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

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Summary

Polygalacturonase (EC 3.2.1.15) produced by *Fursarium oxysporum* f. sp. *lycopersici* was purified by chromatography on DEAE-cellulose, CM-cellulose, and hydroxyapatite. The purified enzyme consisted of two electrophoretically distinct "isozymes", that behaved as charge isomers during electrophoresis in several different concentrations of polyacrylamide gel. The two isozymes had similar "endo" modes of action on polygalacturonic acid, as determined by comparison of viscosity reduction, reducing group release, and thin-layer chromartography of oligomeric hydrolysis products. Both isozymes hydrolzyed 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. Removal of large amounts of carbohydrate during purification did not affect heat stability of the enzymes. A large proportion of the remaining carbohydrate appeared to be covalently linked to the enzyme protein.

Introduction

Polygalacturonase(poly, α-1,4-galacturonide glycanohydrolase, EC 3.2.1.15) plays an important role in a number of plant diseases [1—4], and has been implicated in vascular wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici*. Polygalacturonases produced by this fungus and other fungi ex-

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tensively degrade plant cell walls [5,6] and "prepare" cell wall components for degradation by other enzymes [5,7]. These extracellular enzymatic processes are especially important to saprophytes and facultative saprophytes involved in the decay of plant litter.

Knowledge of the chemical properties of polygalacturonases is necessary for a full understanding of their behavior in living and dead plant materials. Polygalacturonases from a number of fungi have been purified and characterized. They show a great deal of variation in physical and chemical properties and mode of substrate hydrolysis [4,5,8–15]. Compositions of polygalacturonases from *Aspergillus niger* [14] and *Verticillium albo-atrum* [15] have been determined, but the polygalacturonase produced by the *Fusarium* fungus has not been characterized at this level. Previous work established a scheme for purifying this enzyme [16], and although the enzyme appeared homogeneous in disc gel electrophoresis there was a significant amount of carbohydrate in the purified enzyme preparation.

The present study was initiated to improve the purification of the polygalacturonase produced by *F. oxysporum lycopersici*, to investigate the possibility that the 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. *F. oxysporum* f. sp. *lycopersici* (Sacc.) Snyd. 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 $MgSO_4 \cdot 7H_2O$, 40 mg $FeCl_3 \cdot 6H_2O$, 20 mg $ZnSO_4 \cdot 7H_2O$ and 10 g pectin N.F. (Sunkist product no. 3442)per l. Initial culture pH was 4.4. After 6 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 μm) and NaN_3 was added to a concentration of 0.02%.

Enzyme assays. For routine determination of polygalacturonase activity during fractionation procedures, a cup-plate assay [17] was used. A 1.5% agar gel containing 1.0% sodium polypectate (Sunkist product no. 6024) and 0.5% ammonium oxalate buffered at pH 5.0 with 0.1 M sodium acetate was incubated for 16 h at 40°C after adding 0.1 ml enzyme samples to wells in the gel. The diameters of zones developed by flooding plates with 5 M HCl were linearly related to the logarithm of enzyme concentration. With the standard curve used, 20- and 27-mm zones corresponded to 25 and 725 units of polygalacturonase activity, respectively. This assay, although using a substrate that was only about 50% uronide, gave results that were highly correlated with assays for the release of reducing groups from polygalacturonic acid.

Polygalacturonase activity was quantified by measuring the release of reducing groups from polygagacturonic acid (Sunkist product no. 3491) using the Somogyi assay [18] with Nelson's arsenomolybdate reagent [19]. The zinc- and barium-deproteinizing treatments were omitted, and precipitated substrate was removed by filtration through Whatman No. 4 filter paper after reaction with the arsenomolybdate. Equivalents of reducing groups released were

based on a standard curve relating the absorbance at 540 nm to μmol of galacturonic acid monohydrate (Pfanstiel). One unit of activity was defined as the amount of enzyme that releases 1 μmol of galacturonic acid per min at 30°C. Before use, the polygalacturonic acid was purified by precipitation from a filtered 5% solution with 80% ethanol containing 50 mM HCl, and extraction of the precipitate with $7 \cdot 10^{-4}$% dithizone [20] in the acidified ethanol [21]. The purified product was over 99% galacturonic acid by the carbazole [22] and phenol-sulfuric acid [23] assays. Measurement of reducing ends by the modified Somogyi assay gave a M_N of 13 900 ± 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 [24] using 1.0% pectin as the substrate and 0.04% bromcresol purple as the indicator. The standard curve relating diameter of the yellow zones developed after 16 h 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 enzyme activity.

