Degradation of Corn Cell Walls by Extracellular Enzymes Produced by Helminthosporium maydis Race T

D. F. Bateman, T. M. Jones, and O. C. Yoder

Department of Plant Pathology, Cornell University, Ithaca, New York 14850.

ABSTRACT

Endopolygalacturonase and endoxylanase were produced by *Helminthosporium maydis* race T growing on a mineral salts-glucose medium at 23 C. Pectin lyase (endopectin methyl-*trans*-eliminase) with a pH optimum of about 8.5 was produced by the fungus on potato broth-Na polypectate or mineral salts-glucose-Na polypectate media. The endopolygalacturonase exhibited a pH optimum of 4.8, a pI of 8.3, and released mono-, di-, tri-, and higher oligomers of D-galacturonic acid from Na polypectate; this enzyme induced a 50% loss in viscosity of 1% Na polypectate at 30 C with about 0.1% hydrolysis. The xylanase system had a pH optimum between 4 and 6. It released D-xylose and oligomers of D-xylose from xylan. Within 2 hr at 30 C, culture filtrates of *H. maydis* race T grown on the mineral salts-glucose medium released 31% of the galacturonate, 45% of the xylose, and 75% of the arabinose from the trifluoroacetic acid-*Sclerotium rolfsii* enzyme hydrolyzable portion of cell walls from leaves of 10-day-old corn seedlings.

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The abilities of fungi pathogenic to monocots to produce cell wall-degrading enzymes and the significance of such enzymes in pathogenesis are not well documented. *Helminthosporium maydis* (Nisikado & Miyake) race T is a pathogen of corn (*Zea mays* L.) cultivars which contain Texas male sterile cytoplasm. The disease caused by this pathogen is characterized by distinct lesions on susceptible leaves that may enlarge and coalesce, causing foliage blight. Histological studies have shown that the fungus ramifies inter- and intracellularly in areas of infection loci (9). In later stages of infection, cell walls collapse (32). These observations suggest involvement of cell wall-degrading enzymes during pathogenesis. Production of such enzymes by other *Helminthosporium* spp. has been observed. *H. carbonum* is known to produce a polygalacturonase (21). *H. sativum* has been reported to produce polymethylgalacturonase, pectin lyase, and cellulase (Cx) (18), and *H. atypicum* is known to produce a pectic enzyme capable of causing tissue maceration (10). The pectic enzymes of these *Helminthosporium* spp. appear to be produced in an inductive manner.

This report deals with the ability of *H. maydis*

race T to produce cell wall-degrading enzymes in vitro. Characteristics of a constitutively produced endopolygalacturonase and an endoxylanase are described. In addition, crude culture filtrates of *H. maydis* race T are shown to release galacturonic acid, xylose, arabinose, galactose, and glucose from isolated corn cell walls. A preliminary report of this work has appeared (5).

MATERIALS AND METHODS.—The isolate of *H. maydis* used in this study was identified as race T by its specificity to corn containing Texas male sterile cytoplasm and by its ability to produce host-specific toxin in culture (11). The fungus was maintained on potato-dextrose agar or in dried, infected corn leaves.

The basal medium for enzyme production was potato broth (filtrate from 200 g of potato autoclaved for 15 min in 1,000 ml water) or a salts solution containing per liter: 181 mg $MgSO_4$, 149 mg KCl, 1,000 mg NH_4SO_4, 650 mg KH_2PO_4, 3.5 mg $ZnSO_4 \cdot H_2O$, 6.2 mg $MnSO_4$, 2.0 mg $FeCl_3$, 2.0 mg $CuSO_4$, and 2.0 g yeast extract. The carbon source added as a supplement to basal media was 2.0% glucose, 2.0% Na polypectate (Sunkist Growers, Inc., Ontario, Calif.), or 1.0% glucose plus 1.0% Na polypectate. Cultures were grown in darkness at 23 C in 300-ml Erlenmeyer flasks containing 50 ml of sterile medium. The media were seeded with 5- to 7-mm diam plugs of *H. maydis* race T growing on PDA or with a spore suspension (500/ml, final concn) prepared from young cultures grown on PDA. After incubation, mycelium was removed from culture fluid with a double layer of cheesecloth. Fungal fragments and spores were removed from filtrates by centrifugation at 20,000 *g* for 20 min at 4 C. After dialysis against several hundred volumes of distilled water for 12 to 18 hr at 4 C, filtrates were either used immediately in experiments or frozen at −20 C until needed.

