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ISOZYMES PRODUCED BY FUSARIUM OXYSPORUM

f. sp. LYCOPERSICI

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Polygalacturonase produced by Fusarium oxysporum f. sp. lycopersici was purified by chromatography on DEAE cellulose, CM cellulose, and hydroxylapatite. Removal of large amounts of carbohydrate by chromatography on hydroxylapatite did not affect heat stability of the enzyme. A large proportion of the remaining carbohydrate appeared to be covalently linked to the enzyme protein. The purified enzyme consisted of two electrophoretically distinct "isozymes." The two had similar "endo" modes of action on polygalacturonic acid, as determined by comparison of viscosity reduction, reducing group release, and thin layer chromatography of oligomeric hydrolysis products. Both isozymes hydrolyzed 5% of the substrate

bonds in reaching 50% viscosity reduction. The amino acid compositions of the isozymes were similar and their molecular weights were about 37,000 as determined by sedimentation equilibrium. Electrophoresis in several different concentrations of polyacrylamide gel indicated the two isozymes were charge isomers.

Characterization of Two Endopolygalacturonase
Isozymes Produced by Fusarium oxysporum
f. sp. lycopersici

by

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CHARACTERIZATION OF TWO ENDOPOLYGALACTURONASE
ISOZYMES PRODUCED BY FUSARIUM OXYSPORUM
f. sp. LYCOPERSICI

INTRODUCTION

Polygalacturonase (poly, α -1, 4-galacturonide glycanohydrolase, E. C. 3.2.1.15) plays an important role in a number of plant diseases (2, 3, 6, 39). This enzyme has been implicated in vascular wilt of tomato caused by Fusarium oxysporum f. sp. lycopersici, but its importance in this disease has been questioned (4, 40). Recent work has provided evidence for induction of foliar symptoms of cotton wilt, a syndrome different from that of Fusarium wilt of tomato, by purified Verticillium endopolygalacturonase (29). Polygalacturonases produced by F. oxysporum f. sp. lycopersici and other fungi are able to degrade cell walls extensively (16, 19) and to "prepare" cell wall components for degradation by other enzymes (16, 20). These enzymatic processes are especially important to saprophytes and facultative saprophytes involved in the decay of plant litter.

Basic to understanding the behavior of polygalacturonases in living and dead material is a knowledge of the chemical properties of these enzymes. Compositions of polygalacturonases from Verticillium albo-atrum (41) and Aspergillus niger (34) have been determined. The polygalacturonase produced by F. oxysporum f. sp. lycopersici has not been characterized at this level. Previous work (24) established

a scheme for purifying this enzyme. The purified enzyme appeared homogeneous in disc gel electrophoresis and behaved as an endopolygalacturonase in combined hydrolysis and viscosity reduction assays. Carbohydrate was present in the purified preparation, and large amounts of carbohydrate were separated from the enzyme during purification. Carbohydrate non-covalently associated with some enzymes influences their heat stability (17, 22). The present study reports the presence of two charge isomers ("isozymes") of Fusarium polygalacturonase and characterizes their modes of action, amino acid compositions, and molecular weights. Also investigated were the possibilities that carbohydrate in the purified preparation is covalently linked to the enzyme, and that carbohydrate associated with the enzyme but removed during purification has a stabilizing influence on the enzyme.

MATERIALS AND METHODS

Growth of Fungus

Fusarium oxysporum f. sp. lycopersici (Sacc.) Snyder and Hans. race 1 was grown in shake culture on a medium containing 5.0 g NH_4NO_3 , 2.5 g KH_2PO_4 , 1.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 40 mg $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 20 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 10 g pectin N. F. (Sunkist product no. 3442) per liter. Initial culture pH was 4.4. After six days growth at room temperature under diurnal light, the culture filtrate was harvested by centrifugation. This and all subsequent enzyme solutions were sterilized by filtration through a Millipore HA filter (0.45 μ) and addition of sodium azide to 0.02%.

Enzyme Assays

For routine determination of polygalacturonase activity during fractionation procedures, a cup-plate assay (9) was used. A 1.5% agar gel containing 1.0% sodium polypectate (Sunkist product no. 6024) buffered at pH 5.0 with 0.1 M sodium acetate buffer was incubated for 16 hours at 40°C after adding 0.1 ml enzyme samples to wells in the gel. The diameters of zones developed with 5 N HCl were linearly related to the logarithm of enzyme concentration. With the standard curve used, 20 mm and 27 mm zones corresponded to 25 units and 725 units, respectively. This assay, although using a

substrate that was only about 55% uronide, gave results that were highly correlated with assays for the release of reducing groups from polygalacturonic acid.

Polygalacturonase activity was quantitated by measuring the release of reducing groups from polygalacturonic acid (Sunkist product no. 3491) using the Somogyi assay (35) with Nelson's arsenomolybdate reagent (30). The zinc and barium deproteinizing treatments were omitted, and precipitated substrate was removed by filtration after reaction with the arsenomolybdate. Equivalents of reducing groups released were based on a standard curve relating the absorbance at 540 nm to μ moles galacturonic acid monohydrate (Pfanstiel), and one unit of activity was defined as that amount of enzyme releasing one μ mole galacturonic acid reducing equivalents per minute from 1.0% polygalacturonic acid at 30°C. Before use, the polygalacturonic acid was purified by precipitation from a filtered 5% solution with acidified 80% ethanol, and extraction of the precipitate with 7×10^{-4} % dithizone (37) in acidified 80% ethanol (21). The purified product was 100% galacturonic acid by carbazole (27) and phenol-sulfuric acid (10) assays. Measurement of reducing ends by the modified Somogyi assay gave a \bar{M}_N of $13,900 \pm 200$ for the purified polygalacturonic acid, assuming it to be 100% galacturonide. This is an average chain length of 80 galacturonic acid residues.

Pectinesterase activity was estimated with a modification of a cup-plate method (32) using 1.0% pectin as substrate and 0.04% bromocresol purple as the indicator. The standard curve relating diameter of the yellow zones developed after 16 hours at 40°C to logarithm of the enzyme concentration was not linear. A smooth curve fitted to the points was used, with a 30 mm zone defined as representing 1000 units of activity.

Protein Assay

Relative protein concentrations during fractionations were followed by reading absorption at 280 nm. The Lowry assay (26) with a bovine serum albumin (Sigma fraction V) standard curve was used to quantitate protein in enzyme fractions.

Carbohydrate Assay

To follow carbohydrate during fractionation and to quantitate carbohydrate in the various enzyme fractions, the phenol-sulfuric acid assay (10) was used.

Gel Electrophoresis

A modification of the polyacrylamide gel electrophoresis method of Reisfeld et al. (33) was used for homogeneity determinations and isomer studies. Running gels (7%) buffered at pH 4.3 and stacking

gels (2.5%) buffered at pH 6.0 with potassium acetate were used. Samples were layered onto the stacking gels in 1 M sucrose, and gels were run for three hours at a current of 4 mA per tube with a 0.35 M β -alanine-acetate buffer of pH 4.3. Gels were fixed in 12.5% trichloroacetic acid for one-half hour, stained for one-half hour with 0.05% Coomassie Brilliant Blue R-250 in the trichloroacetic acid, and destained with the trichloroacetic acid. To determine presence of polygalacturonase activity, unstained duplicate gels were sectioned and the sections were incubated for at least four hours with 1 ml portions of 0.1 M acetate buffer, pH 5.0, and then assayed in cup-plates.

