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A Cell Wall-degrading Endopolygalacturonase Secreted by *Colletotrichum lindemuthianum*[1]

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ABSTRACT

Cultures of *Colletotrichum lindemuthianum* (Saccardo and Magnus) Scribner have been induced to secrete an endopolygalacturonase (polygalacturonide glycanohydrolase EC3.2.1.15). This enzyme has been brought to a high state of purity by ion exchange, gel filtration, and agarose affinity chromatography. The enzyme has optimal activity at pH 5, has an apparent molecular weight as determined by gel filtration of about 70,000, and prefers polygalacturonic acid to pectin as its substrate. The enzyme, while hydrolyzing only 1% of the glycosidic bonds, reduces the viscosity of a polygalacturonic solution by 50%. Nevertheless, the initial as well as the final products of polygalacturonic acid hydrolysis are predominantly tri- and digalacturonic acid and, to a lesser extent, monogalacturonic acid. The purified enzyme catalyzes the removal of about 80% of the galacturonic acid residues of cell walls isolated from suspension-cultured sycamore cells (*Acer pseudoplatanus*) as well as from the walls isolated from 8-day-old Red Kidney bean (*Phaseolus vulgaris*) hypocotyls.

Most polysaccharide-degrading enzymes are unable to attack effectively their substrates within isolated cell walls. Evidence has been presented which suggests that the "wall-modifying enzyme" purified from the culture filtrate of *Aspergillus niger* is a polygalacturonic acid-degrading enzyme (13). Treatment of walls with the wall-modifying enzyme permits other enzymes to degrade their substrates within the wall. Additional evidence that polygalacturonic acid-degrading enzymes are probably able, without assistance, to degrade cell walls comes from numerous efforts to demonstrate that these enzymes macerate plant tissues (2, 4, 5, 7, 12, 18, 20). That polygalacturonic acid-degrading enzymes are able to attack some plant cell components, although not necessarily cell walls, has been established by demonstrating that purified preparations of such enzymes can initiate cell killing (10, 15).

The mixture of enzymes secreted by *C. lindemuthianum*, when this fungus is grown on isolated cell walls as the carbon source, has been shown to degrade isolated cell walls (8). The first detectable enzyme secreted when this fungal pathogen is grown under these conditions is an endopolygalacturonase (9). The present paper describes the production, purification, and partial characterization of this enzyme and demonstrates the ability of this purified enzyme to degrade isolated cell walls.

MATERIALS AND METHODS

Growth of Fungus and Production of Endopolygalacturonase. The α strain of *Colletotrichum lindemuthianum* was cultured as reported (9). The endopolygalacturonase secreted by this fungus was induced by growing the fungus in shake culture on minimal salts media containing 1% citrus pectin (the gift of Sunkist Growers, Inc.). A standard induction culture contained 1 liter of medium in a 2.8-liter Fernbach flask. The cultures were initiated with 10^6 spores and were incubated at 23 C on a rotary shaker at a shaking rate of 60 to 70 rpm.

Cultures were harvested after 8 days by filtering the culture fluid through a coarse sintered glass funnel. The mycelial residue was discarded. The solution was filtered again, this time through two layers of 4.25-cm GF/C glass paper. Only about 200 ml can be filtered at a time, as the glass filters become plugged. The solution which passed through the filter was exhaustively dialyzed against 50 mM sodium acetate, pH 5.2. This solution represents the "crude" endopolygalacturonase (Table I).