Pectic enzyme activities were measured by loss in viscosity of Na polypectate and/or pectin N.F. (3), by increase in free reducing groups in reaction mixtures (20), by increase in absorbance of reaction products at 230 nm (19), and by the thiobarbituric acid (TBA) method (24). Size 300, Ostwald-Fenske viscometers containing 5.0 ml of 1.2% substrate plus 1.0 ml of enzyme solution were used to determine rate of viscosity loss of substrates at 30 C. Results are reported in relative activity units (reciprocal of the time in min for 50% viscosity loss $\times 10^3$) per ml or per mg protein. Reaction mixtures for reducing group and TBA assays contained 1.0 ml of 1.2% substrate plus 1.0 ml of an appropriately diluted enzyme solution. Reaction mixtures were incubated at 30 C. Assays were made in duplicate on 0.2-ml aliquots of reaction mixtures. All reactions were buffered with 0.05 M buffer (Na citrate, pH 3 to 4; Na acetate, pH 4 to 5; citric acid-Na phosphate, pH 5 to 6; Na phosphate, pH 6 to 7; and tris-HCl, pH 8 to 9).

Xylanase activity was estimated by determining the release of reducing groups from a β-1,4-D xylan (Pfaltz & Bauer, Inc., Flushing, N.Y.) as described above for polygalacturonase activity. The same buffer systems were employed.

Products released during enzymatic depolymerization of Na polypectate and xylan were identified by paper chromatography. Reaction mixtures contained 0.5 ml of enzyme and 0.5 ml of 1.2% substrate in 0.1 M Na acetate buffer (pH 4.8) and were incubated at 30 C. Controls consisted of reaction mixtures without substrate and reaction mixtures without enzyme. After incubation, 50-μl aliquots of reaction mixtures were applied to 25 × 55 cm sheets of Whatman No. 1 paper. Reference standards (50 μg of each) were D-glucose, D-galacturonic acid, and/or D-xylose. Chromatograms were irrigated in a descending manner with butanol-acetic acid-water (2+1+1) for 24 hr at 21 C and developed with silver nitrate (2, 28).

Dialyzed culture filtrates were subjected to ammonium sulfate fractionation and to diethylaminoethyl cellulose (DEAE cellulose-Cl form) column chromatography at pH 8.0 using procedures described by Mount et al. (17). Gel filtration was performed in 2.5 × 34 cm columns of Sephadex G-75 as described by Andrews (1). The molecular weight of polygalacturonase was estimated in this system using bovine serum albumin (mol wt 67,000), horseradish peroxidase (mol wt 40,000), and cytochrome C (mol wt 12,400) as reference proteins. Carboxymethyl cellulose (CM-cellulose) chromatography was done in 1.5 × 20 cm columns containing 5.0 g washed CM-cellulose suspended in 0.05 M Na acetate buffer at pH 4.0. Twenty to 50 ml of dialyzed culture filtrate, adjusted to pH 4.0, was added to the columns, followed by 0.05 M Na acetate buffer (pH 4.0) and a NaCl gradient (0 to 0.5 M) in buffer. Five-ml fractions were collected, dialyzed against water, and assayed for enzyme activity. Enzyme preparations were subjected to isoelectric focusing in a LKB 8101 Ampholine apparatus equipped with a 110-ml column (LKB-Produkter AB, Bromma, Sweden) containing pH 3 to 10 Ampholine carriers (17). Electrofocusing was carried out for approximately 48 hr, at which time the pH gradient had formed and the current had stabilized (500 v, 0.8 ma). The pH of each 5-ml fraction was measured, then each fraction was dialyzed against water for about 24 hr and assayed for enzyme activity. All of the above procedures, except enzyme assays, were carried out at 4 C.

Protein determinations.—Protein concns were estimated using the method of Lowry et al. (16); crystalline bovine serum albumin was used as the reference protein.