Protein assay. Relative protein concentrations during fractionations were followed by reading absorption at 280 nm. The Lowry assay [25] with a bovine serum albumin (Sigma fraction V) standard curve was used to quantify protein in enzyme fractions.

Carbohydrate assay. To follow carbohydrate during fractionation and to quantify carbohydrate in the various enzyme fractions, the phenol-sulfuric acid assay [23] was used.

Gel electrophoresis. A modification of the polyacrylamide gel electrophoresis method of Reisfeld et al. [26] 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 3 h 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 0.5 h, stained for 0.5 h 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 4 h with 1 ml portions of 0.1 M acetate buffer, pH 5.0, and then assayed in cup-plates.

Results

Enzyme purification

25 l of culture filtrate were dialyzed against 10 mM potassium phosphate buffer, pH 6.0, and passed through DEAE-cellulose (Sigma) regenerated with 1 M NaOH and equilibrated in the same buffer. 50 g of DEAE-cellulose were used for one half of the dialyzed culture filtrate. The unadsorbed enzyme (>99%) was dialyzed against 10 mM sodium acetate buffer, pH 4.0, and chromatographed on CM-cellulose (Bio-Rad Cellex CM). Polygalacturonase activity was eluted with the major protein and carbohydrate peaks (Fig. 1A). An additional carbohydrate peak was obtained at the beginning of the elution.

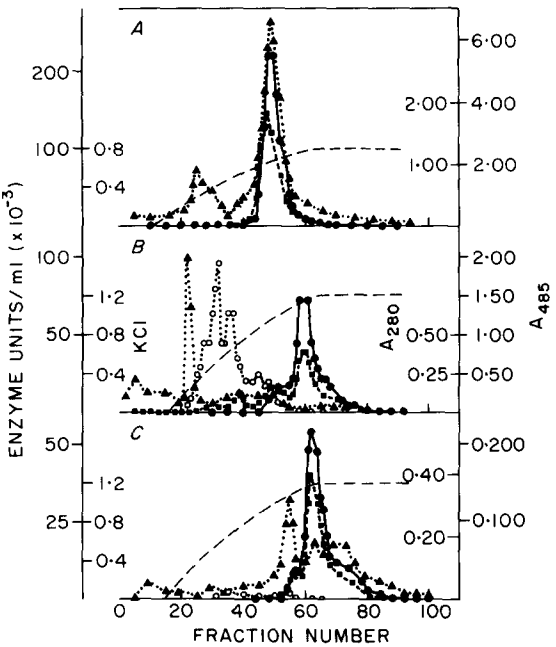

Fig. 1. Column chromatographic fractionation of the polygalacturonase produced by *F. oxysporum* f. sp. *lycopersici*. ●———●, polygalacturonase activity by cup-plate assay; ○ · · · · · · ○, pectinesterase activity by cup-plate assay; ■ - - - - - - ■, protein as absorbance at 280 nm; ▲ · · · · · · ▲, carbohydrate as absorbance at 485 nm in the phenol-sulfuric acid assay; - - - - - -, molarity of KCl. Fraction volumes were 10 ml. (A) Elution of polygalacturonase activity, protein, and carbohydrate from a CM-cellulose column (2.5 × 55 cm) with a 0—0.8 M KCl gradient in 10 mM sodium acetate buffer, pH 4.0. Elution rate was 2.0 ml/min. CM-cellulose was regenerated with 1 M HCl and equilibrated in the acetate buffer. (B) Elution of polygalacturonase and pectinesterase activities, protein, and carbohydrate from an hydroxyapatite column (2.5 × 39 cm) with a 0—1.2 M KCl gradient in 10 mM potassium phosphate buffer, pH 6.0. Elution rate was 0.5 ml/min. Hydroxyapatite was regenerated with 1.0 M potassium phosphate buffer, pH 6.0, and equilibrated with the 10 mM phosphate buffer. (C) Elution of polygalacturonase and pectinesterase activities, protein, and carbohydrate from an hydroxapatite column (2.5 × 33.5 cm) as in (B).