Assays. Endopolygalacturonase was assayed by the method of Nelson (16) as modified by Somogyi (19). A standard reaction contained 100 μl of enzyme and 1 ml of 0.1% polygalacturonic acid dissolved in 50 mM sodium acetate, pH 5.2. The polygalacturonic acid was synthesized from citrus pectin by base-catalyzed de-esterification (pH 12, 0 C, 2 hr) and sodium borohydride reduction (10 mg/liter, pH 12, 0 C, 22 hr). A standard reaction was incubated for 30 min at 28 C. One unit of endopolygalacturonase is defined as that amount which hydrolyzes 0.5 μmole of galacturonic acid glycosidic linkages under these conditions. This amount is equivalent to 10% of the galacturonic linkages present in the substrate of a standard reaction. To assure linearity, we diluted all enzyme solutions before assay, such that each reaction contained no more than 2.3 units of endopolygalacturonase activity. Since the endopolygalacturonase is relatively unstable in dilute solutions, the enzyme was diluted as required with 50 mM sodium acetate, pH 5.2, containing 100 μg/ml of bovine serum albumin (Armour Pharmaceutical Co.).

The activity of the endopolygalacturonase was also measured viscometrically (9).

Cell walls, isolated from 8-day-old Red Kidney bean hypocotyls and from sycamore cells grown in suspension culture, were prepared as described (17) except that 500 rather than 100 mM phosphate buffer was used and the walls were washed

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with water five times rather than once. The release of sugars from the cell walls upon treatment with endopolygalacturonase was followed either by the Nelson-Somogyi (16, 19) assay for reducing sugars or by the carbazole assay (6) for uronic acids. The neutral sugar composition of cell walls both before and after endopolygalacturonase treatment as well as the neutral sugar composition of the material solubilized by endopolygalacturonase was determined by gas chromatography (3). The galacturonic acid content of these preparations was determined both by the carbazole method (6) and by gas chromatography (T. M. Jones and P. Albersheim, unpublished results).

Proteins, with bovine serum albumin as a standard, were measured by the method of Lowry *et al.* (14).

All experiments were carried out at 2 C unless otherwise specified.

RESULTS

Purification of the Endopolygalacturonase. The crude enzyme solution (3 liters) was dialyzed for 48 hr against 14 liters of 5 mM sodium acetate, pH 5.2. The buffer was changed after 12, 24, and 36 hr. Five hundred grams of DE-52 (Whatman diethylaminoethyl cellulose) which had been pre-equilibrated with 5 mM sodium acetate, pH 5.2, were added to the nondialyzable enzyme solution. This suspension was stirred mechanically for 8 hr. The DE-52 was removed by filtration through a coarse sintered glass funnel and was washed two times with 500 ml of 5 mM sodium acetate, pH 5.2.

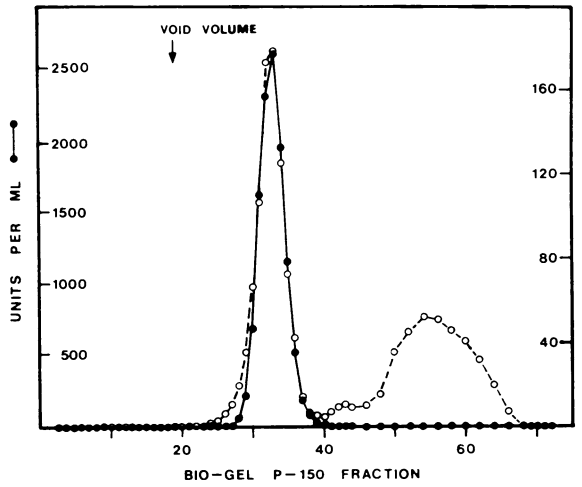

FIG. 1. Gel filtration of the endopolygalacturonase to separate this enzyme from smaller molecular weight substances. This step in the purification follows elution of the enzyme from carboxymethyl acrylic polymer.

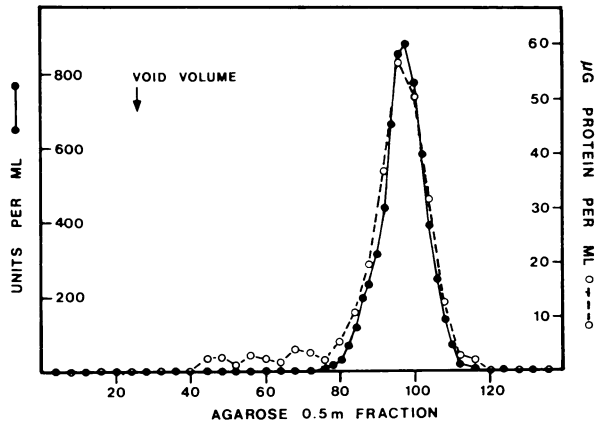

FIG. 2. Purification of endopolygalacturonase by affinity chromatography on Agarose 0.5 m. This step in the purification follows gel filtration through Bio-Gel P-150.