RESULTS

Enzyme Purification

Twenty-five liters of culture filtrate were dialyzed against 10 mM potassium phosphate buffer, pH 6.0, and passed through DEAE cellulose (Sigma) regenerated with 1 N NaOH and equilibrated in the same buffer. The unadsorbed enzyme (> 99%) was dialyzed against 10 mM acetate buffer, pH 4.0, and chromatographed on CM cellulose (Bio-Rad Cellex CM) regenerated with 1 N HCl and equilibrated in the pH 4.0 buffer. The CM cellulose was eluted with a 0 to 0.8 M KCl gradient. A major protein peak associated with the polygalacturonase activity and the major carbohydrate peak was obtained (Figure 1A). An additional carbohydrate peak was obtained at the beginning of the elution.

Polygalacturonase peaks from eight CM columns (e.g., fractions 44-65 in Figure 1A) were pooled and divided into six equal portions. Each portion was dialyzed against distilled water, and adsorbed onto hydroxylapatite (Bio-Rad HTP) that had been regenerated with 1.0 M phosphate buffer, pH 6.0, and equilibrated with 10 mM phosphate buffer, pH 6.0. Enzyme was eluted with a 0 to 1.2 M KCl gradient in the buffer. Two minor protein peaks were obtained from the HTP in addition to the major polygalacturonase peak (Figure 1B), and most of the carbohydrate and pectinesterase was separated from

Figure 1. Column chromatographic fractionation of the polygalacturonase produced by *F. oxysporum* f. sp. *lycopersici*.

●—● = polygalacturonase activity by cup-plate assay

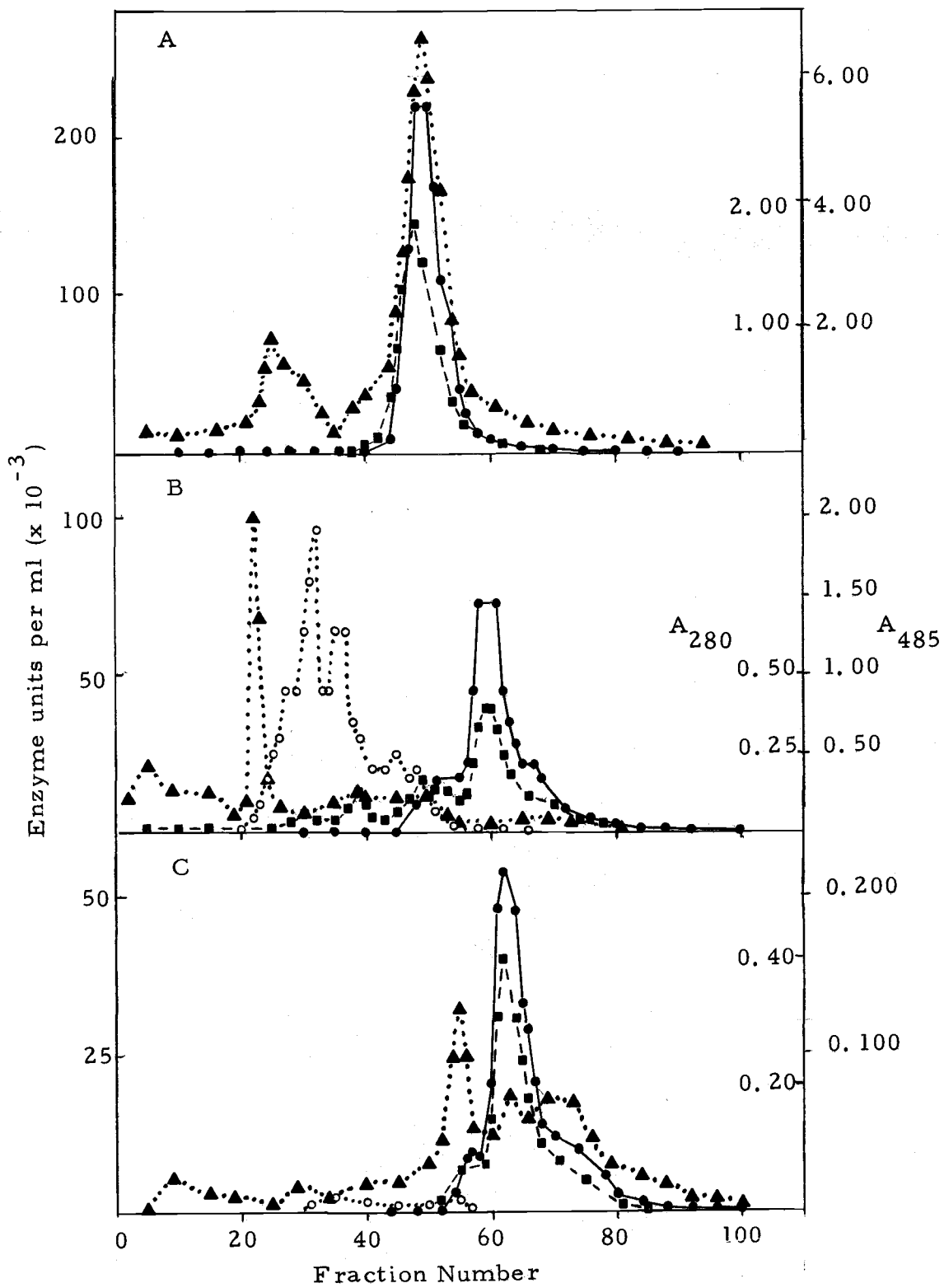
○····○ = pectinesterase activity by cup-plate assay

■---■ = protein as absorbance at 280 nm

▲····▲ = carbohydrate as absorbance at 485 nm in the phenol-sulfuric acid assay

Fraction volumes were 10 ml.

- (A) Elution of polygalacturonase activity, protein, and carbohydrate from a CM cellulose column (2.5 x 55 cm) with a 0 to 0.8 M KCl gradient in 10 mM acetate buffer, pH 4.0.
- (B) Elution of polygalacturonase and pectinesterase activities, protein, and carbohydrate from an hydroxylapatite column (2.5 x 39 cm) with a 0 to 1.2 M KCl gradient in 10 mM phosphate buffer, pH 6.0.
- (C) Elution of polygalacturonase and pectinesterase activities, protein, and carbohydrate from an hydroxylapatite column (2.5 x 38.5 cm) with a 0 to 1.2 M KCl gradient in 10 mM phosphate buffer, pH 6.0.



the polygalacturonase. The polygalacturonase peaks from the six columns (e. g., fractions 51-72 in Figure 1B) were pooled, and the HTP procedure repeated. During the second HTP chromatography, no pectinesterase activity was detected in the major polygalacturonase peak (Figure 1C), but there was still carbohydrate associated with the polygalacturonase. The peak fractions that were pooled after this step (e. g., fractions 59-83 in Figure 1C) did not include the minor shoulder peak of polygalacturonase (e. g., fractions 55-58 in Figure 1C) that contained pectinesterase. Thus, only the major polygalacturonase fraction was further characterized.

The second HTP chromatography represents the final step of the purification scheme used by Harman and Corden (24). Their final preparation was homogeneous by electrophoresis in 15% polyacrylamide containing 6.25 M urea and 0.9 M acetic acid. However, when the polygalacturonase obtained here was run in a 7% gel at pH 4.3, two protein bands with polygalacturonase activity, and a minor band with no polygalacturonase activity, were obtained (e. g., see Figure 5B). Thus, it appeared that the major polygalacturonase produced by F. oxysporum f. sp. lycopersici consisted of two components that migrated differently in disc gel electrophoresis. The slower-moving band was labelled PG₁, and the faster-moving PG₂. The difference in migration behavior of PG₁ and PG₂ may have been eliminated in the urea-acetic acid system used by Harman and Corden (24).