Polygalacturonase peaks from eight CM columns (e.g. fractions 44—65 in Fig. 1A) were pooled and divided into six equal portions. Each portion was dialyzed against distilled water until Cl^- free and then adsorbed onto hydroxyapatite (Bio-Rad HTP). Two minor protein peaks were eluted from the hydroxyapatite in addition to the major polygalacturonase peak (Fig. 1B), and most of the carbohydrate and pectinesterase was separated from the polygalacturonase. The polygalacturonase peaks from the six columns (e.g. fractions 51—72 in Fig. 1B) were pooled, and the hydroxyapatite procedure repeated. No pectinesterase activity was detected in the major polygalacturonase peak (Fig. 1C), but carbohydrate was still associated with the polygalacturonase. The peak fractions that were pooled after this step (e.g. fractions 59—83 in Fig. 1C) did not include the minor shoulder peak of polygalacturonase (e.g. fractions 55—58 in Fig. 1C) that contained pectinesterase. Thus, only the major polygalacturonase fraction was further characterized.

The second hydroxyapatite chromatography represents the final step of the purification scheme used by Harman and Corden [16]. 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. Fig. 4B). Thus, it appeared that the major polygalacturonase produced by *F. oxysporum lycopersici* consisted of two components that migrated differently in disc gel electrophoresis. The slower moving band was labelled polygalacturonase$_1$, and the faster moving polygalacturonase$_2$. The difference in migration behavior of polygalacturonase$_1$ and polygalacturonase$_2$ may have been eliminated in the urea-acetic acid system used earlier [16].

To attempt the separation of polygalacturonase$_1$ from polygalacturonase$_2$, and to remove the non-polygalacturonase protein, a portion of the enzyme from hydroxyapatite was subjected to isoelectric focusing in a pH gradient of 3—10 using LKB Ampholine equipment. The focusing was run for 72 h at about 18°C, during which the power dropped from 0.12 W to a constant 0.012 W. 6-ml fractions were collected and their pH, polygalacturonase activity, and protein content (A_{280nm}) were measured. A single major peak of protein and polygalacturonase activity was obtained at pH 7.0. The peak samples were dialyzed against five 15-volume changes of 1 M NaCl in 10 mM sodium acetate buffer, pH 4.0, to remove Ampholine [27], then against the acetate buffer alone, and finally assayed by gel electrophoresis. All three bands previously seen were present. Thus, electrofocusing under these conditions gave no increased purification or separation, and indicated an isoelectric point (p*I*) of about 7.0 for polygalacturonase$_1$ and polygalacturonase$_2$. There may be a small difference between the p*I* values of polygalacturonase$_1$ and polygalacturonase$_2$ that was not detected in the pH gradient used. An apparent minor peak of activity and A_{280nm} 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 polygalacturonase$_1$ and polygalacturonase$_2$. A portion of the enzyme preparation from hydroxyapatite concentrated by freeze-drying, was chromatographed on Bio-Gel P-100 (Bio-Rad Laboratories, 100—200 mesh). A single peak of polygalacturonase activity was obtained from the column (Fig. 2A). When the central fraction of the peak ($V_E : V_O = 1.8$) was electrophoresed, it contained polygalacturonase$_1$ and polygalacturonase$_2$, but not the non-polygalacturonase protein, which may have been the smaller, second protein peak from the column. These results indicate that polygalacturonase$_1$ and polygalacturonase$_2$ are about the same size. The P-100 was calibrated with globular protein standards (cytochrome *c*, chymotrypsinogen, ovalbumin, and bovine serum albumin), and from a plot of log (molecular weights) vs. $V_E : V_O$, a molecular weight of 35 000 was estimated for both polygalacturonases. This is in agreement with the weight obtained with Sephadex G-75 by Harman and Corden [16].

Gel filtration under denaturing conditions was used to see if dissociation of the enzyme into smaller subunits and carbohydrate from the enzyme would occur. Guanidine hydrochloride (Mann Ultra-Pure) was dissolved in one half of the concentrated enzyme to make a 2 M solution, a concentration that com-