The filtrate and DE-52 washings were combined and passed through a Bio-Rex 70 (Bio-Rad Labs, carboxymethyl acrylic polymer) column (1 × 13 cm) that had been pre-equilibrated with 50 mM sodium acetate, pH 5.2. The enzyme solution was allowed to flow through the column at about 0.3 ml/min. The column was then washed with 200 ml of 50 mM sodium acetate, pH 5.2. All of the endopolygalacturonase binds to the column during these procedures. When the column is next washed with 200 ml of 0.33 M sodium acetate, pH 5.2, about 10% of the endopolygalacturonase elutes. This activity may represent an endopolygalacturonase isozyme which has not been studied further. The Bio-Rex 70 column is then subjected to a linear gradient elution in which 100 ml of 0.33 M sodium acetate, pH 5.2, is the starting buffer and 100 ml of 1.5 M sodium acetate, pH 5.2, is the final buffer. Two-milliliter fractions were collected.

Fractions 20 through 50 of the Bio-Rex 70 column were pooled and dialyzed overnight against 50 mM sodium acetate, pH 5.2. The enzyme was concentrated by the following procedure. The dialyzed enzyme solution was passed over a second Bio-Rex 70 column which had been pre-equilibrated in 50 mM sodium acetate, pH 5.2. This column (0.4 × 4.0 cm) had a bed volume of only 0.5 ml. The endopolygalacturonase was eluted from the column with 20 ml of 1.5 M sodium acetate, pH 5.2. This solution was dialyzed overnight against 50 mM sodium acetate, pH 5.2. The dialysis bag was then placed on Parafilm, and dry Sephadex G-200 (Pharmacia) was poured over the bag. In this manner, the enzyme solution could be concentrated in about 6 hr to about 1 ml. The enzyme was removed from the dialysis membrane, and the inside of the dialysis bag was washed two times with 0.5 ml of 50 mM sodium acetate, pH 5.2. The enzyme solution and washes were combined.

The concentrated enzyme was further purified by filtration through a Bio-Gel P-150 (Bio-Rad Labs) column (1.4 × 91 cm; measured void volume: 38 ml). The column was eluted with 50 mM sodium acetate, pH 5.2. Two-milliliter fractions were collected and assayed for endopolygalacturonase activity and for protein (Fig. 1). The peak of the endopolygalacturonase eluted at 66 ml while lysozyme (mol wt: 14,400), used as a standard, eluted at 109 ml. With this information, the calculated molecular weight of the endopolygalacturonase is 78,000.

Fractions 28 through 38 from the Bio-Gel P-150 column were combined, placed in a dialysis bag, and concentrated to 1 ml with Sephadex G-200. This concentrated enzyme solution was further purified by affinity chromatography on an Agarose 0.5 m (Bio-Rad Labs) column (1.4 × 98 cm; calculated void volume: 57 ml). The column was eluted with 50 mM sodium acetate, pH 5.2. Two-milliliter fractions were collected and assayed for endopolygalacturonase activity and for protein (Fig. 2). The endopolygalacturonase elutes after the inclusion volume of this column. The affinity of the endopolygalacturonase for the agarose may result from the fact that the anhydrogalactose residues of the agarose resemble the enzyme's substrate.