To attempt the separation of PG₁ from PG₂, and to remove the non-polygalacturonase protein, a portion of the enzyme from HTP was subjected to isoelectric focusing in a pH gradient of 3 to 10 using LKB 8102 Ampholine equipment. The focusing was run for 72 hours at about 18°C, during which the power dropped from 0.12 w to a constant 0.012 w. Six-ml fractions were collected and their pH, polygalacturonase activity, and protein content (A₂₈₀) were measured. A single major peak of polygalacturonase activity was obtained at pH 7.0 (Figure 2). This peak (fractions 37-41) was dialyzed against 1 M NaCl to remove Ampholine (31), then against 10 mM acetate buffer, pH 4.0, and assayed in gel electrophoresis. All three bands previously seen were present. Thus, electrofocusing under these conditions gave no increased purification or separation. An apparent minor polygalacturonase peak at pH 9.8 was probably an artifact caused by Ampholine reagents, because neither activity nor protein bands in electrophoresis were found after dialysis and concentration.

Gel filtration chromatography was used to estimate the molecular weights of PG₁ and PG₂. A portion of the enzyme preparation from HTP was concentrated by freeze-drying and redissolving in 50 mM acetate buffer, pH 5.1. One-half the concentrate was chromatographed on Bio-Gel P-100 (Bio-Rad Laboratories, 100-200 mesh) equilibrated with the buffer. A single peak of polygalacturonase activity was obtained from the column (Figure 3A). The central fraction of the

Figure 2. Isoelectric focusing of polygalacturonase from HTP. One percent ampholine ampholyte, pH 3-10, was used in a 0-40% sucrose gradient in the 440 ml LKB 8102 column. The anode was at the bottom of the column, and enzyme was added in the dense solution.

- = polygalacturonase activity by cup-plate assay
- = protein as absorbance at 280 nm
- = pH of fractions measured at the column temperature (about 18°C)

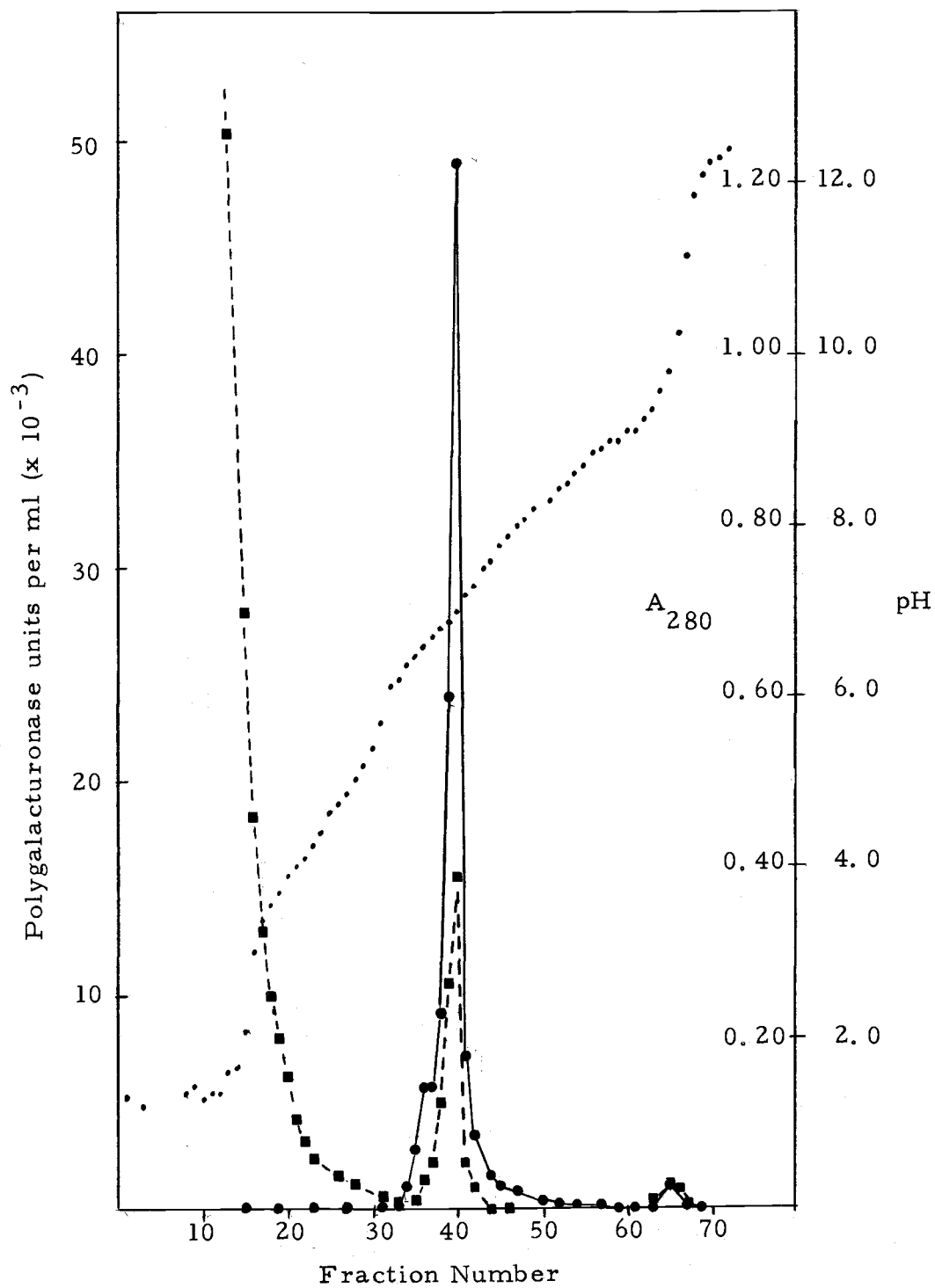


Figure 3. Gel filtration chromatography on Bio-Gel P-100 of the polygalacturonase preparation obtained from HTP.

