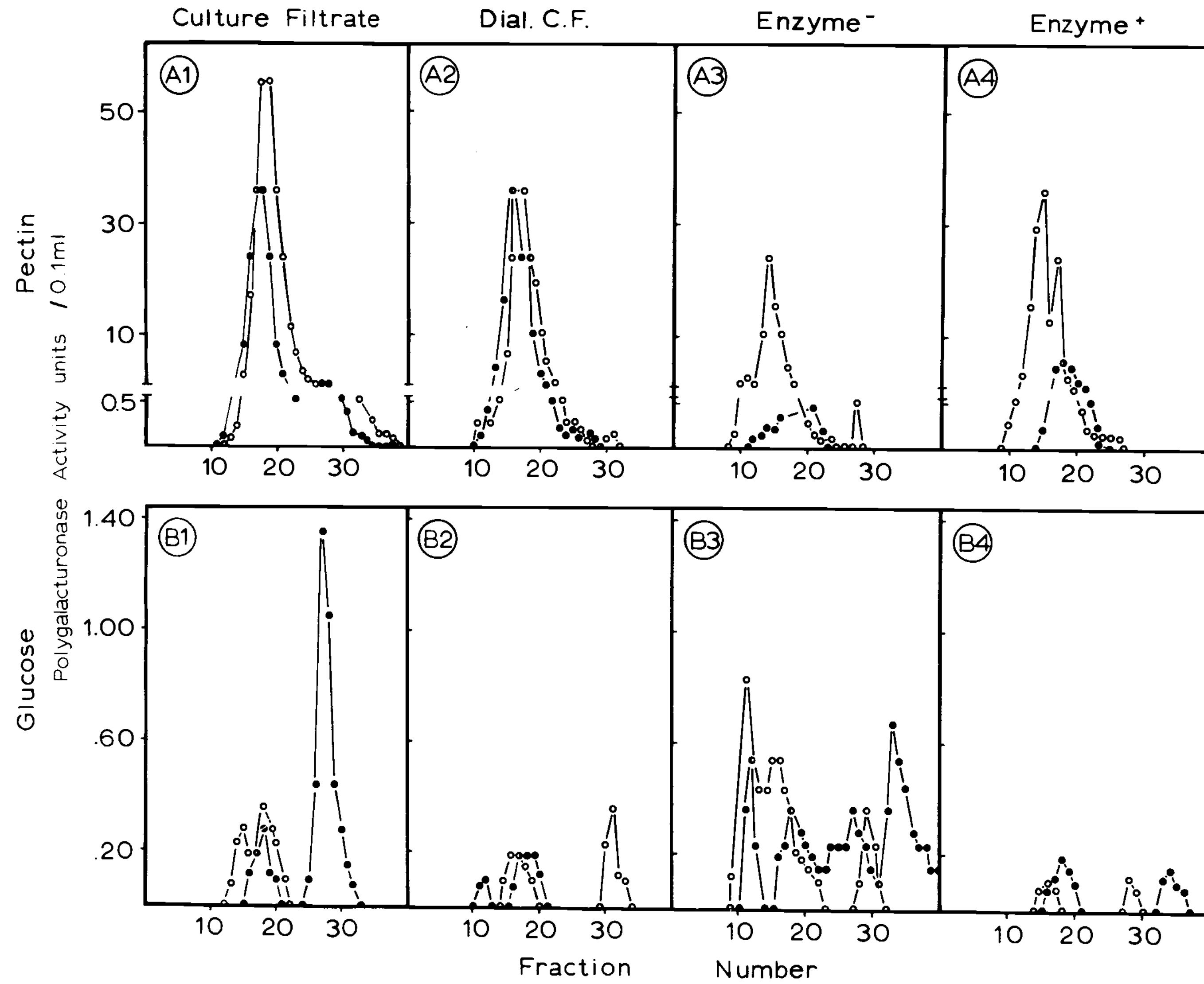
The major carbohydrate released from the NaPP-reaction-mixtures was an unidentified aldohexose that was probably either glucose or galactose, but traces of mono- and digalacturonic acid were also detected. Consequently, the "pectolytic enzyme" produced by _F_. _oxysporum_ f. sp. _lycopersici_ on glucose was probably not PG, but an enzyme related to the α-L-arabinofuranosidase reported by Gremli and Neukom (34). This arabinase hydrolyzed both NaPP and pectin, while Narasaki (59) using a purified hemicellulase detected glucose, galactose, fucose, and galacturonic acid as some of the hydrolytic products released from a purified soybean hemicellulose. These results suggest that the enzyme produced on glucose by _F_. _oxysporum_ f. sp. _lycopersici_ as reported by Patil and Dimond (74) is probably a mixture of enzymes and not just PG, and it would also seem probable that the enzymes reported by Bateman (10) and Winstead and McCombs (97) are related to hemicellulases.

Column Chromatography of PG

The enzyme fractions obtained from the pectin cultures were chromatographed on a Sephadex G-75 column to determine their homogeneity and estimate their molecular weights (Figure 5A). The culture filtrates (Figure 5A1) were characterized by the production of two peaks of PG activity (e.g. four day C.F.). The major peak contained 90% of the activity and had a Kav (distribution coefficient of the enzyme on the column) of 0.249, while the minor peak contained 3% of the total activity and had a Kav of 0.91. On the same column, the dialyzed culture filtrate produced a shoulder and two peaks of PG activity (Figure 5A2) in the four day fractions with Kav's of 0.0, 0.249, and 1.02. A shift in the chromatographic pattern of the dialyzed enzyme fractions, as depicted by the 17 day fraction (Figure 5A2), was evident by an increase in the number of peaks with Kav's of 0.0, 0.249, 1.08, and 1.58. However, the major portion of the PG activity was located in the second peak (Kav = 0.249) for both the dialyzed fractions and culture filtrates (Figure 5A1).

The Enz^- fractions from the pectin cultures exhibited a wide distribution of molecular sizes which increased with the incubation period of the cultures (Figure 5A3). The Enz^- fractions produced three peaks containing PG activity with a definite shift toward larger

Figure 5. Characterization of the polygalacturonases obtained from the pectin and glucose time course experiments by reverse flow Sephadex G-75 column chromatography with: A) enzyme fractions from the pectin cultures (void volume = 57.6 ml) and B) enzyme fractions from the glucose cultures (void volume = 55 ml). The numbers represent: 1) culture filtrate, 2) dialyzed culture filtrate, 3) enzyme$^-$, and 4) enzyme$^+$. The two day pectin and five day glucose enzyme fractions = ●——●, while the 17 day pectin and 11 day glucose enzyme fractions = ○——○. PG activity was determined by the cup plate assay (30).