Preparation of corn cell walls.—The distal half of leaves from 10-day-old corn (W64A T) seedlings grown in a greenhouse at about 27 C were harvested and frozen. Cell walls were prepared from this tissue by a modification of the procedure of Nevins et al. (22). Tissue was ground in a mortar in liquid nitrogen until cells were disrupted. The pulverized tissue was extracted with 2.5 volumes (w/v) of cold 500 mM Na phosphate buffer (pH 7.0) by grinding in a Waring Blendor for 2.0 minutes. The resulting brei was filtered with a no. 60 (U.S. Standard) sieve and the residue retained was extracted two more times with

2.5 volumes (w/v) of 500 mM Na phosphate buffer. The residue from these extractions was extracted once with 2.5 volumes (w/v) of chloroform-methanol (1+1) and twice with acetone, using 2.5 volumes (w/v) for each extraction. The residue (cell wall preparation) was washed with a large volume of acetone, air-dried, and stored at room temperature in a capped vial.

Assay of cell wall degradation.—Cell wall hydrolysis by *H. maydis* race T enzymes was assayed by a modification of the procedure of Jones and Albersheim (13). Reaction mixtures contained 10.0 mg isolated corn cell walls, 1.0 ml of 0.1 M Na acetate buffer (pH 4.8), and 1.0 ml dialyzed culture filtrate. Water was substituted for enzyme in the control treatment. Incubation was for 2 hr at 30 C. Two ml of water was added to each reaction mixture; the preparation was centrifuged for 5 min (1,150 g) and the supernatant was decanted. The pellets were washed twice with 5 ml water and the washings were discarded. The washed residues were subjected to depolymerization by treatment with 0.2 N trifluoroacetic acid (TFA) followed by enzymolysis with a *Sclerotium rolfsii* enzyme preparation (13, 30). Acetate derivatives of the liberated monosaccharides were prepared and analyzed gas chromatographically by the procedure described by Jones and Albersheim (13). The decrease in monosaccharides released by TFA and *S. rolfsii* enzymes after exposure of walls to culture filtrate was attributed to wall solubilization by *H. maydis* race T enzymes.

RESULTS.—*Production of extracellular enzymes by H. maydis race T.*—Culture filtrates of *H. maydis* race T grown on several media exhibited pectolytic activity (Table 1). The most active preparations were obtained from cultures grown on mineral salts-glucose medium. The pectic enzyme in these filtrates readily released reducing groups (Fig. 1), oligomers, and mono-D-galacturonic acid, from Na polypectate. The

pH optimum for enzyme activity was near 5.0 (Fig. 2). This enzyme caused a 50% decrease in viscosity of a 1% solution of Na polypectate at 30 C with only about 0.1% hydrolysis. Reaction products did not absorb light at 230 nm or react with TBA to yield a chromagen that absorbs maximally at 548 nm. It was concluded that *H. maydis* race T produces an endopolygalacturonase.

In some experiments, maximum enzyme activity was observed after 9 days of fungal growth while in others maximum activity was not reached until after 12 days. Maximum endopolygalacturonase was recovered from cultures grown on the salts-glucose medium; there was less recovery of this enzyme from cultures containing potato broth as the basal medium or pectic polymers as a carbon source. Apparently the *H. maydis* race T enzyme is produced in a constitutive manner. In contrast, other *Helminthosporium* spp. (10, 18, 21) produce pectic enzymes in an adaptive manner.

Significant pectin lyase [pectin methyl-*trans*-eliminase (6)] activity was detected in filtrates from cultures grown in mineral salts-glucose-Na polypectate and potato broth-Na polypectate media (Table 1). This enzyme did not affect Na polypectate but degraded pectin N.F. in a *trans*-eliminative manner with a pH optimum of about 8.5 (Fig. 3). Reaction products absorbed light maximally at about 230 nm and, upon reaction with TBA, yielded a red chromagen with a maximum light absorbance at 548 nm. Although lyase activity was low, it was demonstrated with certainty using all four assay methods. For example, product was formed at the rate of 56 nmoles/ml per hr when filtrate from a 9-day-old culture grown on potato broth-Na polypectate medium was incubated with 0.12% pectin N.F. in 0.05 M Tris-HCl at pH 8.5, and assayed by increase in absorbance at 230 nm (19). The slow rate of product appearance compared with the rate of substrate viscosity loss indicates that the lyase produced by *H. maydis* race T is of the endo type (6).