●—● = polygalacturonase activity by reducing group assay

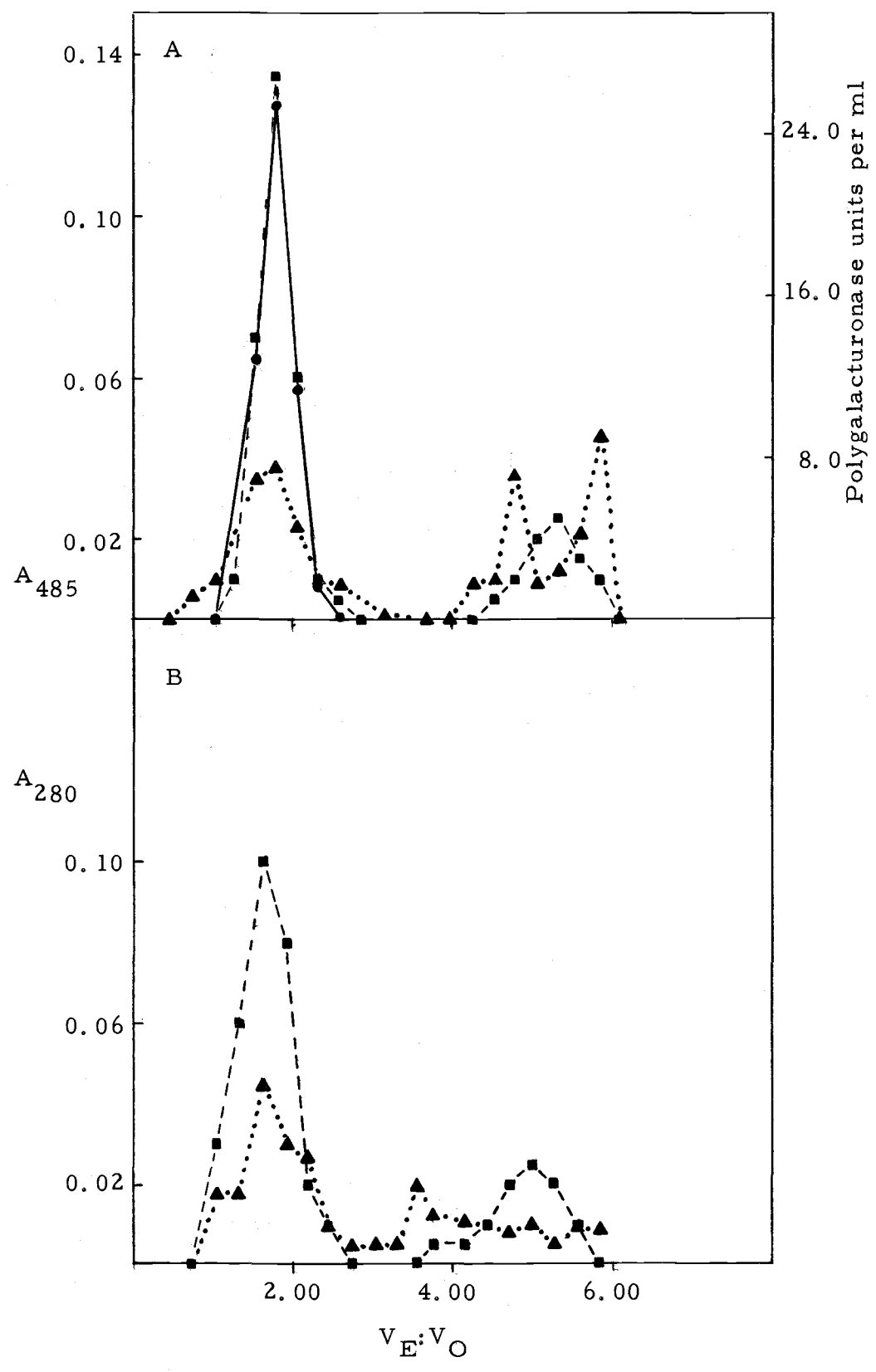
■---■ = protein as absorbance at 280 nm

▲.....▲ = carbohydrate as absorbance at 485 nm in the phenol-sulfuric acid assay.

Columns were eluted at 0.2 ml per min., and 3-ml fractions collected.

(A) Sample (2.0 ml, $A_{280} = 0.45$) applied to a P-100 column (1.5 x 28 cm) eluted with 50 mM acetate buffer, pH 5.1.

(B) Sample (2.4 ml, $A_{280} = 0.38$) containing 2 M guanidine hydrochloride applied to a P-100 column (1.5 x 28 cm) eluted with 2 M guanidine hydrochloride in the acetate buffer.



peak ($V_E:V_O = 1.8$) contained both PG bands in gel electrophoresis, but not the non-PG protein. The smaller, second peak from the column may have been the contaminant. These results indicate that PG_1 and PG_2 are about the same size. The P-100 column was calibrated with globular protein standards (cytochrome C, chymotrypsinogen, ovalbumin, and bovine serum albumin), and from a plot of $\log(\text{molecular weight})$ vs. $V_E:V_O$ a molecular weight of 35,000 was estimated for PG_1 and PG_2 . This is in agreement with the result of Harman and Corden (24) using Sephadex G-75.

Gel filtration under denaturing conditions was used to see if dissociation of enzyme into smaller subunits and of carbohydrate from enzyme would occur. Guanidine hydrochloride (Mann Ultra-Pure) was dissolved in one-half of the polygalacturonase concentrate to 2 M, a concentration that completely eliminated polygalacturonase activity. The denatured enzyme was chromatographed on a column of P-100 (100-200 mesh) equilibrated with 2 M guanidine hydrochloride in the pH 5.1 acetate buffer. Protein and carbohydrate peaks from this column (Figure 3B) were similar to those from the column without denaturant, except that the peaks were shifted to lower $V_E:V_O$ ratios. This shift would be expected for globular proteins unfolding in the presence of a denaturant, and was seen when the protein standards were chromatographed on the column containing guanidine hydrochloride. The results indicate that PG_1 and PG_2 are not composed of

subunits. The fact that guanidine hydrochloride, a denaturant that breaks hydrophobic, ionic, and hydrogen bonding, did not dissociate the carbohydrate from the polygalacturonase suggests that the carbohydrate is covalently linked to enzyme protein.

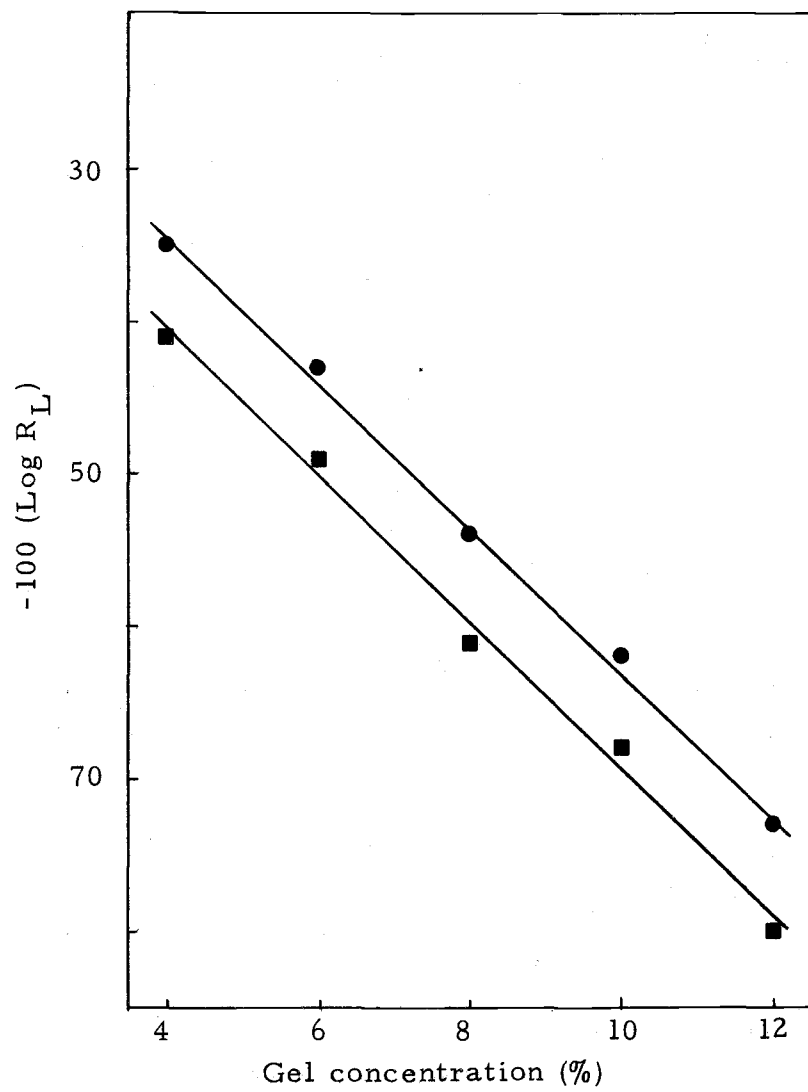
Size and charge isomers can be differentiated by electrophoresis in different concentrations of polyacrylamide gel (25). Therefore, the mixture of PG₁ and PG₂ was run in five different concentrations of the pH 4.3 running gel prepared by dilution from one polyacrylamide solution before polymerization. Relative mobilities (R_L) were determined with respect to total gel length. The plots of $\log R_L$ vs. gel concentration for PG₁ and PG₂ were parallel (Figure 4). This indicated that charge differences and not size differences were responsible for the different mobilities of PG₁ and PG₂, thus supporting the results of the gel filtration experiments. Separation of PG₁ from PG₂ by a fractionation procedure based on charge differences was suggested by these results.