Culture Filtrate
Dial. C.F.
Enzyme⁻
Enzyme⁺
A1
A2
A3
A4
B1
B2
B3
B4
Pectin
Glucose
Polygalacturonase Activity units / 0.1ml
50
30
10
0.5
1.40
1.00
.60
.20
10
20
30
Fraction Number

molecular weights occurring in the 17 day Enz$^-$ fraction as exemplified by Kav's of 0.249, 0.748, and 1.36 vs. Kav's of 0.0, 0.137, and 0.858 for the four and 17 day Enz$^-$ fractions, respectively. The major portion of the enzyme activity was located in the primary peak (Kav = 0.249) for the four day Enz$^-$ fraction while the second peak (Kav = 0.137) contained the majority of the PG activity from the 17 day Enz$^-$ fraction.

The Enz$^+$ fractions obtained from the pectin cultures all produced single peaks (Figure 5A4) on Sephadex G-75 columns except for the 17 day fractions. However, there was a decrease in the Kav's from 0.368 (four day) to 0.193 (17 day) for the major peak indicating an increase in molecular weight with increasing incubation period.

The Sephadex G-75 chromatography of the early (three day) PGA cultures produced a single peak of PG activity with a Kav of 0.32. However, with continued incubation (seven day) two peaks were detected in the PGA culture filtrates with Kav's of 0.051 and 0.254.

The results obtained from the Sephadex G-75 chromatography of the glucose culture filtrates (Figure 5B1) indicated the presence of two molecular species in these samples (e.g. five day) with Kav's of 0.378 and 0.709. The majority of the PG activity was located in the second peak (Kav = 0.709). The estimated molecular weight of the PG in these culture filtrates increased with increasing incubation time as

exemplified by the 11 day cultures (Figure 5B1). The shift in the molecular weights for these two peaks were shown by the decrease in Kav's of 0.203 to 0.378. The majority of the PG activity was detected in the second peak (Kav = 0.378) and was identical to the results reported for the 11 day culture filtrate from pectin.

The dialyzed glucose culture filtrates produced two peaks of PG activity on Sephadex. The molecular weights of the fractions appeared to decrease with increased incubation time (Figure 5B2). The five day sample had Kav's of 0.051 and 0.304 while the peaks from the 11 day fraction had Kav's of 0.253 and 1.01. The majority of the PG activity was located in the second peak or smaller molecular fractions while the reverse was true for the dialyzed pectin cultures.

The number of peaks produced by the glucose Enz^- fractions decreased after five days of incubation and this was also related to a decrease in the amount of carbohydrate material associated with the enzyme. The five day Enz^- fraction from the glucose cultures produced five peaks (Kav's of 0.0, 0.051, 0.304, 0.709, and 1.12), while the 11 day Enz^- fractions produced only three activity peaks (Kav's of 0.0, 0.203, and 0.912) with an overall increase in molecular weight. The same phenomena was reported for the Enz^- fractions obtained from the pectin cultures (Figure 5A3).

Although there was only a trace of PG activity in the glucose

Enz^+ fractions, they also produced two peaks on Sephadex chromatography with Kav's of 0.378 and 1.16 (Figure 5B4). Similar results were obtained with the glucose culture filtrates, but the majority of the PG activity in the Enz^+ samples was located in the first (Kav = 0.378) and not the second peak (Figure 5B1). The glucose Enz^+ fractions from the glucose cultures (Figure 5B4) also differed from the Enz^+ fractions obtained from the pectin cultures (Figure 5A4), i.e. the early pectin fractions produced a single peak of PG activity.

All of the enzyme fractions chromatographed on Sephadex G-75 indicated that the molecular weights of the enzyme fractions increased with the increasing age of the cultures with the exception of the dialyzed glucose fractions. These results are contrary to those of Akinrefon (1) who found the molecular weight of PG produced by Phytophthora palmivora decreased as the incubation period increased.

The actual detection of low molecular weight ($\leq$ 2,000) enzyme molecules during Sephadex G-75 chromatography of the pectin and glucose enzyme fractions was substantiated by the detection of PG in the filtrates that passed through the U.M. 2 Diaflo ultrafiltration membrane which has a molecular weight cutoff of 1,000.

The PG present in the enzyme fractions obtained from the pectin cultures were characterized by their gradient elution patterns