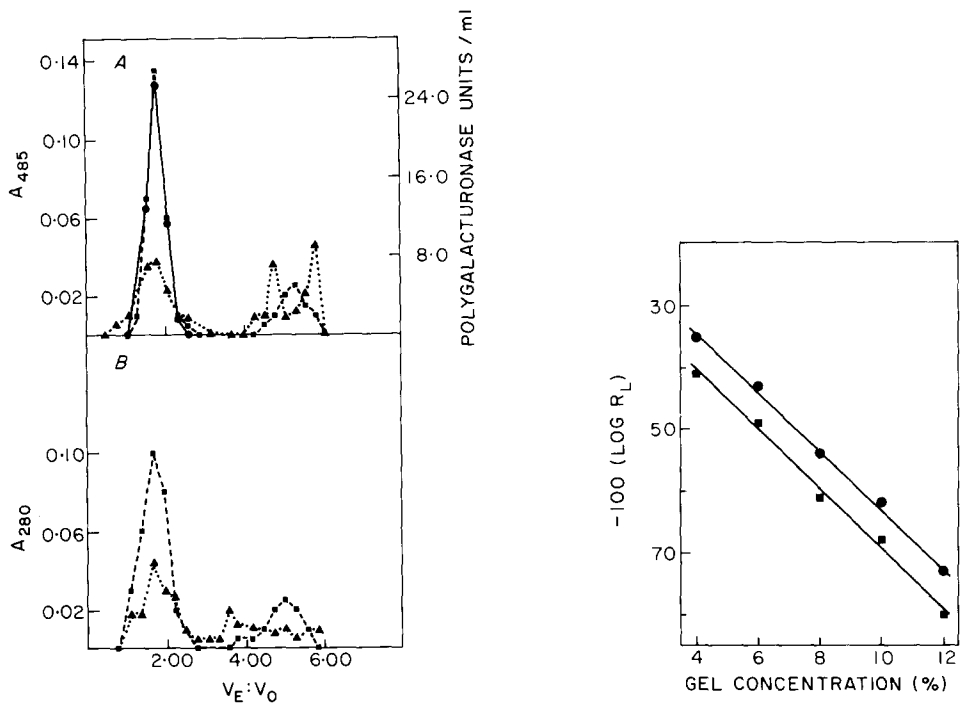

Fig. 2. Gel filtration chromatography on Bio-Gel P-100 of the polygalacturonase preparation obtained from hydroxyapatite and concentrated by lyophilizing and redissolving in 50 mM sodium acetate buffer, pH 5.1. ●———●, polygalacturonase activity by reducing group assay; ■ - - - - - - ■, protein as absorbance at 280 nm; ▲ . . . . . ▲, carbohydrate as absorbance at 485 nm in the phenol-sulfuric acid assay. Columns were eluted at 0.2 ml/min, and 3-ml fractions collected. (A) Sample (2.0 ml, A_{280nm} = 0.45) applied to a P-100 column (1.5 × 28 cm) eluted with 50 mM sodium acetate buffer, pH 5.1. (B) Sample (2.4 ml, A_{280nm} = 0.38) containing 2 M guanidine hydrochloride applied to a P-100 column (1.5 × 28 cm) eluted with 2 M guanidine hydrochloride in the acetate buffer.

Fig. 3. Relative electrophoretic mobilities (R_L = distance moved by band divided by length of gel) of the polygalacturonase$_1$ and polygalacturonase$_2$ bands in different concentrations of polyacrylamide gels. The pH 4.3 system was used. Gels were run for 3 h at 4 mA per tube, and bands were stained with Coomassie brilliant blue R-250. ■———■, polygalacturonase$_1$; ●———●, polygalacturonase$_2$. Regression slopes for both lines are —4.8.

pletely eliminated polygalacturonase activity. The denatured enzyme was chromatographed on a column of P-100 equilibrated with 2 M guanidine hydrochloride. Protein and carbohydrate peaks from this column (Fig. 2B) 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 denatured protein standards were chromatographed on the column containing guanidine hydrochloride. The results indicated that polygalacturonase$_1$ and polygalacturonase$_2$ were 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 [28]. Therefore, the mixture of polygalacturonase$_1$ and polygalacturonase$_2$ 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 polygalacturonase$_1$ and polygalacturonase$_2$ were parallel (Fig. 3). This indicated that charge differences and not size differences were responsible for the different mobilities of polygalacturonase$_1$ and polygalacturonase$_2$ thus supporting the results of the gel filtration experiments. Separation of polygalacturonase$_1$ from polygalacturonase$_2$ by a fractionation procedure based on charge differences was suggested by these results.