Filtrates of 9- and 12-day-old cultures grown on mineral salts-glucose medium were examined for arabinosidase, cellulase (Cx), galactanase, and xylanase activities. Reaction mixtures contained 0.5 ml of 0.25% substrate in 0.1 M Na acetate buffer (pH 4.8) and 0.5 ml of dialyzed filtrate. Assays for increase in reducing groups were done at 30 C over a 2-hr period. Aside from the endopolygalacturonase, the only other cell wall-degrading enzyme that could be consistently demonstrated in these culture filtrates was xylanase (Fig. 4). The optimum pH for the xylanase system was between 4.0 and 6.0 (Fig. 5). Paper chromatography of reaction products revealed that D-xylose as well as oligomers of D-xylose, including di-, tri-, tetra-, and penta-D xylose, were released from xylan. Thus, it appears that *H. maydis* race T is capable of producing an endoxylanase and, based on the relatively large amount of monomer released, possibly a β-xylosidase.

Unlike the cell wall-degrading enzymes produced by many plant pathogens (3, 4, 6, 7, 12, 17, 19), the enzymes produced by *H. maydis* race T proved to be

TABLE 1. Relative pectic enzyme activities produced by *Helminthosporium maydis* race T in various media[a]

Substrate	Assay pH	Enzyme activity[b]					
		SG	PBG	PBGP	SGP	SP	PBP
Na polypectate	4.9	140	7	17	24	W	W
Na polypectate	8.3	W[c]	W	W	W	W	W
Pectin N.F.	4.7	25	W	4	7	W	W
Pectin N.F.	8.1	W	W	W	10	W	13

[a] Dialyzed filtrates from 9-day-old cultures grown at 23 C were assayed by the viscosity-loss method. Results are reported as the reciprocal of the time in min for 50% viscosity loss at 30 C × 10^3.

[b] The culture media are described in the text. The abbreviations are: SG = salts-glucose-yeast extract; PBG = potato broth-glucose; PBGP = potato broth-glucose-Na polypectate; SGP = salts-glucose-yeast extract-Na polypectate; SP = salts-Na polypectate-yeast extract; and PBP = potato broth-Na polypectate.