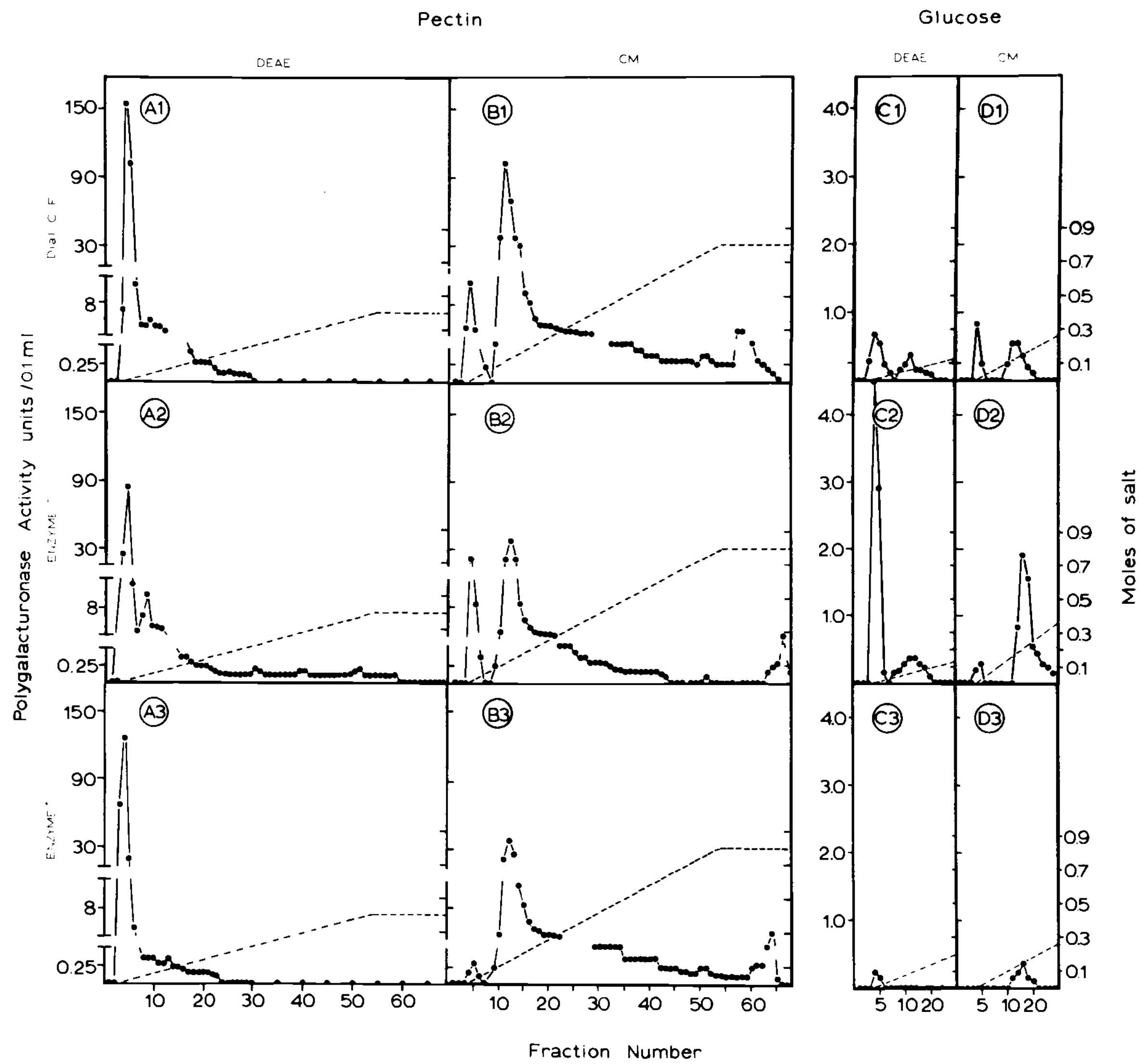
from DEAE- and CM-cellulose ion-exchangers (Figure 6A and B). The dialyzed pectin culture filtrates when chromatographed on DEAE gave two peaks of PG activity (Figure 6A1). The first and major peak was eluted with the void volume of the column (Figure 6A1) while the second peak or trailing shoulder was adsorbed.

When the Enz$^-$ fractions from the pectin cultures were chromatographed on DEAE, the results showed two strong peaks for the early fractions (two and four day) with three additional later peaks being detected in the 17 day Enz$^-$ fraction (Figure 6A2). The initial and major peak was eluted with the void volume while the later minor peaks indicated the presence of an increasing amount of enzyme that was adsorbed on the column. The production of peaks on DEAE was proportional to the increasing incubation period of the cultures (Figure 6A2) and the increasing carbohydrate concentration of the Enz$^-$ fractions from the pectin cultures (Figure 4C6).

The Enz$^+$ fractions produced two peaks on DEAE (Figure 6A3) with the majority of the PG activity being eluted with the void volume. These chromatographic patterns were similar to those obtained for the dialyzed culture filtrates (Figure 6A1).

The elution pattern of the dialyzed pectin culture filtrates on CM-cellulose showed that two peaks were produced by the early cultures (two and four day) while three peaks of PG activity were detected in the 17 day fractions (Figure 6B1). The first peak was

Figure 6. Characterization of the polygalacturonases obtained from the pectin and glucose time course experiments by gradient elution ion-exchange column chromatography with: A) DEAE, pectin enzyme fractions; B) CM, pectin enzyme fractions; C) DEAE, glucose enzyme fractions; and D) CM, glucose enzyme fractions. The numbers represent the stage of purification with: 1) dialyzed culture filtrate, 2) enzyme$^-$, and 3) enzyme$^+$ fractions. PG activity was determined by the cup plate assay (30).



Pectin
Glucose
DEAE
CM
DEAE
CM
A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3
150
90
30
8
0.25
4.0
3.0
2.0
1.0
0.9
0.7
0.5
0.3
0.1
10 20 30 40 50 60
5 10 20
Polygalacturonase Activity units /0.1 ml
Moles of salt
Fraction Number

eluted while the second and third peaks were adsorbed by the exchanger. The second peak contained the majority of the PG activity. These results corroborate those reported for the DEAE dialyzed pectin fractions (Figure 6A1), i. e. the majority of the PG in the dialyzed pectin fractions was probably positively charged.

The results obtained from the CM-cellulose chromatography of the Enz^- fractions from the pectin cultures (Figure 6B2) were similar to the results reported for the dialyzed pectin fractions (Figure 6B1).

When the Enz^+ fractions from the pectin cultures were rechromatographed on CM-cellulose, two strong peaks which were adsorbed were detected as expected, with the exception that a trace (0.4%) of enzyme activity was eluted with the void volume from the 17 day Enz^+ sample (Figure 6B3). The majority of the enzymatic activity was detected in fractions from the adsorbed peak. Production of additional peaks in the 17 day Enz^+ fraction was correlated with increased carbohydrate concentration of this fraction (Figure 4D6).

Chromatography of the dialyzed glucose fractions on DEAE-cellulose resulted in the production of two peaks one of which was eluted with the void volume while the other was adsorbed. The majority of the activity was detected in the fraction that was eluted with the void volume which is in contradiction to the results obtained for the dialyzed pectin fractions (Figure 6A1).

The Enz^- fractions obtained from the glucose cultures also produced enzyme that was eluted with the void volume and produced fractions that were adsorbed on DEAE. The eluted fraction contained most of the PG activity (Figure 6C2). The production of a strong eluted peak by the glucose Enz^- fractions corresponded to their carbohydrate concentrations. The DEAE chromatography of the Enz^+ fractions from the glucose cultures revealed a single adsorbed peak containing PG activity (Figure 6C3). However, the activity of these fractions was so low that a peak in the void volume, if it existed, could not have been detected.

The dialyzed (Figure 6D1) and Enz^- (Figure 6D2) fractions from the glucose cultures produced several peaks on CM-cellulose. The major portion of the PG activity was located in the adsorbed peaks which contradicts the results obtained for both of these fractions on DEAE (Figure 6C1 and C2).