The enzyme preparation from HTP was rechromatographed on CM cellulose in the same manner as before. Ten-ml fractions taken at points A, B, and C (Figure 5A) were electrophoresed and the stained gels were scanned at 620 nm using an SD3000 Spectrodensitometer and SDC Density Computer (Schoeffel Inst. Co.). The results of the scans and of activity determinations in duplicate gels were combined (Figure 5B). Fraction B contained PG₁ and PG₂ free of the non-PG protein, and fraction C contained PG₂ free of PG₁.

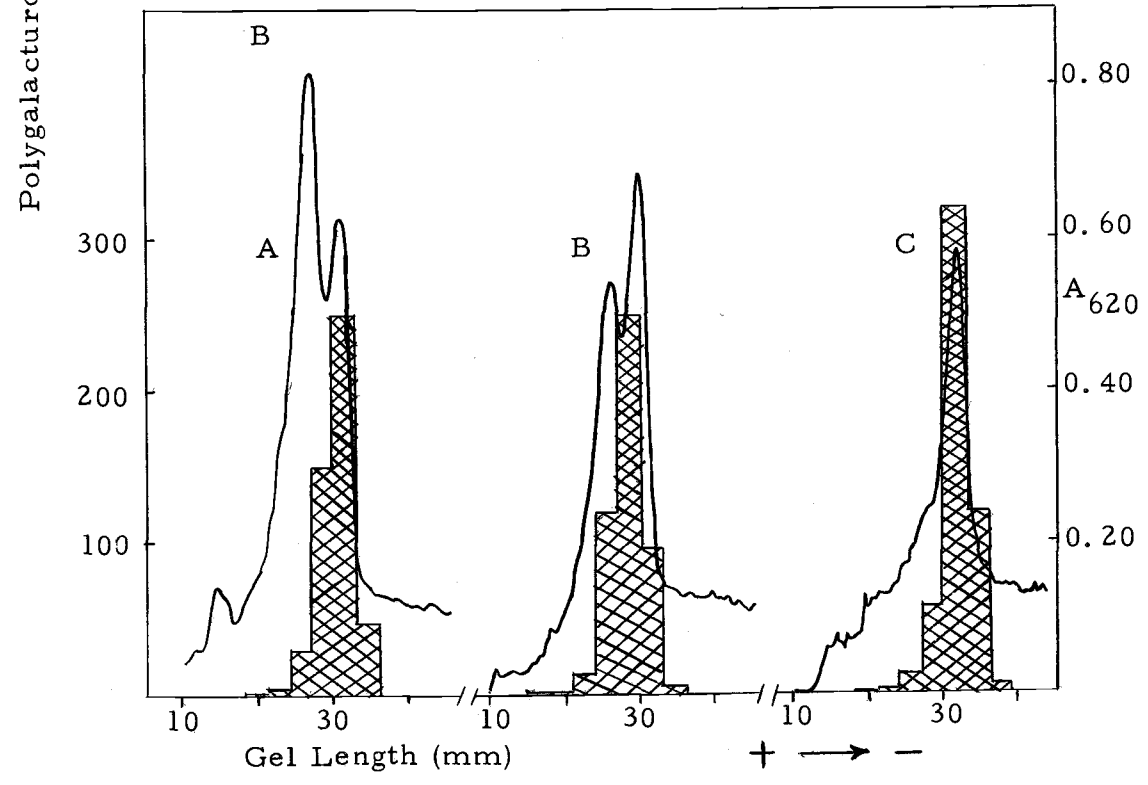
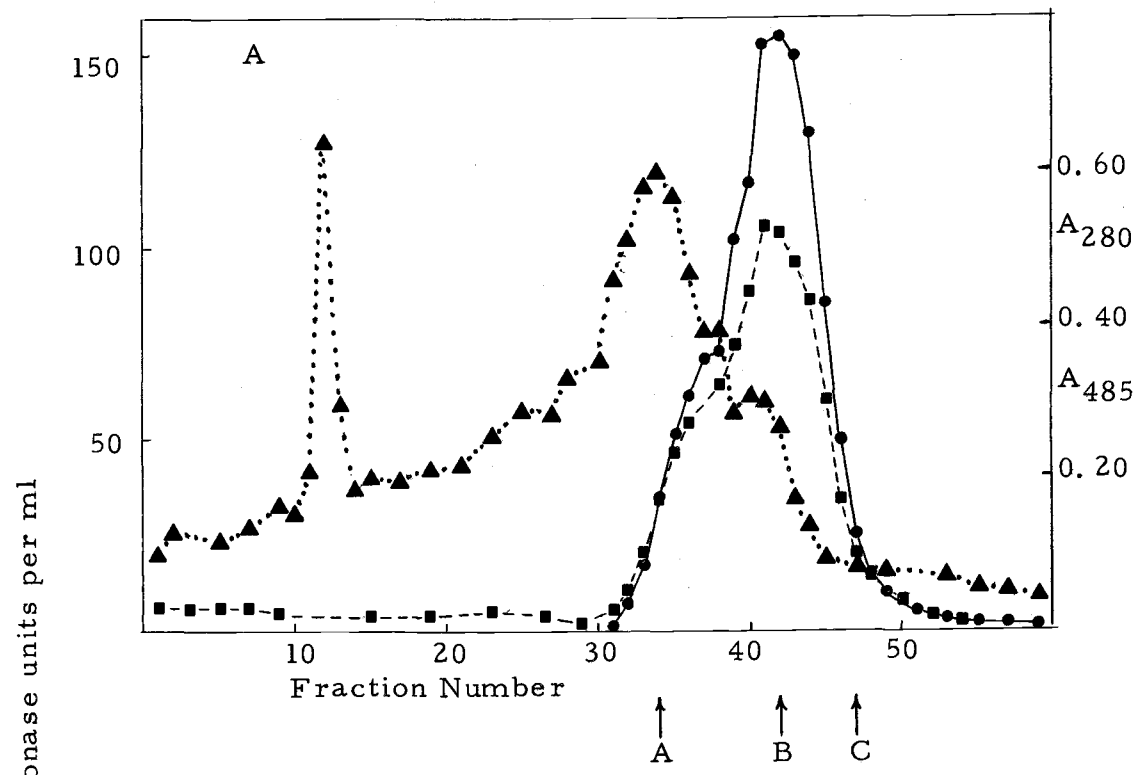
Figure 4. Relative electrophoretic mobilities (R_f = distance moved by band divided by length of gel) of the PG_1 and PG_2 bands in different concentrations of polyacrylamide gels. The pH 4.3 system was used. Gels were run for three hours at 4 mA per tube, and bands were stained with Coomassie Brilliant Blue R-250.

■ — ■ = PG_1
● — ● = PG_2

Regression slopes for both lines are -4.8.