Fractions 78 through 112 from the Agarose 0.5 m column were combined and concentrated on a Bio-Rex 70 (0.5-ml bed volume) column with the aid of Sephadex G-200 as described above. The concentrated enzyme was then chromatographed

on a Bio-Gel P-300 (Bio-Rad Labs) column (1.4 × 108 cm; measured void volume: 50 ml). The column was eluted with 50 mM sodium acetate, pH 5.2. Two-milliliter fractions were collected and assayed for endopolygalacturonase activity and for protein (Fig. 3). The endopolygalacturonase elutes at 2.2 void volumes, which, according to the Bio-Rad charts, indicates that the endopolygalacturonase has a molecular weight of 62,000. Fractions 48 to 62 were combined and used for further studies.

The data in Table I summarize a typical purification of the endopolygalacturonase.

Characterization of the Purified Endopolygalacturonase. The results presented in Figure 4 demonstrate that polygalacturonic acid is a far better substrate for the purified enzyme than is the 70% methyl esterified polymer, pectin. The optimal activity of the enzyme in sodium acetate buffer is about pH 5 (Fig. 5). A similar pH optimum is obtained when the enzyme is assayed viscometrically.

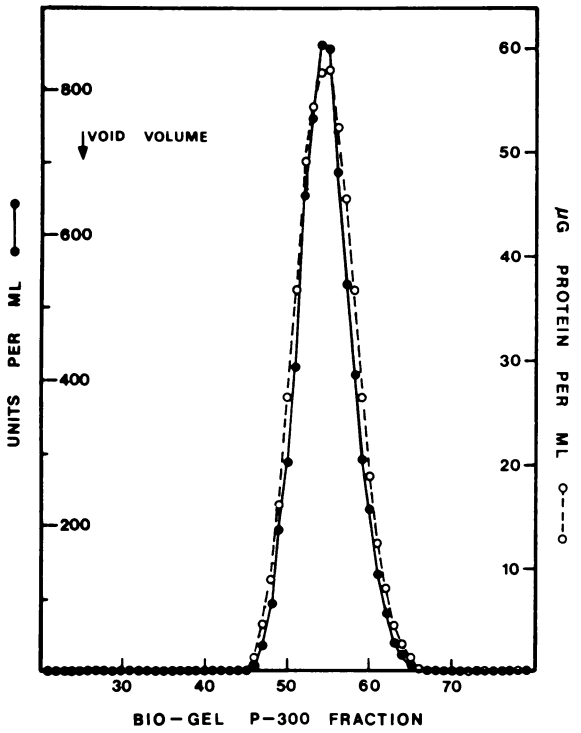

FIG. 3. Gel filtration of the endopolygalacturonase purified by affinity chromatography on agarose 0.5 m. This procedure was used to confirm that the enzyme obtained from the agarose column is a homogeneous protein.

Table I. *Summary Purification of C. lindemuthianum Endopolygalacturonase*

Fraction	Activity	Volume	Total Activity	Yield	Protein	Specific Activity	Purification
	units/ml[1]	*ml*	*units*	%	*μg/ml*	*units/μg protein*	*relative*
Crude	24.7	3475	85,800	100	78.0	0.32	1
DE-52	15.7	3980	62,500	73	21.6	0.73	2.3
Bio-Rex 70	572	62	35,500	41	80.5	7.10	22
Bio-Gel P-150	1020	22	22,400	26	76.6	13.3	42
Agarose 0.5m	239	62	15,800	18	17.1	14.0	44
Bio-Gel P-300	302	36	10,300	12	21.5	14.0	44

[1] One unit of enzyme hydrolyzes 1 μmole of polygalacturonic acid glycosidic bonds in 1 hr at 28 C when the substrate is 0.1% citrus polygalacturonic acid and the reaction is carried out in 1 ml of 50 mM sodium acetate, pH 5.2.