[c] The letter "W" indicates that the enzyme activity was too weak to be measured accurately by the method used.
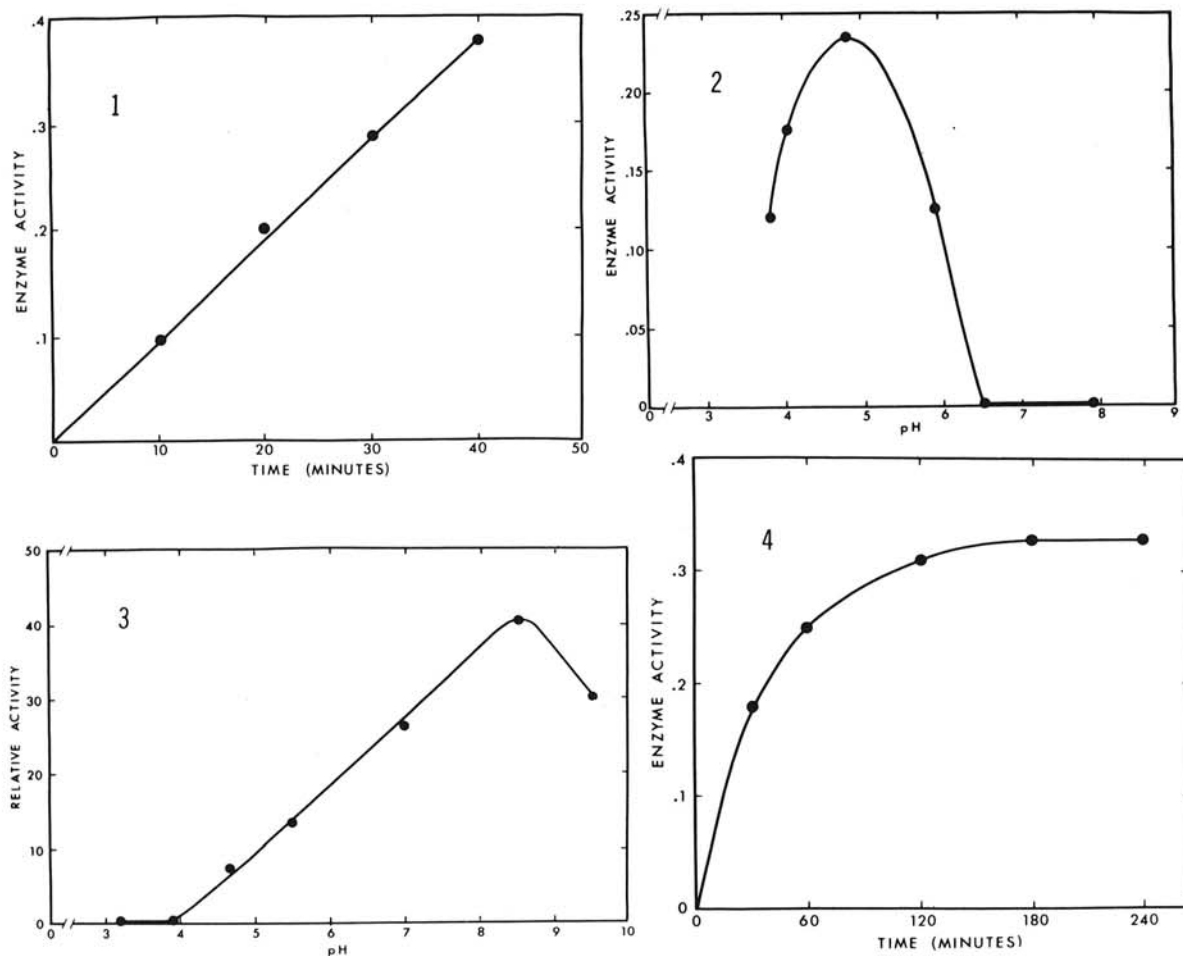
Fig. 1-4. Characteristics of pectolytic and xylolytic enzymes produced by *Helminthosporium maydis* race T. **1)** Release of reducing groups from Na polypectate by dialyzed culture filtrate of *H. maydis* race T grown on a salts-glucose medium. Reaction mixtures contained 4.0 ml of 1.2% Na polypectate in 0.1 M acetate buffer (pH 4.8) and 2.0 ml of filtrate. Results are expressed as μmoles of reducing groups released/ml of filtrate. **2)** Effect of pH on activity of *H. maydis* race T polygalacturonase. Reaction mixtures were similar to those in [1]; Tris-HCl buffer was used in the alkaline pH range. Results are expressed as μmoles of reducing groups released/ml of filtrate/20 min. **3)** Effect of pH on activity of *H. maydis* race T pectin lyase. Results are expressed as relative viscosity loss of reaction mixtures containing 4.0 ml of 1.2% pectin N.F. in 0.1 M buffer (Na acetate or Tris-HCl), 1.0 ml of filtrate (from cultures grown in a potato broth-Na polypectate medium), and 1.0 ml of water; the procedure for calculating relative activity is described in Table 1. **4)** Release of reducing groups from xylan by dialyzed culture filtrate of *H. maydis* race T grown on salts-glucose medium. Reaction mixtures contained 1.0 ml of 0.25% xylan in 0.1 M Na acetate buffer (pH 4.8) and 1.0 ml of filtrate. Results are expressed as in [1].

difficult to work with and were often readily inactivated when subjected to routine purification procedures. When culture filtrates were dialyzed, it was not uncommon to lose up to one-third of the endopolygalacturonase activity. Attempts to fractionate batches of several hundred ml of culture filtrates by ammonium sulfate at 40, 60, 80, and 95% saturation (17) were unsuccessful. Less than 10% of the original endopolygalacturonase or xylanase activity could be recovered in any one ammonium sulfate fraction and no increase in specific activity for either enzyme could be demonstrated. Also, the relatively low xylanase activity in crude culture filtrate, coupled with its instability, made it difficult to purify and characterize by conventional procedures.