The enzyme preparation from hydroxyapatite was rechromatographed on CM-cellulose in the same manner as before. Samples of 10-ml fractions taken at points 1, 2, and 3 (Fig. 4) 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 (Fig. 4B). Fraction 2 contained polygalacturonase$_1$ and polygalacturonase$_2$ free of the non-polygalacturonase protein, and fraction 3 contained polygalacturonase$_2$ free of polygalacturon-

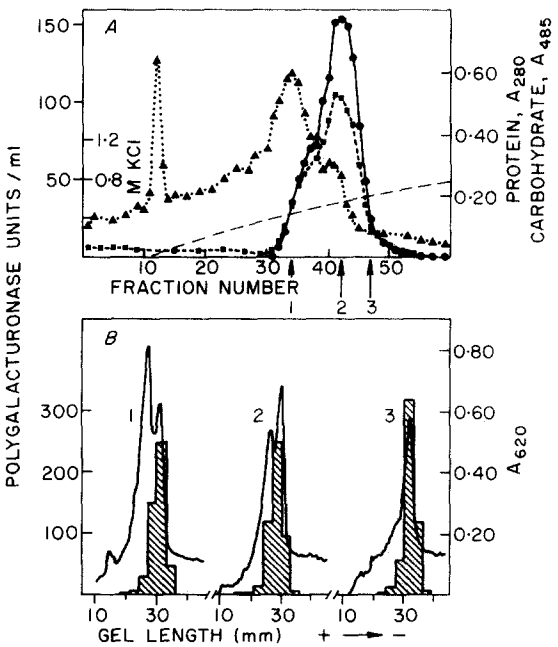

Fig. 4. (A) Polygalacturonase from hydroxyapatite applied to a CM-cellulose column (2.5 × 53 cm) and eluted with a 0—0.8 M KCl gradient in 10 mM sodium acetate buffer, pH 4.0. (B) Polyacrylamide gel electrophoresis (pH 4.3) of fractions taken at points 1, 2, and 3 from the CM-cellulose column. Gels were 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; - - - - - -, molarity of KCl.

TABLE I

PURIFICATION OF POLYGALACTURONASE FROM *F. OXYSPORUM* f. sp. *LYCOPERSICI*

Step	Volume (ml)	Activity [a] ($\times 10^{-3}$)	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
Hydroxyapatite [e]	2 465	36.2	142	430	84.6	8.9	0.021
Second hydroxyapatite [e]	2 750	21.6	79	290	74.6	4.1	0.017
				146 [f]	148 [f]		0.028 [f]
Second CM-cellulose							
Fraction polygalacturonase$_{1+2}$	116	6.3	23	95	65.8	1.5	0.016
				33 [f]	194 [f]		
Fraction polygalacturonase$_2$	101	1.1	4.5	23	52.4	0.36	0.016
				8.3 [g]	148 [g]		0.043 [g]

[a] One unit = enzyme releasing 1 μmol reducing groups per min from 1.0% polygalacturonate.

[b] Lowry assay [25], bovine serum albumin standard curve.

[c] Specific activity, μmol reducing groups released per min per mg protein.

[d] Phenol/sulfuric acid assay [23], galactose standard curve.

[e] Quantities corrected for volumes set aside during purification.

[f] Based on $E^{1\%}_{280}$ of 11.4 from amino acid analysis of polygalacturonase$_{1+2}$.

[g] Based on $E^{1\%}_{280}$ of 12.7 from amino acid analysis of polygalacturonase$_2$.

ase_1. Column fractions 41–43 were pooled to give a mixture of polygalacturonase$_1$ and polygalacturonase$_2$ (polygalacturonase$_{1+2}$) and fractions 46–48 were pooled to give polygalacturonase$_2$.

The various steps in purification are summarized in 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 [16,29]. 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 [30]. Thus, the carbohydrate in the polygalacturonase$_1$ and polygalacturonase$_2$ fractions should not be considered as all covalently bound.