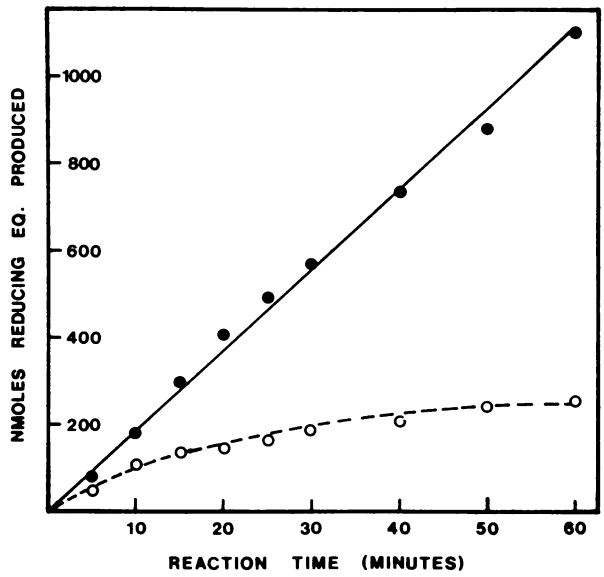

FIG. 4. Hydrolysis of 0.1% polygalacturonic acid (de-esterified pectin) (●) and of 0.1% pectin (○) by 1.1 units of purified endopolygalacturonase in 50 mM sodium acetate, pH 5.2 at 28 C.

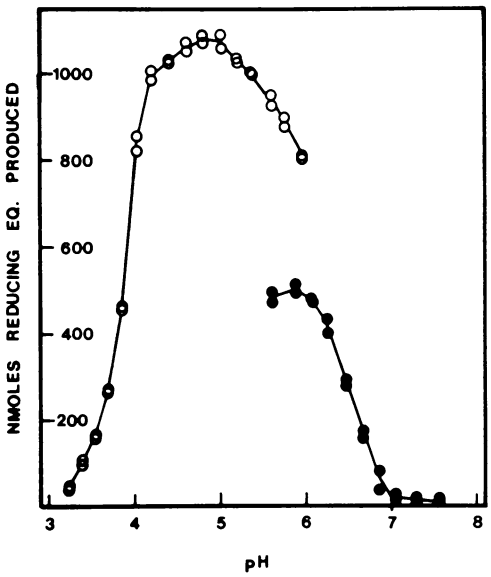

FIG. 5. Effect of various hydrogen ion concentrations on the hydrolysis of 0.1% polygalacturonic acid by 2.2 units of purified endopolygalacturonase in 30 min at 28 C. The buffers are 90 mM sodium acetate (○) and 90 mM sodium phosphate (●).

The data of Figure 6 show that the endopolygalacturonase hydrolyzes polygalacturonic acid in an endo- rather than exo-fashion. The enzyme, while hydrolyzing only 1% of the glycosidic bonds, reduces the viscosity of a polygalacturonic acid solution by 50%.

The product of exhaustive enzymic hydrolysis of both citrus and sycamore polygalacturonic acid contains almost exclusively mono-, di-, and trigalacturonic acid. This was shown both by paper chromatography in 1-butanol-acetic acid-water (5:2:3) (11) and by gel filtration through Bio-Gel P-2 (Fig. 7). The data of Figure 7 demonstrate that the predominant products are tri- and digalacturonic acid. The monogalacturonic acid peak represents less than 50% of either of the other two components. That the peaks in Figure 7 repre-