The endopolygalacturonase of *H. maydis* race T did not bind to DEAE-cellulose at pH 8.0, but it did adsorb to CM-cellulose at pH 4.0. When dialyzed filtrates were subjected to CM-cellulose column chromatography, enzyme was eluted with 0.05 M NaCl in 0.05 M Na acetate buffer (Fig. 6). This procedure resulted in about a 10-fold increase in specific activity. In numerous experiments over a one-year period, the recovery of endopolygalacturonase from CM-cellulose columns varied from 38 to 88%. Endopolygalacturonase in dialyzed filtrates was purified up to 16-fold by
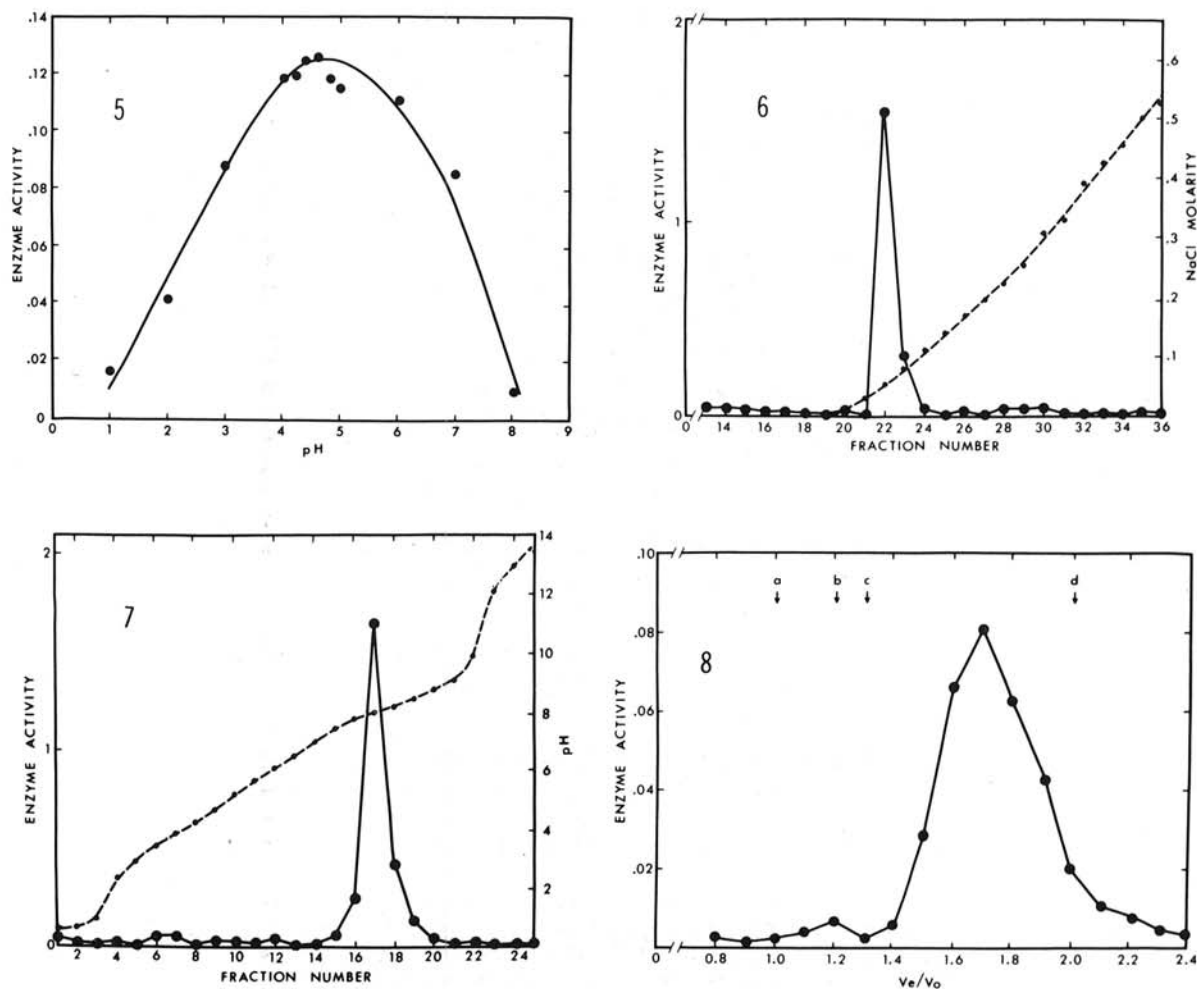
Fig. 5-8. Characteristics of pectolytic and xylolytic enzymes produced by *Helminthosporium maydis* race T. **5)** Effect of pH on activity of *H. maydis* race T xylanase. Reaction mixtures contained 0.5 ml of 0.25% xylan in 0.1 M buffer (see Materials and Methods) and 0.5 ml of dialyzed culture filtrate. Results are expressed as in [2]. **6)** Carboxymethylcellulose column chromatography of *H. maydis* race T polygalacturonase. Dialyzed culture filtrate (50 ml) was adjusted to pH 4.0 and applied to a 1.5 × 17 cm column of CM-cellulose equilibrated with 0.05 M Na acetate buffer (pH 4.0). The column was washed with 20 ml of buffer followed by a NaCl gradient in buffer. Fractions (5 ml) were dialyzed and a 0.5-ml aliquot of each fraction was incubated with 0.5 ml of 1.2% Na polypectate in 0.1 M Na acetate (pH 4.8) and assayed for release of reducing groups. Results are expressed as in [2]. **7)** Isoelectric focusing of polygalacturonase in a 50-ml sample of dialyzed *H. maydis* race T culture filtrate. After focusing, fractions (5 ml) were assayed as described in [6]. **8)** The elution pattern of *H. maydis* race T polygalacturonase from a Sephadex G-75 column (2.5 × 34 cm) in the presence of 0.05 M Na acetate (pH 4.8) and 0.1 M NaCl. Fractions (5 ml) were assayed as described in [6]. The markers a, b, c, and d represent the elution peaks of blue Dextran, bovine serum albumin, horseradish peroxidase, and cytochrome C, respectively.