The chromatography on CM-cellulose of the Enz^+ fractions obtained from the glucose cultures resulted in the production of a single adsorbed peak (Figure 6D3) which corroborated the results obtained by DEAE chromatography (Figure 6C3).

The amount of enzyme eluted with the void volume ion-exchange chromatography and the number of peaks and shifts in molecular weights produced on gel filtration column chromatography was correlated with the increasing carbohydrate concentration and age

of the enzyme fractions obtained from the pectin and glucose cultures. The degree of polymerization of the carbohydrate associated with the Enz^+ fractions from the pectin cultures, as determined by the phenol-sulfuric acid (95, p. 388-389) and reducing group assays as based on galacturonic acid standard curves, was calculated to be a dimer or trimer.

DISCUSSION

In previous pectic enzyme work by Barash (8), Bateman (10-12), Keen and Horton (46, 47), Patil and Dimond (70-74), Sherwood (79), Swinburne and Corden (88), Waggoner and Dimond (92) and Young (101), there has been a failure to use sufficiently purified substrates in their reaction-mixtures. The commercially available NaPP varies from 60 to 68% anhydro-galacturonic acid while commercial PGA varies from 80 to 85% anhydro-galacturonic acid as determined by the carbazole assay (52). It is unrealistic to try to determine the mode of action of an enzyme using different substrates at different concentrations especially since the NaPP is known to contain polymers other than galacturonic acid (araban, glucans, galactomanans, etc.) (48, p. 78-89), which could serve as substrates for hemicellulases.

In this connection, Hancock (38) reports the use of two different substrates in determining the mode of action of polygalacturonases, i.e., NaPP for viscosity reduction analysis and a partially purified PGA (commercial preparation) for the reducing group assay.

Various researchers have used only one assay to characterize PG, such as the viscosity reduction (66, 73, 74, 71, 71, 84, 85, 90, 94, 97, 100) or the reducing group assay (39, 40, 43, 44, 75, 79,

87, 103) while employing NaPP as the substrate. In these studies no attempt was made to determine the percentage of substrate bonds hydrolyzed at specific levels of viscosity reduction.

One point which needs to be made concerning the assay methods used for characterizing PG deals with procedures used in the paper or TLC chromatography of the hydrolytic products from enzyme reaction-mixtures. The majority of the chromogenic reagents used were not qualitative and failed to detect a variety of carbohydrates. Some of these non-specific reagents are ammoniacal $AgNO_3$ (8, 9, 57), alkaline permanganate, bromophenol blue (6, 12, 53, 57, 88, 101, p. 24-28), and aniline phthalate (6, 37). Along with the fact that these reagents are not specific, the Rf's obtained for the spots on the chromatograms are also misleading since the presence of the buffer salts in the reaction-mixtures retards the movement of the breakdown products (102), thus, masking the presence of other sugars which might be released from impure substrates. This phenomenon was demonstrated by the detection of an aldohexose as the major hydrolytic product in the NaPP-reaction-mixtures with the "pectolytic enzyme" from _F. oxysporum_ f. sp. _lycopersici_ growing on glucose medium.

In this regard, Narasaki (59), reported the hydrolytic products of a purified hemicellulose isolated from soybean cotyledons to be galacturnoic acid, galactose, arabinose, rhamnose, glucose

and fucose. Wood (98, p. 137-138) also mentions several instances where hemicellulases have hydrolyzed their respective substrates resulting in the production of glucose and other pentoses. Furthermore, Gremli and Neukom (34) reported the isolation of an α-L-arabinofuranosidase which is capable of hydrolyzing NaPP and pectin. This would also suggest that the enzymes isolated by Bateman (10) and by Winstead and McCombs (97) which released glucose from NaPP-reaction-mixtures may be hemicellulases.

Based on the above results, it is suggested that the majority of the glucose induced pectolytic enzyme synthesis, which has been reported in the literature (4, 8, 9, 53, 70, 72-74) where their respective activities were determined using commercial NaPP, was probably due to a mixture of enzymes primarily containing hemicellulase activity. In addition, these results would support the contention of Joslyn (13) that glucose or its polymers are interspersed between the galacturonic acid polymers in NaPP.

All of the papers reviewed (40, 53, 59, 69, 93, 101, p. 22-24) concerning the alkaline-copper method of reducing group determination, referred to one of two papers, either Nelson's (63) or Somogyi's (83) as the method which was used for their reducing group assays.

The above researchers failed to mention alterations or changes in either Nelson's (63) or Somogyi's (83) original procedure during the performance of the tests. Therefore, it must be assumed that the procedures used in these papers were exactly the same as those reported in the original papers (63, 83). As the results in Figure 1A-D show, not only does the $Ba(OH)_2$ precipitate the enzyme, but it also results in the precipitation of large and small uronides, as well as galacturonic acid. This precipitation results in a marked decrease in reducing ends available for reaction, thereby, producing samples with extremely low readings. This explains some of the differences reported for results obtained with different assays.

The applicability of the DNS method as a means of determining the mode of action of pectolytic enzymes by reducing group liberation is severely questioned. The results presented in Figure 2 leave little doubt that all of the pectic materials used in the DNS experiments were degraded to smaller compounds when the reagents for the DNS assay were added to the reaction mixtures. In fact, Kertesz (48, p. 145) pointed out that the molecular weight for some pectic substances which have been determined by the DNS method were in the range of 2500 to 8000, while other workers reported ranges of 20,000 to 300,000 for the same compounds with other reducing group techniques. This decrease in molecular weight was undoubtedly the result of substrate decomposition during the performance of the DNS

test. This is further documented by the report of Barash (8) where he found that 36% of the bonds in a NaPP-reaction-mixture were hydrolyzed in 20 minutes using the DNS method for the reducing group determination. He suggested that this was an exo-PG while in reality he was undoubtedly measuring reagent degradation of his substrate.

The data obtained for the enzyme fractions from the pectin cultures by the cup plate (Figure 4A1 to D1), reducing group, and viscosity reduction (Figure 4A2-D2) assays demonstrated a correlation, with the exception of the results obtained from the 17 day assays. This deviation in the 17 day fractions was undoubtedly due to the increase in the specific activity and concomittant increase in the endo activity of these fractions. The demonstration of this correlation between the reducing group assays and cup plate assay justifies the use of the cup plate assay as a reliable method of measuring PG activity. However, these results differ from those reported by Swinburne and Corden (88) who reported a correlation between the cup plate assay and viscosity reduction assay, but not necessarily with the reducing group assay. The discrepancy which exists in the correlation between these assays may be explained from the standpoint that the PG was synthesized by different fungi and may not be the same enzyme or more probably because Swinburne and Corden (88) used the DNS assay to measure PG activity.