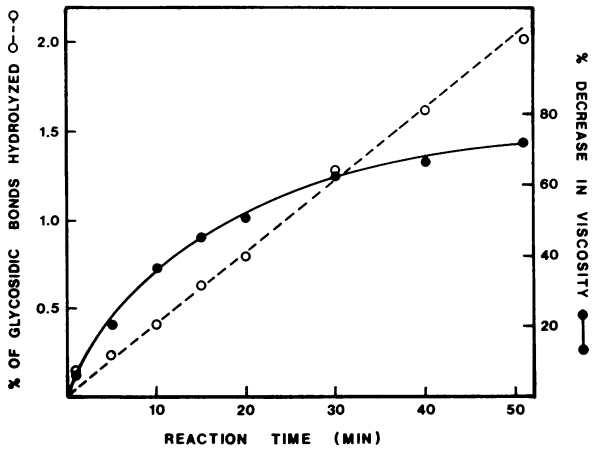

FIG. 6. Comparison of the ability of the purified endopolygalacturonase to hydrolyze the glycosidic linkages of polygalacturonic acid with the ability of the enzyme to reduce the viscosity of the solution containing the polygalacturonate. The data were obtained by incubating 50 units of the enzyme (200 μl) with 16 ml of 0.9% polygalacturonic acid in 50 mM sodium acetate, pH 5.2, at 28 C. One ml aliquots were removed for viscosity measurements while 0.5 ml aliquots were used for reducing group assays. The initial solution had a relative viscosity of 8.5, and the solution after 50 min incubation had a relative viscosity of 2.4.

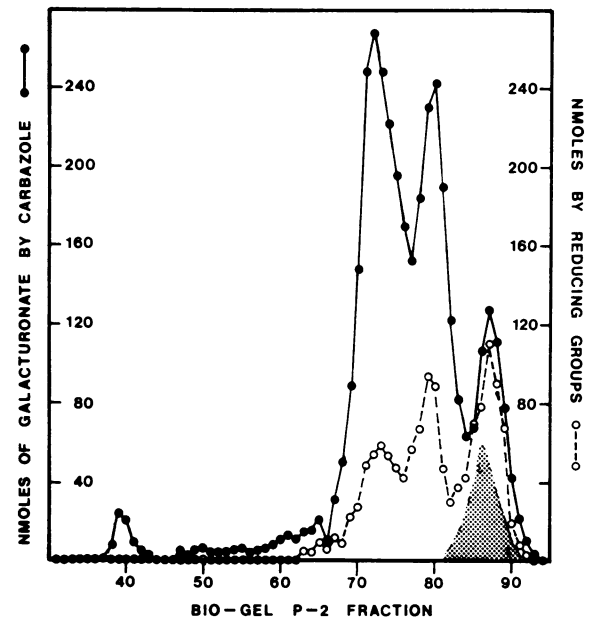

FIG. 7. One mg of polygalacturonic acid was subjected to hydrolysis for 3 hr at 28 C by 20 units of purified endopolygalacturonase. The products were chromatographed in 50 mM sodium acetate, pH 5.2, on a Bio-Gel P-2 column (1.4 × 72 cm, void volume 38 ml), and 1 ml fractions were collected. The fractions were assayed for uronic acids by the carbazole method and for reducing sugars by the Nelson-Somogyi method. A sample of monomeric galacturonic acid (stippled area) was chromatographed separately.

sent the monomer, dimer, and trimer is supported by comparison in each peak of the ratio of reducing groups to uronic acids. This ratio in the monomer peak (fractions 86–88) was 0.85. On this basis, the dimer peak (fractions 79 and 80) should have a ratio of 0.43 and the trimer peak (fractions 72–74) should have a ratio of 0.28. The values measured were 0.39 and 0.22, respectively. The values may be somewhat lower than expected because the dimer and trimer may not react as efficiently as monomers in the reducing group assay. However, the values are accurate enough to say that the two larger peaks are more likely di- and trigalacturonic acid than tri- and tetragalacturonic acid. The rate of migration of these two components in paper chromatography as compared to the rate for monogalacturonic acid confirms that they are indeed di- and trigalacturonic acid (11). The small peak at the void volume of this column (fractions 37–40) contains a high proportion of neutral sugars and a low content of galacturonic acid. The citrus polygalacturonic acid used in these experiments contains about 5% neutral sugars. This portion of the polysaccharide is apparently not susceptible to the action of the endopolygalacturonase.

It is an interesting and unusual fact that the initial as well as the terminal products of the enzymic catalyzed hydrolysis of polygalacturonic acid by the endopolygalacturonase are the mono-, di-, and trigalacturonic acids. We have demonstrated both by paper chromatography and by Bio-Gel P-2 gel filtration (data not presented) that even when only a small percentage of the galacturonide glycosidic linkages are hydrolyzed by the endopolygalacturonase, the tri-, di-, and monogalacturonic acids are the predominant products. And the ratio of these three compounds does not change significantly throughout the hydrolysis; at all times, the ratios are approximately those seen in Figure 7.