electrofocusing in a pH 3 to 10 gradient at 4 C; the pI of this enzyme was about 8.3 (Fig. 7). Sephadex G-75 gel filtration of crude preparations resulted in about a two-fold purification of endopolygalacturonase. Using this technique, the molecular weight of the enzyme was estimated to be about 30,000 (Fig. 8). Attempts to use any of the above purification procedures in sequence to further purify endopolygalacturonase resulted in essentially complete inactivation of the enzyme.

Degradation of isolated corn cell wall by H. maydis race T enzymes.—When cell walls isolated from corn seedlings were subjected to hydrolysis by 0.2 N TFA and enzymolysis by *S. rolfsii* enzymes as described by Jones and Albersheim (13), about 16% of the cell wall was recovered as monomeric carbohydrate. Cellulose is not degraded significantly by this procedure (27). The cellulose content of isolated walls averaged about 22% as determined by the method of Updegraff (29). The remainder of the cell wall was not accounted for in our analyses. Our incomplete recovery of monosaccharides from corn cell walls is not unlike that of Nevins et al. (22). Under the conditions employed, corn cell walls do not appear to be as susceptible to hydrolysis as are dicot cell walls (22).

Pretreatment of corn cell walls with *H. maydis* race T culture filtrate (known to contain endopolygalacturonase and xylanase) for 2 hr at 30 C resulted in significant removal of arabinose, xylose, galactose, and galacturonic acid from the 0.2 N TFA-*S. rolfsii* enzyme-soluble wall fraction (Fig. 9). Essentially all of the galactose detectable in untreated walls was solubilized by *H. maydis* race T enzymes; the depletion of arabinose, xylose, and galacturonic acid from this wall fraction was 75, 45, and 31%, respectively. The amount of noncellulosic glucose in these walls was not appreciably affected. These studies have clearly demonstrated the removal of polymeric carbohydrate constituents from corn cell walls by enzymes produced by *H. maydis* race T.

DISCUSSION.—*H. maydis* race T was shown to possess the ability to produce an endopolygalacturonase, a pectin lyase, and xylanase. The endopolygalacturonase and xylanase systems examined in this study were obtained from culture filtrates of the fungus grown on a mineral salts-glucose medium. Glucose is known to repress cell wall-degrading enzyme synthesis in a number of phytopathogenic organisms (7, 12, 23), but *H. maydis* race T does not appear to share this characteristic. In this study we obtained less enzyme production (recovery) on media containing Na polypectate as a carbon source.