Characterization

The mode of substrate hydrolysis by fractions polygalacturonase$_{1+2}$ and

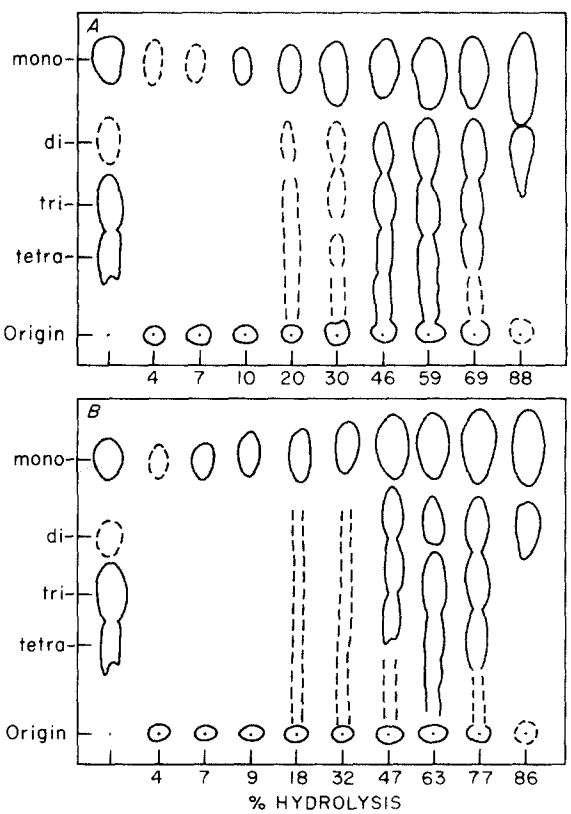

Fig. 5. Chromatographic separation of hydrolysis products following incubation of 1.0% polygalacturonic acid in 0.1 M sodium acetate buffer, pH 5.0, with (A) polygalacturonase$_{1+2}$ and (B) polygalacturonase$_2$. 1-ml samples of reaction mixtures were boiled to stop the reactions, returned to 1.0 ml with distilled water, and shaken with Dowex 50 (H^+ form) to remove Na^+, which interferes with the chromatography [31]. 10 μl of each sample was spotted on the thin-layer plates, 0.5 mm MN cellulose. Plates were developed with an ethanol/formic acid/sodium formate solvent [31]. Outlined areas represent zones of colorization by the "CD-1" 2-aminobiphenyl reagent [32]. The 69 (A) and 77% hydrolysis (B) occurred after about 0.5 h incubation while the 88 (A) and 86% hydrolysis (B) required 24 h.

polygalacturonase$_2$ 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% galacturonide with a molecular weight of 13 900. With 1—2 enzyme units per ml, hydrolysis at 50% viscosity reduction was 5.0% for polygalacturonase$_2$ and 5.4% for polygalacturonase$_{1+2}$.

In a separate experiment, thin-layer chromatography was used to follow appearance of oligogalacturonides during hydrolysis. Higher concentrations of enzyme (110 units per ml) were used to attain more complete hydrolysis. Samples were taken periodically and assayed for hydrolysis and oligomer release. The hydrolysis curves for polygalacturonase$_{1+2}$ and polygalacturonase$_2$ leveled off at 87—88% hydrolysis, and the patterns of oligomer release were essentially the same (Figs. 5A and 5B). 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.

Polygalacturonases produced by *F. oxysporum lycopersici* showed a mode of substrate hydrolysis unlike that reported for other polygalacturonases. The early appearance of monogalacturonic acid as the only reaction product detectable until 20% hydrolysis was like patterns reported for exopolygalacturonases [12,13]. However, the 5—5.4% hydrolysis at 50% viscosity reduction was similar to values reported for endopolygalacturonases [2,5,9—11], and much lower than the 40% hydrolysis required for an exopolygalacturonase to acheive 50% viscosity reduction [12].

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 polygalacturonase fractions were analyzed for amino acids. Amino acid compositions of the two preparations were nearly identical (Table II). These enzymes were somewhat unusual in containing large amounts of aspartic acid and no methionine, but their compositions were similar to those determined for *Aspergillus* [14] and *Verticillium* [15] endopolygalacturonases. In all three cases aspartic acid was the most prevalent amino acid, followed by glycine and serine. None of three polygalacturonases contained methionine. The p*I* of about 7.0 determined for the *F. oxysporum lycopersici* 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).

Sedimentation analysis

Sedimentation equilibrium [35] was used to obtain measurements of the

molecular weights of polygalacturonase$_1$ and polygalacturonase$_2$. A Spinco model E ultracentrifuge was used with 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. Equilibrium was reached after 21 h at 36 000 rev./min. The plot of ln C vs. $(R^2—R_a{}^2)$ for the polygalacturonase$_{1+2}$ fraction was linear, indicating that both components had the same or nearly the same molecular weight. This is in agreement with the gel filtration and electrophoresis studies. The results with polygalacturonase$_2$ showed more variation because of the low concentration used, but a linear plot was obtained using fringe data from the bottom of the cell. A partial specific volume of 0.723, calculated from the amino acid compositions by the method of Cohn

TABLE II

AMINO ACID COMPOSITION OF ENDOPOLYGALACTURONASES FROM *F. OXYSPORUM* f. sp. *LYCOPERSICI* [a]

Amino acid	Polygalacturonase$_{1+2}$		Polygalacturonase$_2$	
	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
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	4.3		3.9 [d]	
Tyrosine [f]	3.9		4.7	
Average tyrosine	4.1	9	4.3	10
Phenylamine	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. [33]. Samples were hydrolyzed for 20 and 70 h. 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-h value.