- Figure 5. (A) Polygalacturonase from HTP applied to a CM cellulose column (2.5 x 53 cm) and eluted with a 0 to 0.8 M KCl gradient in 10 mM acetate buffer, pH 4.0.
- (B) Polyacrylamide gel electrophoresis (pH 4.3) of fractions from the CM cellulose column. Gels were stained with 0.05% Coomassie Brilliant Blue R-250, and polygalacturonase activity was determined in sections of duplicate gels by the cup-plate assay.
- = polygalacturonase activity by reducing group assay
 - = protein as absorbance at 280 nm
 - ▲····▲ = carbohydrate as absorbance at 485 nm in the phenol-sulfuric acid assay
 - = 620 nm scan of stained gels
 -  = polygalacturonase activity by cup-plate assay



The various steps in purification are summarized (Table I). Recoveries greater than 100% from chromatography on DEAE and CM cellulose may result from removal of inhibiting materials such as phenolics, or they may be due to the dissociation of enzyme units or enzyme-substrate complexes (24, 38). The increased carbohydrate content relative to protein after the second CM cellulose chromatography suggests the possibility of carbohydrate "leakage" from the exchanger. "Leakage" of carbohydrate from DEAE cellulose has been reported (18). Thus, the carbohydrate in the PG₁ and PG₂ fractions should not be considered as all covalently bound.

Characterization

The mode of substrate hydrolysis by PG₁₊₂ and PG₂ was determined by measurement of viscosity reduction, release of reducing groups, and appearance of oligomers in polygalacturonic acid reaction mixtures. Viscosity was measured using a Brookfield viscometer with an ultra-low viscosity adapter. Percent viscosity reduction was determined relative to the difference between the viscosity of 1.0% polygalacturonate and 1.0% monogalacturonate. Percent hydrolysis was determined using the reducing group assay and assuming the substrate was 100% galacturonic acid with a molecular weight of 13,900. Using enzyme concentrations of 1-2 reducing group units per ml, hydrolysis at 50% viscosity reduction was 5.0% for PG₂ and 5.4% for PG₁₊₂.

Table I. Purification of Polygalacturonase from *Fusarium oxysporum* f. sp. *lycopersici*.

Step	Volume (ml)	$10^{-3} \times$ Activity ^a	Recovery (%)	Protein ^b (mg)	Specific Activity ^c	Carbohydrate ^d	Carbohydrate: Protein
Dialyzed culture filtrate	25,000	27.5	100	2625	12.6	3050	1.16
DEAE-cellulose	27,630	37.2	135	2215	16.8	1800	0.81
CM-cellulose	2,440	56.1	202	985	56.0	150	0.15
Hydroxylapatite ^e	2,465	36.2	142	430	84.6	8.9	0.021
Second Hydroxylapatite ^e	2,750	21.6	79	290 146 ^f	74.6 148 ^f	4.1	0.017 0.028 ^f
Second CM-cellulose ^e							
Fraction PG ₁₊₂	116	6.3	23	95 33 ^f	65.8 194 ^f	1.5	0.016 0.045 ^f
Fraction PG ₂	101	1.1	4.5	23 8.3 ^g	52.4 148 ^g	0.36	0.016 0.043 ^g

^aOne unit = enzyme releasing 1 μ mole reducing groups per min. from 1.0% polygalacturonate

^bLowry assay (26), bovine serum albumin standard curve

^cSpecific activity = μ moles reducing groups released per min. per mg protein

^dPhenol-sulfuric acid assay (10), galactose standard curve

^eQuantities corrected for volumes set aside during purification

^fBased on E₂₈₀^{1%} of 11.4 from amino acid analysis of PG₁₊₂

^gBased on E₂₈₀^{1%} of 12.7 from amino acid analysis of PG₂

In a separate experiment, higher concentrations of enzyme (110 units per ml) were used to attain more complete hydrolysis. Samples taken periodically were boiled to stop the reaction, returned to their original volumes, and assayed for hydrolysis by reducing groups and for oligomeric products by thin-layer chromatography. Before TLC, the samples were shaken with Dowex 50 (H⁺ form) to remove Na⁺, which interferes with the chromatography (43). The hydrolysis curves for PG₁₊₂ and PG₂ were the same (Figure 6A and 6B), both leveling off at 87-88% hydrolysis. The patterns of oligomer release were also essentially the same (Figure 6C and 6D). Monogalacturonic acid was the only product detectable until about 20% hydrolysis. The final reaction products were mono- and digalacturonic acids and a small amount of material that failed to move from the origin.

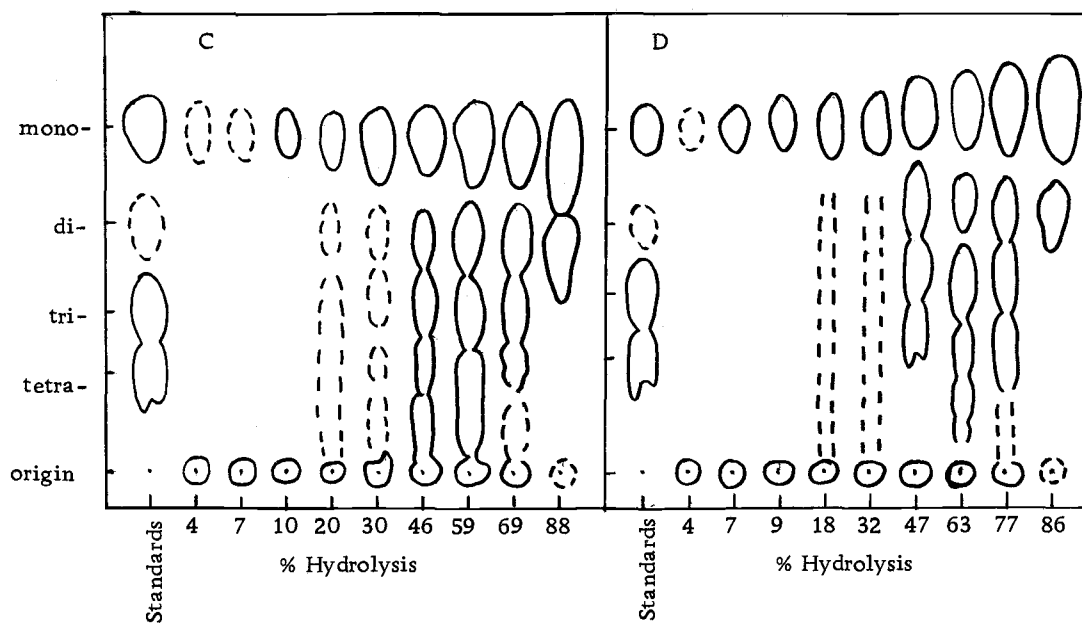
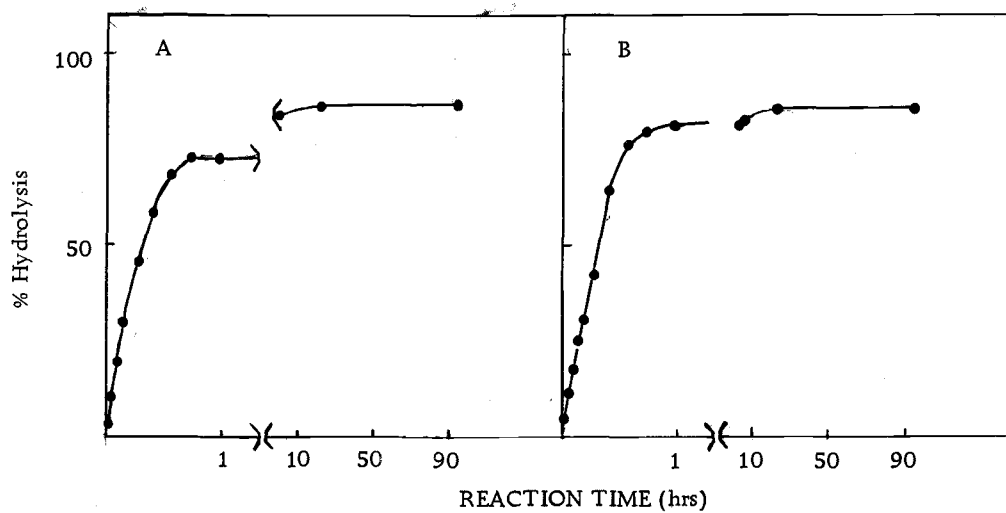
The 5% and 5.4% hydrolyses at 50% viscosity reduction indicate that both PG₁ and PG₂ are endopolygalacturonases. These values are similar to the 4-5% obtained for endopolygalacturonases from Coniothyrium diplodiella (12, 13, 14), but are higher than the 1% values for Colletotrichum lindemuthianum endopolygalacturonase (16) and Verticillium albo-atrum endopolygalacturonase estimated from the data of Mussell and Strouse (28). The early appearance of monogalacturonic acid as the only reaction product detectable until 20% hydrolysis is more like the patterns reported for exopolygalacturonases (15, 28). However, the hydrolysis required to reach 50%