The pectic enzymes and xylanase obtained from cultures of *H. maydis* race T were relatively unstable when compared to similar extracellular enzymes produced by many other plant pathogenic organisms (3, 4). This instability and the relatively low enzyme concns, particularly with the xylanase system, led to considerable experimental difficulty in working with cell wall - degrading enzymes produced by this pathogen. The stability problem may be related to the low protein content of our enzyme preparations. Filtrates of 8- to 12-day-old *H. maydis* race T cultures grown on the mineral salts-glucose medium contained between 95 and 115 µg protein/ml. Upon subjecting these filtrates to various fractionation procedures such as electrofocusing, CM-cellulose column chromatography, or Sephadex G-75 gel filtration, the protein content of fractions containing the enzymes was reduced to 12-60 µg/ml. Development of a system that would permit greater enzyme synthesis by *H. maydis* race T, and/or developing a means to stabilize the enzymes produced, would greatly aid work on cell wall degradation by this pathogen.

We were unable to demonstrate significant cellulase (Cx), galactanase, or arabanase activities in filtrates of *H. maydis* race T grown in the mineral salts-glucose medium. Larson and Riedel (14) have reported pectic and cellulolytic (Cx) enzyme activities in *H. maydis* race T filtrates taken from shake cultures grown on potato broth-glucose medium supplemented with pectin N.F. or Na carboxymethyl cellulose. They also demonstrated these enzyme activities in extracts of blighted corn seedlings. These observations, coupled with the data reported in this paper, demonstrate that *H. maydis* race T is capable of degrading many of the polymeric
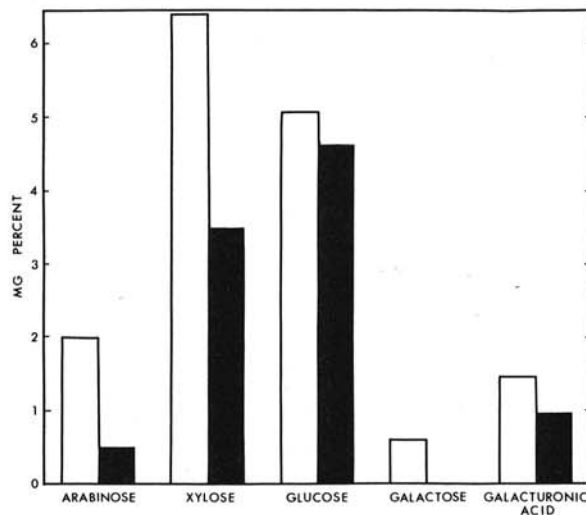

Fig. 9. Neutral sugar and galacturonic acid content of control (open bars) and culture filtrate-treated (solid bars) isolated corn cell walls. Filtrate was from *Helminthosporium maydis* race T cultures grown on the salts-glucose medium. The ordinant indicates mg of constituent released per 100 mg of cell wall.

carbohydrates of corn cell walls. It is not known whether the pattern of degradation of walls from resistant corn differs from that of susceptible corn.

Histological studies of infection by *H. maydis* race T have revealed that this organism ramifies its host both inter- and intracellularly and eventually causes cell wall collapse (9, 32). We have demonstrated that culture filtrates of *H. maydis* race T, shown to contain endopolygalacturonase and xylanase, released significant amounts of arabinose, xylose, galactose, and galacturonic acid from isolated corn cell walls within a 2-hr period at 30 C (Fig. 9). Pectic enzymes that split the α-1,4 bond of uronic acid polymers are known to be key factors in the process of tissue maceration (4, 17, 25). There is also evidence that enzymes which degrade the polyuronide of cell walls render the other wall polysaccharides susceptible to enzymatic attack (27). It would appear that the cell wall-degrading enzymes produced by *H. maydis* race T could aid its ability to degrade host tissue.

H. maydis race T produced host specific toxin activity in culture and in diseased plants (8, 15, 31). Although the role of this toxin in disease development has not been fully evaluated, other host specific toxins are known to play primary roles in disease initiation and disease development (26). *H. maydis* race T toxin may be found to fit the same pattern. However, it is possible that events such as tissue degradation and restriction of lesion size will not be explained in terms of toxin activity. Factors such as cell wall-degrading enzymes and resistance mechanisms may be important in the total disease syndrome. Because the genetics of both host and pathogen can be readily manipulated, this disease may serve as a model to investigate possible roles of

toxins, enzymes, and induced resistance mechanisms in a common host-pathogen system.

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