The PG produced by F. oxysporum f. sp. lycopersici chromatographed, by gel filtration (Figure 5A), quite differently than was reported by Swinburne and Corden (89) despite the fact that they used Sephadex G-200. Their results indicated the detection of two PG peaks with molecular weights of approximately 200,000 and 50,000 while the data reported here for similar culture filtrates by gel filtration (Sephadex G-75) resulted in the detection of two peaks of PG activity, however, the molecular weights were 22,800 and $\leq$ 2,000, respectively. The results obtained by Swinburne and Corden (89) may have been due in part to ion exchange since the concentration of the buffer (0.01 M) elutant was insufficient to overcome the native charge of the dextran. According to the manufacturer, buffer concentrations greater than 0.02 M are required to negate this charge.

The results reported here for the characterization of PG by DEAE-cellulose column chromatography were similar to those reported by Young (101, p. 34-61) and Swinburne and Corden (89), i.e. 90% of the PG activity was eluted with the void volume while the remainder of the activity was adsorbed to the exchanger. Furthermore, Swinburne and Corden reported that chromatography of the dialyzed culture filtrate resulted in the production of a single adsorbed peak on Duolite CS-101 (cation exchanger). The rechromatography of this Duolite peak also resulted in the production of a single peak. However, the results reported here showed a small peak eluted with

the void volume and a large adsorbed peak for the dialyzed culture filtrate (Figure 6B1) when chromatographed on CM-cellulose, but a single adsorbed peak was detected on chromatography of the Enz^{+} fraction obtained from the CM-batchwise treatment. The detection of a nonadsorbed peak of PG activity on CM columns from the pectin culture filtrates was confirmed by the detection of an adsorbed peak following DEAE column chromatography.

The number of peaks produced by PG fractions from the pectin and glucose cultures on ion exchange chromatography was correlated to the increasing carbohydrate concentration and age of any given enzyme fraction. The number of peaks and shifts to larger molecular weights which these enzyme fractions produced on Sephadex G-75 columns was also found to be correlated to the increasing carbohydrate concentration and age of the cultures. Therefore, these results suggest that the alteration in the chromatographic pattern of the PG reported by Swinburne and Corden (89) as due to the disassociation of an enzyme substrate complex could be achieved by the removal of some of the carbohydrate associated with the enzyme. The failure to detect an increase in the reducing groups in the enzyme fractions during storage further documents the formation of a stable enzyme-substrate complex.

Swinburne and Corden (88) also reported the alteration in the mode of action of endo-PG, produced by _Penicillium expansum_, was

due to a modification in the secondary or tertiary structure of the enzyme during ion exchange chromatography. However, based on the results reported here, it would seem that this increased activity was probably due to the removal of some of the carbohydrate material associated with the enzyme with a possible increase in the number of binding sites. Furthermore, McClendon and Kreisher (51) attributed the failure to effectively separate PG from other enzymes by a pH gradient on a cellulose-phosphate column was due to the presence of other (carbohydrate) compounds in their enzyme preparations. This would also explain the inability to separate PE from PG reported here and by Young (101, p. 34-61).

SUMMARY

1. The results of the pectin, PGA, and glucose time course studies showed that *F. oxysporum* f. sp. *lycopersici* produced polygalacturonase, pectin methyl trans-eliminase and pectin esterase but not polygalacturonate trans-eliminase on these carbon sources. Pectin induced the maximum synthesis of PG and PE. When PGA was used as the sole carbon source, the major enzyme synthesized was PMTE with only trace amounts of PG and PE being detected. Trace amounts of PG and PMTE were produced on glucose, but more PE was produced on glucose than on PGA.

2. Using improved viscosity reduction, reducing group, and TLC assays, the pectic enzymes isolated from the pectin, PGA, and glucose cultures were characterized as being endo-PG and endo-PMTE. The results obtained from the TLC of the PG-reaction-mixtures indicated the preferential release of monogalacturonic acid which has been used as the criterion for the determination of exo activity. However, this theory was rejected as only a small number of the available α-1,4 linkages were hydrolyzed at high levels of viscosity reduction.

3. The use of the cup plate assay as a method of measuring PG activity was established by showing that the results obtained from this assay were directly related to the results obtained

from the improved viscosity reduction and reducing group assays.

4. The CM-cellulose batchwise purification showed that 90% of the pectin endo-PG was adsorbed (Enz^+) and represented a 24-30 fold increase in specific activity. The majority of the pectin produced PE activity was also found in the Enz^+ fraction while the majority of the small quantity of endo-PMTE which remained following dialysis was found in the fractions eluted (Enz^-) with the void volume. All of the pectolytic enzyme activity in the PGA culture filtrates was lost following dialysis against acetate buffer (pH 4.0). However, the majority of the endo-PG present in the glucose cultures was found in the Enz^- fractions.

5. A heretofore undescribed enzyme was isolated from the glucose cultures. This enzyme preferentially hydrolyzed NaPP, and an unidentified aldohexose that was probably either glucose or galactose was the major hydrolytic product present in these enzyme-reaction-mixtures. It is suggested that this NaPP hydrolyzing enzyme has been misclassified as a PG. It is suggested that this enzyme is responsible for the pectolytic enzyme activity reported in the literature for PG produced on glucose media. This enzyme would appear to be related to the hemicellulases.

6. The chromatographic patterns obtained from the characterization of the pectin, PGA, and glucose produced enzyme fractions by gel filtration and ion exchange chromatography showed a correlation with the amount of carbohydrate material remaining attached to the enzymes. The molecular weight of the enzyme and the number of peaks produced on Sephadex G-75 and ion-exchange columns increased with increasing carbohydrate concentration and incubation period. Thus, the dialyzed and Enz^- fractions normally produced more peaks on the Sephadex G-75 and ion exchange columns than did the culture filtrate (only Sephadex) or the Enz^+ fractions. The accurate determinations of the molecular weight and ion exchange properties of these enzyme fractions including the individual pectolytic enzymes present in each fraction will not be achieved until this attached carbohydrate material is removed or shown to be required for enzymatic activity.