Figure 6. Hydrolysis of 1.0% polygalacturonic acid by (A) PG_{1+2} and (B) PG_2 enzyme fractions.

●—● = % hydrolysis determined by reducing group assay

Chromatographic separation of hydrolysis products following incubation of 1.0% polygalacturonic acid with (C) PG_{1+2} and (D) PG_2 .

Outlined areas = zones of colorization by the CD-1 2-amino biphenyl reagent (23) on 0.5 mm MN cellulose thin-layer plates developed with an ethanol:formic acid:sodium formate solvent (43)



viscosity reduction is much less than the 40% hydrolysis for Coniothyrium exopolygalacturonase (15) and the 30% hydrolysis estimated from data on Verticillium exopolygalacturonase (28).

Amino Acid Analysis

To determine if differences in amino acid constituents might account for the two forms of polygalacturonase, and to gain insight into the basic chemical composition of this enzyme, both PG fractions were analyzed for amino acids. Amino acid compositions of the two preparations were nearly identical (Table II). These enzymes are somewhat unusual in containing large amounts of aspartic acid and no methionine, but their compositions are very similar to those determined for Verticillium (41) and Aspergillus (34) endopolygalacturonases. In all three cases aspartic acid is the most prevalent amino acid, followed by glycine and serine. None of the three polygalacturonases contains methionine. The isoelectric point (pI) of about 7.0 determined for the Fusarium polygalacturonases indicates that much of the aspartic and glutamic acid is probably present as asparagine and glutamine. Differences in amounts of asparagine and glutamine might account for the two electrophoretic mobilities. These two suggestions are supported by the amounts of ammonia found (Table II).

Table II. Amino Acid Composition of Endopolygalacturonases from Fusarium oxysporum f. sp. lycopersici.^a

Amino Acid	PG ₁₊₂		PG ₂	
	g/100 g protein	residues/ 37,000 g ^b	g/100 g protein	residues/ 37,000 g ^b
Lysine	7.4	21	7.8	22
Histidine	2.5	7	2.8	8
Ammonia ^c	3.0	65	3.2	70
Arginine	1.0	2	1.0	2
Aspartic acid	18.8	60	19.3	62
Threonine ^d	9.4	34	9.9	36
Serine ^d	9.1	39	9.4	40
Glutamic acid	4.7	13	4.7	13
Proline	3.9	13	3.9	13
Glycine	7.2	47	7.3	47
Alanine	3.4	18	3.3	17
One-half cystine	1.9	7	2.0	7
Valine ^e	7.5	28	7.7	29
Methionine	0	0	0	0
Isoleucine ^e	8.1	26	8.3	27
Leucine	6.0	20	6.1	20
Tyrosine ^f	4.3		3.9 ^d	
Tyrosine ^f	3.9		4.7	
Ave. tyrosine	4.1	9	4.3	10
Phenylalanine	4.8	12	4.9	12
Tryptophan ^f	2.8	6	2.1	4
Total	102.6	362	104.6	369
\bar{v}	0.723		0.723	

^a Analysis was with Spinco model 120B amino acid analyzer using the method of Spackman *et al.* (36). Samples were hydrolyzed for 20 and 70 hours. Except where noted, values are averages of those obtained at these two times.

^b Assumed molecular weight

^c Values not included in totals

^d Values obtained by a first-order rate extrapolation to zero time of hydrolysis.

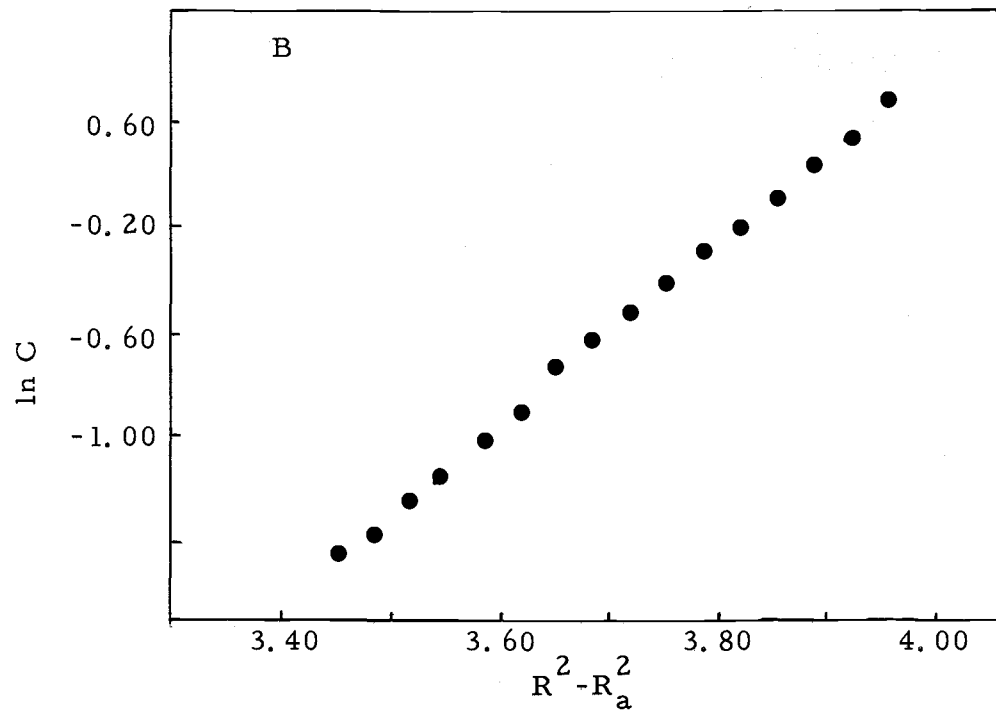
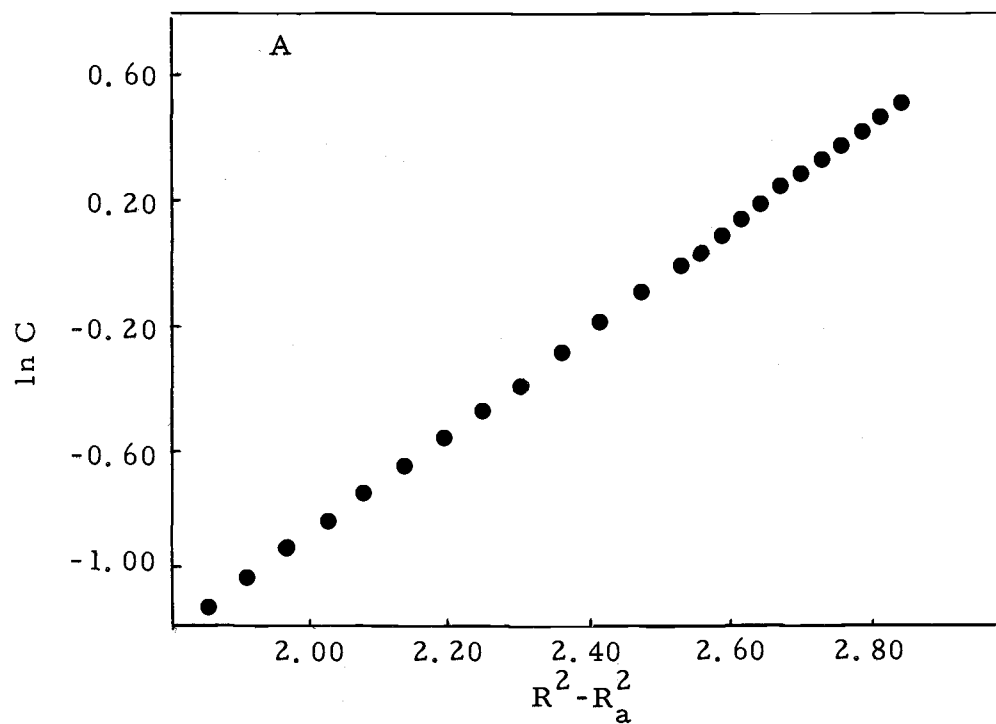
^e 70-hour value