The Purified Endopolygalacturonase-catalyzed Degradation of Cell Walls. The endopolygalacturonase releases as uronic acid in oligo- and monosaccharides about 9% of the cell walls isolated from sycamore cells and about 10% of the walls isolated from 8-day-old Red Kidney bean hypocotyls (Fig. 8). The ratio of total uronic acid to reducing sugars in the products released is 2.2 (Fig. 8). This would indicate that the average chain length of this material is between two and three sugars long. This is in good agreement with the results of the degradation of citrus polygalacturonic acid (Fig. 7). That the products released contained large amounts of tri-, di-, and monogalacturonic acids was confirmed both by paper chromatography in 1-butanol-acetic acid-water (5:2:3) (11) and by Bio-Gel P-2 chromatography.

The data of Table II show that the material removed from

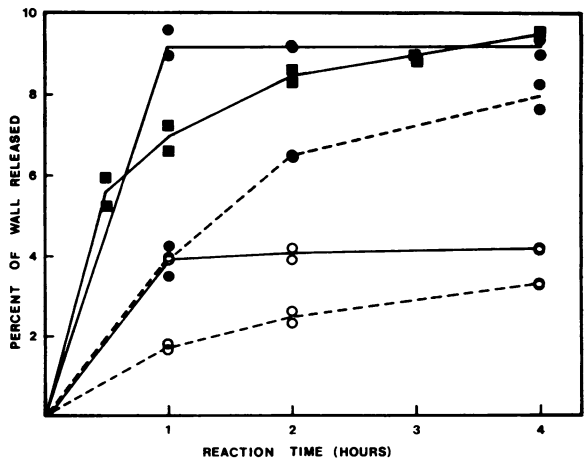

FIG. 8. Cell walls (5-mg samples) isolated from suspension cultured sycamore cells (○;●) and from 8-day-old Red Kidney bean hypocotyls (■) were subjected at 30 C to either 4.2 units (dotted lines) or 21 units (solid lines) of the purified endopolygalacturonase. The material released from the walls was assayed by the Nelson-Somogyi method for reducing sugars (○) or by the carbazole method for uronic acids (●;■).

both the sycamore and bean cell wall preparations is predominantly galacturonic acid. This material also contains some arabinose, galactose, and rhamnose, sugars which have previously been shown to be covalently linked in pectin (21).

DISCUSSION

The endopolygalacturonase secreted by *C. lindemuthianum* has been isolated in a highly purified state. The evidence suggesting that the protein is homogeneous or nearly homogeneous includes the constant specific activity of the active fractions obtained upon affinity chromatography on agarose 0.5 m (Fig. 2). This unusual affinity for agarose undoubtedly permitted the enzyme to be freed of remaining contaminants. Further evidence of purity was obtained upon gel filtration through Bio-Gel P-300 (Fig. 3). The active fractions from this column again have a constant specific activity, and the specific activity of these fractions is the same as that obtained for the agarose column (Table I). The elution volumes on both Bio-Gel P-150 and P-300 indicate that the endopolygalacturonase has a molecular weight of about 70,000 provided that the enzyme is a normal globular protein.

It should be noted that a significant loss in activity occurred every time it was necessary to concentrate the endopolygalacturonase. We believe most of the loss in enzyme activity is caused by a tendency of the enzyme to precipitate irreversibly at high concentrations. A precipitate was observed after concentration of the enzyme in a dialysis bag. This explanation is made more plausible by the fact that very poor recoveries of the enzyme were obtained after concentration by lyophilization, by ultrafiltration, and by ultracentrifugation. On the other hand, the endopolygalacturonase is very unstable in solutions containing less than 50 units (3 μg of enzyme) per ml. The enzyme's stability in such dilute solutions was improved by the addition of bovine serum albumin. The expected losses of enzyme due to manipulation of the protein solution are also encountered.