BIBLIOGRAPHY

1. Akinrefon, O. A. Influence of isolates and age of cultures on extracellular enzymatic patterns of Phytophthora palmivora. (Butl.) Butl. Biochemical Journal 106:38. 1968.

2. Albersheim, P. and Ursula Killias. Studies relating to the purification and properties of pectin transeliminase. Archives of Biochemistry and Biophysics 97:107-115. 1962.

3. Albersheim, P., H. Neukom and H. Deuel. Splitting of pectin chain molecules in neutral solutions. Archives of Biochemistry and Biophysics 90:46. 1960.

4. Ali, M. M. and M. A. Islam. Pectic enzymes of Penicillium frequentans involved in the retting of jute. Pakistan Journal of Science and Industrial Research 8:47-51. 1965.

5. Andrews, P. Estimation of the molecular weights of proteins by Sephadex gel filtration. Biochemical Journal 91:222-233. 1964.

6. Arima, K., M. Yamasaki and T. Yasui. Studies on pectic enzymes of microorganisms. Part I. Isolation of microorganisms which specifically produce one of several pectic enzymes. Agricultural Biological Chemistry 28:248-254. 1964.

7. Ayers, W. A., G. C. Papavizas and A. F. Diem. Polygalacturonate trans-eliminase and polygalacturonase production by Rhizoctonia solani. Phytopathology 56:1006-1011. 1966.

8. Barash, I. Liberation of polygalacturonase during spore germination by Geotrichum candidum. Phytopathology 58: 1364-1371. 1968.

9. Barash, I. and L. Klein. The surface localization of polygalacturonase in spores of Geotrichum candidum. Phytopathology 59:319-324. 1969.

10. Bateman, D. F. Alteration of cell wall components during pathogenesis by Rhizoctonia solani. In: The dynamic role of molecular constituents in plant-parasite interaction, ed. by C. J. Mirocha and I. Uritani. St. Paul, Bruce Publishing Company, 1967. p. 58-75.

11. Bateman, D. F. Cellulase and the Rhizoctonia disease of bean. Phytopathology 56:1372-1377. 1966.

12. ________________ Hydrolytic and trans-eliminative degradation of pectic substances by extracellular enzymes of Fusarium solani f. phaseoli. Phytopathology 56:238-244. 1966.

13. Bateman, D. F. and R. L. Millar. Pectic enzymes in tissue degradation. Annual Review of Phytopathology 4:119-146. 1966.

14. Black, H. S. Pectolytic enzyme production by Phymatotrichum omnivorum. (Abstract) Phytopathology 58:1044. 1968.

15. Blackhurst, F. M. and R. K. S. Wood. Verticillium wilt of tomatoes--further experiments on the role of pectic and cellulolytic enzymes. Annals of Applied Biology 52:89-96. 1963.

16. Block, R. J., E. L. Durrum and G. Zweig. Manual of paper chromatography and paper electrophoresis. New York, Academic Press, 1958. 710p.

17. Bush, D. A. and R. C. Codner. The nature of macerating factor of Penicillium digitatum Saccardo. Phytochemistry 7: 863-869. 1968.

18. Byrde, R. J. W. and A. H. Fielding. Pectin methyl-trans-eliminase as the maceration factor of Sclerotinia fructigena and its significance in brown rot of apple. Journal of General Microbiology 52:287-297. 1968.

19. ________________ Resolution of endopolygalacturonase and a macerating factor in a fungal culture filtrate. Nature 196: 1227-1228. 1962.

20. Cappellini, R. A. Growth and polygalacturonase production by Rhizopus stolonifer. Phytopathology 56:734-737. 1966.

21. Capellini, R. A. and J. L. Peterson. Production, in vitro, of certain pectolytic and cellulolytic enzymes by fungi associated with corn stalk rot. Bulletin of The Torrey Botanical Club 93: 52-55. 1966.

22. Clarke, D. D. Production of pectic enzymes by Phytophthora infestans. Nature 211:649. 1966.

23. Corden, M. E., J. E. Elson and D. L. Bell. Inhibition of polygalacturonase by cations. (Abstract) Phytopathology 54: 1432. 1964.

24. Dean, Margaret and R. K. S. Wood. Cell wall degradation by a pectate transeliminase. Nature 214: 408-410. 1967.

25. Deese, D. C. and M. A. Stahmann. Pectic enzymes and cellulase formation by _Fusarium oxysporum_ f. _cubense_ on stem tissues from resistant and susceptible banana plants. Phytopathology 52: 247-254. 1962.

26. ________________ Pectic enzymes in _Fusarium_ susceptible and resistant tomato plants. Phytopathology 52: 255-260. 1962.

27. ________________ Wilt resistance in tomatoes. Pectic enzyme formation by _Fusarium oxysporum_ f. _lycopersici_ on susceptible and resistant tomato stems. Journal of Agricultural and Food Chemistry 10: 145-150. 1962.

28. Demain, A. L. and H. J. Phaff. Recent advances in the enzymatic hydrolysis of pectic substances. Wallerstein Laboratory Communications 20: 119-140. 1957.

29. Dimond, A. E. Physiology of wilt disease. In: The dynamic role of molecular constituents in plant-parasite interaction, ed. by C. J. Mirocha and I. Uritani. St. Paul, Bruce Publishing Company, 1967. p. 100-118.

30. Dingle, J., W. W. Reid and G. L. Solomons. The enzymic degradation of pectic and other polysaccharides. II. Application of the cup-plate assay to the estimation of enzymes. Journal of Food Science in Agriculture 4: 149-155. 1953.

31. Garber, E. D. Genetics of phytopathogenic fungi. XVII. An electrophoretic study of extracellular and intracellular endopolygalacturonases from virulent and avirulent strains of _Penicillium italicum_. Phytopathologische Zeitschrift 59: 147-152. 1967.

32. Garber, E. D. and L. Beraha. Genetics of phytopathogenic fungi. Pectolytic enzymes of virulent and avirulent strains of three phytopathogenic _Penicillia_. Canadian Journal of Botany 44: 1645-1650. 1966.

33. Gordon, H. T., W. Thornburg and L. N. Werum. Rapid paper chromatography of carbohydrates and related compounds. Analytical Chemistry 28: 849-855. 1956.

34. Gremli, H. and H. Neukom. The effect of a purified α-L-arabinofuranosidase on some arabinose-containing polysaccharides. Carbohydrate Research 8: 110-112. 1968.