[f] Determined spectrophotometrically [34].

and Edsall [36], was used in calculating molecular weights of 36 500 for polygalacturonase$_{1+2}$ and 37 000 for polygalacturonase$_2$. These are similar to the sizes reported for some other fungal polygalacturonases [2,13—15], and are close to the 35 000 estimate obtained from gel filtration.

Heat inactivation

Heat stability of some enzymes is influenced by non-covalently associated carbohydrate [37,38]. 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 hydroxyapatite (2% carbohydrate) were compared. Samples of each preparation, 0.6 ml in 10 mM sodium 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 release of reducing groups from polygalacturonic acid.

Both enzymes were relatively stable at 50°C, losing about 10% of their activity in 24 h. At 55°C, the preparations from CM-cellulose and hydroxyapatite decayed with first-order rates of —0.09 and —0.11 per h, respectively. These corresponded to initial rates of inactivation of 15 and 18% per h. Both enzymes lost more than 90% of their activity in 1 h at 60°C. From these results it was concluded that the carbohydrate removed by hydroxyapatite chromatography has little or no influence on the enzyme's heat stability.

Discussion

Multiple forms of polygalacturonase have been reported [2,9—13,39]. The *F. oxysporum lycopersici* enzymes purified in this study are the first reported polygalacturonase isomers indistinguishable on the basis of their modes of action, p*I* values, amino acid compositions, and molecular weights. The preparations were homogeneous in disc gel electrophoresis and sedimentation equilibrium, and represent 10—15-fold purifications over the specific activity of the culture filtrate. It should be noted that, based on the protein quantitation from amino acid analysis (Table I), the Lowry assay is about one-third as sensitive for the *F. oxysporum lycopersici* polygalacturonases as for the bovine albumin standard used.

The two polygalacturonases appear to be charge isomers. Because the nature of the difference between the polygalacturonase isomers is unknown, the operational terms "isozymes" polygalacturonase$_1$ and polygalacturonase$_2$ 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; qualitative differences in carbohydrate composition; or quantitative differences in carbohydrate composition not detected by the phenol-sulfuric acid assay, e.g. hexosamines are not detected by this assay [40]. About 2% by weight or carbohydrate to be covalently linked to the enzyme. Detailed study of the composition of this carbohydrate might show differences between the two isoenzyme that could be responsible for their electrophoretic mobilities.

One difference between the two isozymes is the greater specific activity of polygalacturonase$_1$. The polygalacturonase$_{1+2}$ mixture showed about one-third greater specific activity than polygalacturonase$_2$ (Table I), indicating that polygalacturonase$_1$ had 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 [16], polygalacturonase$_1$, is more efficient at hydrolyzing the highly negatively charged substrate than the more positive isozyme, polygalacturonas$_2$. Polygalacturonase$_1$ may bind substrate less tightly, permitting more rapid turnover.

Both isozymes are endopolygalacturonases that hydrolyze about 5% of the bonds in polygalacturonic acid in reducing the viscosity of polygalacturonate solutions by 50%, but monogalacturonic acid is the only detectable oligomeric product until about 20% hydrolysis is reached. Other endopolygalacturonases produce oligouronides in addition to monogalacturonic acid in the early stages of hydrolysis [8—11,13]. Endopolygalacturonase from *Colletotrichum* [5] produces mono-, di-, and trigalacturonic acids in approximately equimolar amounts during hydrolysis. The difference in pattern of action between *F. oxysporum lycopersici* endopolygalacturonase and other endopolygalacturonases suggests 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 *F. oxysporum lycopersici* 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.

Although the physical studies showed no evidence of heterogeneity in either of the two isozymes, each might consist of a mixture of exo- and endopolygalacturonases that were not separated by the techniques used. Such mixtures could explain the observed breakdown patterns. However, an exopolygalacturonase should breakdown digalacturonic but this did not occur in our studies even after prolonged incubation. Apparently the polygalacturonase form Fol is unable to hydrolyze digalacturonic acid judging form its accumulation in the reaction mixtures.

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