An unexpected and unusual property of this enzyme is that although it attacks its substrate in an endo fashion, as evidenced by its rapid reduction of the viscosity of polygalacturonic acid solutions (Fig. 6), its initial and terminal products are predominantly tri-, di-, and monogalacturonic acid (Fig. 7). Therefore, it appears that the enzyme initially attacks the polygalacturonic acid chain molecules randomly, and then the enzyme progresses from that spot along the chain, releasing trimers, dimers, and monomers, until it reaches a barrier such as a branch point or neutral sugar in the chain. At this point the enzyme may release and attack another chain.

Endopolygalacturonases are secreted by a wide variety of plant pathogens (2, 5). These are the first detectable enzymes secreted by *C. lindemuthianum* (9) and by *Fusarium oxysporum* f. sp. *lycopersici* (T. M. Jones, A. Andersen, and P. Albersheim, in preparation) when these pathogens are grown on isolated cell walls. A central role in pathogenesis for polygalacturonic acid-degrading enzymes has been established by the demonstration that they can induce killing of cells (10, 15). A key role for these enzymes in the disease process has been further established by the finding within plant cell walls of proteins which inhibit pathogen secreted endopolygalacturonases without inhibiting a number of other polysaccharide-degrading enzymes secreted by these pathogens (1). These plant cell wall proteins also can distinguish between the polygalacturonases secreted by different pathogens. The endopolygalacturonase described in this paper is one of the pathogen-secreted enzymes inhibited by proteins present in plant cell walls. Indeed, a protein has been isolated from the cell walls of Red Kidney bean hypocotyls which forms an inactive complex with the purified *C. lindemuthianum* endopolygalacturonase. The dissociation constant of this complex is 2×10^{-9} M or less (1).

Table II. *Degradation of Isolated Cell Walls by Endopolygalacturonase*

Sugar composition of Red Kidney bean hypocotyl and of suspension-cultured sycamore cell walls before and after treatment for 4 hr at 30 C with 4 units of purified endopolygalacturonase per mg of isolated walls. The result of this method does not include the glucose present in cellulose.

Sugar	8-day-old Red Kidney Hypocotyls		Suspension-cultured Sycamore Cells		
	Wall composition		Wall composition		Polygalacturonase products[1]
	Untreated control	Remaining after polygalacturonase	Untreated control	Remaining after polygalacturonase	
	%				
Rhamnose	1.2	0.7	2.7	2.3	3.7
Fucose	0.4	0.3	1.2	0.9	0.3
Arabinose	2.5	1.7	18.5	14.9	13.8
Xylose	8.1	7.1	6.1	5.8	0.8
Mannose	1.0	1.0	0.3	0.3	0.0
Galactose	6.7	4.8	10.8	9.4	13.4
Glucose	1.5	1.4	12.7	12.4	2.8
Galacturonic acid	14.0	3.0	12.0	2.7	65.2

[1] Adjusted to 100%.

Although the above evidence established the importance of polygalacturonic acid-degrading enzymes in the disease process, it had not previously been demonstrated that a highly purified endopolygalacturonase could, by itself, degrade isolated plant cell walls. The results described in this paper establish that the endopolygalacturonase secreted by *C. lindemuthianum* can remove about 75% of the galacturonic acid as well as a small amount of the neutral sugars from the cell walls of sycamore cells and from the cell walls of bean hypocotyls. Walls degraded by this endopolygalacturonase are susceptible to further degradation by partially purified cellulases, although walls not modified by endopolygalacturonase pretreatment are highly resistant to the cellulases (B. Jurale, K. Keegstra, and P. Albersheim, unpublished results). Because of its wall-degrading ability, the endopolygalacturonase described in this paper has proven to be of great value in our laboratory's current effort to elucidate the structure of cell wall polysaccharides.

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