35. Grossmann, F. Therapeutische Wirkung von pektinase-Hemmstoffen gegen die Fusarium-welke der Tomate. Naturwissenischaften 49: 138-139. 1962.

36. ________________ Untersuchungen uber die Hemmung pektolytischer Enzyme von Fusarium oxysporum f. lycopersici I. Hemmung durch definierte substanzen in vitro. Phytopathologische Zeitschrift 44: 361-380. 1962.

37. Gupta, S. C. and G. S. Rautela. Studies in pectic enzymes of parasitic fungi. V. Pectic enzymes produced by Penicillium expansum Link. Indian Phytopathology 27: 191-201. 1964.

38. Hancock, J. G. Degradation of pectic substances associated with pathogenesis by Sclerotinia sclerotiorum in sunflower and tomato stems. Phytopathology 56: 975-979. 1966.

39. ________________ Hemicellulase degradation in sunflower hypocotyls infected with Sclerotinia sclerotiorum. Phytopathology 57: 203-206. 1967.

40. ________________ Pectate lyase production by Colletotrichum trifolii in relation to changes in pH. Phytopathology 56: 1112-1113. 1966.

41. Hancock, J. G. and R. L. Millar. Relative importance of polygalacturonate trans-eliminase and other pectolytic enzymes in southern anthracnose, spring black stem, and stemphylium leaf spot of alfalfa. Phytopathology 55: 346-355. 1965.

42. Hancock, J. G. and M. E. Stanghellini. Calcium localization in Hypomyces-infected squash hypocotyls and effect of calcium on pectate lyase activity and tissue maceration. Canadian Journal of Botany 46: 405-409. 1968.

43. Heitefuss, R., M. A. Stahmann and J. C. Walker. Production of pectolytic enzymes and fusaric acid by Fusarium oxysporum

f. conglutinans in relation to cabbage yellows. Phytopathology 50:367-370. 1960

44. Horton, J. C. and N. T. Keen. Sugar repression of endopolygalacturonase synthesis during pathogenesis by Pyrenochaeta terrestris as a resistance mechanism in onion pink root. Phytopathology 56:908-916. 1966.

45. Jansen, E. F. and L. R. MacDonnell. Influence of methoxyl content of pectic substances on the action of polygalacturonase. Archives of Biochemistry 8:97-112. 1945.

46. Keen, N. T. and J. C. Horton. Induction and repression of endopolygalacturonase synthesis by Pyrenochaeta terrestris. Canadian Journal of Microbiology 12:443-453. 1966.

47. ________________ The polygalacturonase of Pyrenochaeta terrestris. Phytopathology 56:603-609. 1966.

48. Kertesz, Z. I. The pectic substances. New York, Interscience Publishers, 1953. 628p.

49. Lowery, O. H., N. S. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with Folin phenol reagent. Journal of Biological Chemistry 193:265-275. 1951.

50. Lumsden, R. D. and D. F. Bateman. Pectic enzymes detected in culture filtrates of Thielaviopsis basicola and in extracts of Thielaviopsis-infected bean root tissue. (Abstract) Phytopathology 56:585. 1966.

51. McClendon, J. H. and J. H. Kreisher. Chromatography on cellulose phosphate of polysaccharide hydrolases from fungi. Analytical Biochemistry 5:295-312. 1963.

52. McComb, E. A. and R. M. McCready. Colorimetric determination of pectic substances. Analytical Chemistry 24:1630-1632. 1952.

53. McIntyre, G. A. Some characteristics of polygalacturonase produced by Verticillium albo-atrum. (Abstract) Phytopathology 55:130. 1965.

54. Mann, Brenda. Role of pectic enzymes in the Fusarium wilt syndrome of tomato. Transactions of the British Mycological Society 45:169-178. 1962.

55. Miller, G. L. Use of dinitrosalicylic acid reagent for the determination of reducing sugar. Analytical Chemistry 31:426-428. 1959.

56. Moore, L. D. and H. B. Couch. Influence of calcium nutrition on pectolytic and cellulolytic enzyme activity of extracts of highland bentgrass foliage blighted by _Pythium ultimum._ Phytopathology 58:833-838. 1968.

57. Moran, F., S. Nasuno and M. P. Starr. Extracellular and intracellular polygalacturonic acid trans-eliminase of _Erwinia carotovora_. Archives of Biochemistry and Biophysics 123: 298-306. 1968.

58. Mussell, H. W. and R. J. Green, Jr. Production of polygalacturonase by _Verticillium albo-atrum_ and _Fusarium oxysporum_ f. _lycopersici in vitro_ and in vascular tissue of susceptible and resistant hosts. (Abstract) Phyotpathology 58:1061. 1968.

59. Narasaki, T. Enzymatic hydrolysis of soybean polysaccharides. I. Hydrolysis of the hemicellulose B_1 of soybean cotyledons by take-diastase. Technical Bulletin of the Faculty of Agriculture Kagawa University 18:16-22. 1966.

60. Nasuno, S. and M. P. Starr. Pectic enzymes of _Pseudomonas marginalis_. Phytopathology 56:1414-1415. 1966.

61. ________________ Polygalacturonase of _Erwinia carotovora._ Journal of Biological Chemistry 241:5298-5306. 1966.

62. ________________ Polygalacturonic acid trans-eliminase of _Xanthomonas campestris_. Biochemical Journal 104:178-185. 1967.

63. Nelson, N. A photometric adaptation of the Somogyi method for the determination of glucose. Journal of Biological Chemistry 153:375-380. 1944.

64. Neukom, H. Uber Farbreaktionen von Uronsauren mit Thiobarbitursäure. Chima 14:165-167. 1960.

65. Page, O. T. Quantitative paper chromatographic techniques for the assay of products of polygalacturonase activity of fungus cultures. Phytopathology 51:337-338. 1961.

66. Pandey, D. K. and S. C. Gupta. Studies in pectic enzymes of parasitic fungi. VI. Factors affecting the secretion of pectic enzymes by Alternaria tenuis. Biologia Plantarum 8:131-141. 1966.

67. Papavizas, G. C. and W. A. Ayers. Polygalacturonate trans-eliminase production by Fusarium oxysporum and Fusarium solani. Phytopathology 56:1269-1273. 1966.

68. Paquin, R. and L. J. Coulombe. Pectic enzyme synthesis in relation to virulence in Fusarium oxysporum f. lycopersici (Sacc.) Synder and Hansen. Canadian Journal of Botany 40: 533-541. 1962.