^f Determined spectrophotometrically (11)

Sedimentation Analysis

Sedimentation equilibrium (42) was used to obtain measurements of the molecular weights of PG_1 and PG_2 . A Spinco model E ultracentrifuge was used with an Yphantis cell for high-speed equilibrium studies. Portions of the two enzyme fractions were concentrated by ultrafiltration (Amicon UM-2 membrane). The concentrates were dialyzed against 50 mM phosphate buffer, pH 7.0, before placing in the cell, and their final dialysis fluids were used in the reference compartments. At 36,000 rpm, equilibrium was reached after 21 hours. The linear plot of $\ln C$ vs. $(R^2 - R_a^2)$ (Figure 7A) for the PG_{1+2} fraction indicates that both components have the same or nearly the same molecular weight. This is in agreement with the gel filtration and electrophoresis studies. The results with PG_2 showed more variation because of low concentration, but a linear plot was obtained using fringe data from the bottom of the cell (Figure 7B). A partial specific volume of 0.723, calculated from the amino acid compositions by the method of Cohn and Edsall (5), was used in calculating molecular weights of 36,500 for PG_{1+2} and 37,000 for PG_2 . These are similar to the sizes reported for some other fungal polygalacturonases (3, 28, 34, 41), and are close to the 35,000 estimate from gel filtration of the Fusarium enzyme.

- Figure 7. Plots of $\ln C$ vs $R^2 - R_a^2$ calculated by the *MW Fortran computer program developed by Dr. R. D. Dyson. Concentration (C) was determined by measurement of fringe displacement with interference optics. $R^2 - R_a^2$ is in cm^2 . Equilibrium runs were in an Yphantis cell at 36,000 rpm and 22°C.
- (A) PG_{1+2} at a concentration of 0.68 mg per ml.
- (B) Data from bottom of cell with PG_2 at a concentration of 0.15 mg per ml.



Heat Inactivation

To determine if the carbohydrate associated with the polygalacturonase might have a stabilizing effect on the enzyme, the rates of heat inactivation of enzyme preparations from CM cellulose (15% carbohydrate) and from HTP (2% carbohydrate) were compared. Samples of each preparation, 0.6 ml in 10 mM acetate buffer, pH 4.0, were capped tightly in screw-cap test tubes and incubated in an elevated-temperature water bath. Individual tubes were removed periodically, cooled in an ice water bath, and assayed for polygalacturonase activity at 30°C by measuring reducing group release from polygalacturonic acid. Both enzymes were relatively stable at 50°C, losing about 10% of their activity in 24 hours. At 55°C, both preparations decayed at initial rates of 15% and 18% per hour for the enzymes from CM cellulose and HTP respectively. Both enzymes lost more than 90% of their activity in one hour at 60°C. From these results it was concluded that the carbohydrate removed by HTP chromatography has little or no influence on the enzyme's heat stability.

DISCUSSION AND CONCLUSIONS

The two polygalacturonases produced by F. oxysporum f. sp. lycopersici that were purified in this study were indistinguishable on the basis of their modes of action, pI's, amino acid compositions, and molecular weights. The preparations were homogeneous in disc gel electrophoresis and sedimentation equilibrium, and represent 10 to 15-fold purifications over the specific activity of the culture filtrate. It should be noted that, based on protein quantitation using extinction coefficients obtained from amino acid analysis (Table I), the Lowry assay is about one-third as sensitive for the Fusarium polygalacturonases as for the bovine serum albumin standard used.

Because the nature of the difference between the polygalacturonase isomers is unknown, the operational terms "isozymes" PG₁ and PG₂ are suggested. The charge difference may be due to subtle differences in amino acid composition, e. g., the proportions of aspartic and glutamic acids present as asparagine and glutamine; conformational differences; or differences in carbohydrate composition not detected by the phenol-sulfuric acid assay, e. g., hexosamines are not detected by this assay (1). About 2% by weight of carbohydrate appears to be covalently linked to the enzyme. Detailed study of the composition of this carbohydrate might show differences between the two isozymes that could be responsible for their electrophoretic mobilities.

One difference between the two isozymes is the greater specific activity of PG_1 . The PG_{1+2} mixture showed about one-third greater specific activity than PG_2 (Table I), indicating that PG_1 has about 60-70% higher specific activity. This suggests that the isozyme with a lower net positive charge at about the pH optimum of 4.0 (24), PG_1 , is more efficient at hydrolyzing the highly negatively charged substrate than the more positive isozyme, PG_2 . PG_1 may bind substrate less tightly, permitting more rapid turnover.

Both isozymes are endopolygalacturonases that hydrolyze 5 to 5.4% of the bonds in polygalacturonic acid in reaching 50% viscosity reduction, but monogalacturonic acid is the only detectable product until about 20% hydrolysis. Other endopolygalacturonases produce oliguronides in addition to monogalacturonic acid in the early stages of hydrolysis (7, 8, 12, 13, 14, 28). Endopolygalacturonase from Colletotrichum (16) produces mono- di-, and trigalacturonic acids in approximately equimolar amounts during hydrolysis. The pattern of action of the Fusarium polygalacturonases is similar to that described for a complex of exo- and endopolygalacturonases produced by Sclerotium rolfsii (3). However, there is no evidence that the two polygalacturonase isozymes produced by Fusarium differ in their modes of action. The difference in pattern of action between Fusarium endopolygalacturonase and other endopolygalacturonases does suggest some basic difference in mode of action, and a problem in judging the

mode of action of a polygalacturonase. On the basis of products produced, the Fusarium enzyme would be classified as an exopolygalacturonase. However, results of viscosity reduction and reducing group measurements show it to be an endopolygalacturonase--apparently one with more affinity for splitting off terminal residues such that it hydrolyzes five times as many bonds as some other endopolygalacturonases in reaching 50% viscosity reduction.

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