69. Patel, D. S. and H. J. Phaff. Properties of purified tomato polygalacturonase. Food Research 25:47-57. 1960.

70. Patil, S. S. and A. E. Dimond. Effect of phenols and cytokinins on polygalacturonase production by Verticillium albo-atrum in culture. Phytopathology 58:868-869. 1968.

71. ______________ Effect of sugars and sugar alcohols on production of polygalacturonase by Fusarium oxysporum f. sp. lycopersici. (Abstract) Phytopathology 57:825. 1967.

72. ______________ Induction and repression of polygalacturonase synthesis in Verticillium albo-atrum. (Abstract) Phytopathology 57:825. 1967.

73. ______________ Inhibition of Verticillium polygalacturonase by oxidation products of polyphenols. Phytopathology 57:492-496. 1967.

74. ______________ Repression of polygalacturonase synthesis in Fusarium oxysporum f. sp. lycopersici by sugars and its effect on symptom reduction in infected tomato plants. Phytopathology 58:676-682. 1968.

75. Reinganum, C. Pectolytic enzyme production by Sclerotinia fructicola (Wint.) Rehm. and its role in the pathogenesis of stone fruits. Australian Journal of Biological Science 17:705-718. 1964.

76. Rexova-Benkova, L. Characterization of extracellular polygalacturonases of Aspergillus niger. Collection of Czechoslovakia Communications 32:4504-4509. 1967.

77. Rexova-Benkova, L. and A. Slezarik. Isolation of extracellular pectolytic enzymes produced by *Aspergillus niger*. Collection of Czechoslovakia Communications 31:122-129. 1966.

78. ______________ Molecular weight and amino acid composition of *Aspergillus niger* endopolygalacturonase. Collection of Czechoslovakia Communications 33:1965-1967. 1968.

79. Sherwood, R. T. Pectin lyase and polygalacturonase production by *Rhizoctonia solani* and other fungi. Phytopathology 56: 279-286. 1966.

80. Sherwood, R. T. and A. Kelman. Measurement of pectinolytic and cellulolytic enzyme acdivity by rotating spindle viscometry. Phytopathology 54:110-112. 1964.

81. Singh, G. P. and A. Hussain. Relation of hydrolytic enzyme activity with the virulence of strains of *Colletotrichum falcatum*. Phytopathology 54:1100-1101. 1964.

82. Slezarik, A. and L. Rexova. Characterization of extracellular pectolytic enzymes of *Monilia laxa*. Biologia Bratislava 22: 407-413. 1967.

83. Somogyi, M. Notes on sugar determination. Journal of Biological Chemistry 195:19-23. 1952.

84. Smith, W. K. A survey of the production of pectic enzymes of plant pathogenic and other bacteria. Journal of General Microbiology 18:33-41. 1958.

85. Spalding, D. H. Production of pectinolytic and cellulolytic enzymes by *Rhizopus stolonifer*. Phytopathology 53:929-931. 1963.

86. Starr, M. P. and F. Moran. Eliminative split of pectic substances by phytopathogenic soft-rot bacteria. Science 135:920-921. 1962.

87. Starr, M. P. and S. Nasuno. Pectolytic activity of phytopathogenic *Xanthomonads*. Journal of General Microbiology 46:425-433. 1967.

88. Swinburne, T. R. and M. E. Corden. A comparison of the polygalacturonase produced *in vivo* and *in vitro* by *Penicillium*

expansum Thom. Journal of General Microbiology 55: 75-87. 1969.

89. Swinburne, T. R. and M. E. Corden. Dissociation and recombination of a polygalacturonase complex during ion exchange chromatography. Nature 213:286-287. 1967.

90. Unestam, T. Chitinolytic, cellulolytic, and pectinolytic activity in vitro of some parasitic and saprophytic oomycetes. Physiologia Plantarum 19:15-30. 1966.

91. Wager, H. G. An improved copper reduction method for the micro-determination of reducing sugars. Analysist 79:34-38. 1954.

92. Waggoner, P. E. and A. E. Dimond. Production and role of extracellular enzymes of Fusarium oxysporum f. lycopersici. Phytopathology 45: 79-87. 1955.

93. Wallace, J., J. Kuc and E. B. Williams. Production of extracellular enzymes by four pathogens of apple fruit. Phytopathology 52:1004-1009. 1962.

94. Wells, J. M. Growth of Rhizopus stolonifer in low-oxygen atmospheres and production of pectic and cellulolytic enzymes. Phytopathology 58:1598-1602. 1968.

95. Whistler, R. L. and M. L. Wolfrom. Methods in carbohydrate chemistry. Vol. I. New York, Academic Press. 1962. 589p.

96. Wiese, M. V., J. E. DeVay and A. Ravenscroft. Virulence of Verticillium albo-atrum in cotton in relation to colony characteristics and endopolygalacturonase production. (Abstract) Phytopathology 58:1072. 1968.

97. Winstead, N. N. and C. L. McCombs. Production of pectinolytic and cellulolytic enzymes by curcurbit anthrancnose fungi. Phytopathology 53:961-964. 1963.

98. Wood, R. K. S. Physiological plant pathology. Vol. 6. Oxford, Blackwell Scientific Publications. 1967. 570p.

99. Yamasaki, M., T. Yasui and K. Arima. Studies on pectic enzymes of microorganisms. Part II. Production of endo-polygalacturonase with Aspergillus saitoi. Agricultural and

Biological Chemistry 30: 142-148. 1966.

100. Yamasaki, M., T. Yasui and K. Arima. Studies on pectic enzymes of microorganisms. Part III. Endo-polyglacturonase of _Aspergillus saitoi_. Agricultural and Biological Chemistry 30: 1119-1128. 1966.

101. Young, R. J. Characterization of pectic enzymes produced by _Fusarium oxysporum_ f. _lycopersici_. Ph.D. thesis. Corvallis, Oregon State University, 1963. 92 numb. leaves.

102. Young, R. J. and M. E. Corden. Paper chromatography of galacturonic acids to determine polygalacturonase activity. Biochimica et Biophysica Acta 17: 124-125. 1964.

103. Youssef, Y. A. The production of pectic and cellulolytic enzymes and their role in the development of _Myrothecium_ root rot of kidney bean. Phytopathologische Zeitschrift 46: 11-16. 1962.