Purification and Partial Characterization of a Glycoprotein Toxin Produced by *Cladosporium fulvum*

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ABSTRACT

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A heat-stable, high molecular weight toxin isolated from culture filtrates and cell wall preparations of *Cladosporium fulvum* causes callose formation, host cell necrosis, and ion leakage in tomato leaf tissue. The toxin was purified by a combination of dialysis, methanol precipitation, DEAE-cellulose chromatography, affinity chromatography on concanavalin A-Sepharose 4B, and gel chromatography on Sephadex G-100 and Sepharose 4B. The most highly purified toxin preparations contained both protein and carbohydrate, the ratio ranging from 0.09 to 0.23. Mannose, glucose, galactose, and traces of several unidentified sugars were found in hydrolysates. The importance of the glycosyl moiety to toxin activity was indicated by toxin inactivation after periodate oxidation or treatment with α-mannosidase. Incubation of toxin with proteinase K also reduced activity, but dansylation of the protein did not alter activity. The behavior of the toxin during gel chromatography and electrophoresis suggested it was a heterogenous or polydisperse glycoprotein with a molecular weight range of 3×10^4 to 2.5×10^5 daltons.

Additional key words: tomato leaf mold.

Cladosporium fulvum Cooke, causal agent of tomato leaf mold, produces a high molecular weight component that causes callose formation, necrosis, and increased electrolyte loss from treated tissue when injected into tomato leaves (19). This reproduction of some of the symptoms (17,18) of leaf mold disease supports reports by van Dijkman and Kaars Sijpesteijn (27–29) that *C. fulvum* produces a toxin damaging to the host plasma membrane; experiments (19) on the host specificity of the toxin, however, do not support their conclusion that the toxin is cultivar-specific.

The objective of the present study was to purify the toxin, establish its chemical nature, and obtain preliminary information on its origin. Data indicate the toxin is a heterogenous or polydisperse glycoprotein that can be extracted from fungal cell wall material as well as from culture filtrates.

MATERIALS AND METHODS

Toxin production. Isolates of *C. fulvum* Cooke (Cf) were maintained as described previously (17,22). For toxin production, the fungus was grown on modified Fries' medium (19) in liquid culture for 3 wk. Culture conditions and initial processing of the culture filtrates are described elsewhere (19).

Bioassay techniques. Aqueous solutions were injected into the intercellular space of tomato leaflets on plants at the 4–10 leaf stage as described previously (19). A given sample was injected into one or more leaflets at similar positions on four plants. A number of solutions were bioassayed on the same leaflet, since the lateral veins acted as boundaries not traversed by the injected substance. A dilution series was used to determine the minimum amount of a toxin preparation required for activity.

The necrosis-producing ability of a preparation was assessed on a scale of 0 (no necrosis) to 4 (total necrosis) based on macroscopic appearance of the injected site. Callose formation was observed by fluorescence microscopy of aniline blue-stained tissue (19) and was rated on a scale of 0 (no fluorescence) to 4 (extensive fluorescence). Details of the criteria used for ratings are presented elsewhere (19). The cultivar Potentate, which has no genes for resistance to *C. fulvum*, was used in these bioassays.

The ability of toxin preparations to stimulate ion leakage was tested by measuring electrolyte loss from leaf tissue treated 14–16 hr previously with control or test solutions. The electrolyte loss was determined as described previously (19).

Purification techniques. At most stages of the purification procedure, toxin was monitored by necrosis bioassay and by carbohydrate and protein determinations. Protein was determined by the method of Lowry et al (21) with bovine serum albumin as the standard. Carbohydrate was measured by the technique of Dubois et al (11) with the concentration of polysaccharide given as glucose equivalents/ml.

Fractions from column chromatography were analyzed by bioassays and by carbohydrate tests and/or absorbance over a range of 180–320 nm. Biologically active fractions absorbed maximally at 190–220 nm. Although not a specific test, this absorbance was used when carbohydrate determinations would have caused excessive waste of fractions.

Crude culture filtrates (19) were concentrated in vacuo and adjusted with water to 5% of the original volume. Sufficient 100% methanol was slowly added with constant stirring to bring the concentration to 90% methanol. After incubation overnight at 4 C, the precipitate was removed by vacuum filtration, rinsed extensively with 90% methanol, and dissolved in distilled water to 5% of the original volume. Any insoluble material was removed by centrifugation and discarded.

Dialysis of filtrates was sometimes used in place of methanol precipitation. Concentrated filtrate (about 50 ml) was transferred to prewashed dialysis tubing and dialyzed at 4 C with stirring against 4 L of distilled water that was replaced three times during 24 hr. The dialyzed culture filtrate and the water used to rinse the tubing were combined and centrifuged at 11,000 $\times g$ for 10–15 min. The pellet was discarded after initial experiments showed that the portion of the pellet soluble in 20 mM phosphate buffer, pH 6.5, had no biological activity.

For anion exchange chromatography, DEAE-cellulose

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(diethylaminoethyl cellulose) (Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178) with an exchange capacity of 0.8 meq/g (medium mesh) was equilibrated with 20 mM sodium phosphate buffer, pH 8.0. Concentrated dialyzed culture filtrate (about 20 ml) was made up to 20 mM phosphate buffer by additions of 200 mM sodium phosphate buffer, pH 8.0. The filtrate was layered onto a DEAE-cellulose column (1.5 × 30 cm, bed height 20 cm) and eluted with 20 mM phosphate buffer. The first 100 ml was collected and dialyzed as described for crude filtrates. The dialyzed sample was concentrated in vacuo and made up to the desired volume with distilled water.

Gel chromatography on Sephadex G-100 was used at several stages in purification tests. Samples in 2–5 ml of distilled water were placed on the column (column specifications given with data) and eluted with distilled water, and 5–10 ml fractions were collected. The void volume of gel filtration columns was determined using Blue Dextran 2,000 (Pharmacia [Canada] Ltd., Dorval, Quebec, H9P 1H6), and the total volume was calculated from the volume of the gel bed of the columns. Biologically active fractions were pooled and concentrated in vacuo.

Concanavalin A(ConA)-Sepharose 4B (Pharmacia) was used for affinity chromatography of the toxin. ConA-Sepharose (1.5 × 30 cm column, bed height 16.5 cm) was washed with a minimum of 1 L of ConA buffer (100 mM potassium phosphate buffer, pH 7.2, containing 10 μM $MnCl_2$, 10 μM $CaCl_2$, and 0.01% [w/v] thimerosal). The sample (10–75 mg glucose equivalents) was layered on the column in 2–5 ml of ConA buffer and eluted with 100 ml of ConA buffer followed by 80–100 ml of 4% α-methyl-D-mannopyranoside (mannoside) in ConA buffer. The absorbance of the fractions at 190–200 nm was used in determining which fractions were to be pooled. Pooled fractions were dialyzed against distilled water to remove the mannoside, then concentrated in vacuo.

Gel chromatography on Sepharose 4B (Pharmacia) (1.5 × 30 cm column, bed height 26.5 cm) with 50 mM NaCl for elution was used with toxin that had undergone the described purification procedures. Pooled active fractions (1.4 ml) were dialyzed against distilled water to remove NaCl.

Preparation of fungal cell wall enriched material. Partially purified mycelial wall material was prepared by the technique described by Anderson-Prouty and Albersheim (2) with modifications. Dried mycelium, previously weighed and stored at −20 C, was initially homogenized in distilled water (20 ml/g dry wt) with a Virtis 23 homogenizer at top speed for 5 min. The homogenate was centrifuged at 1,610 × g, and the pellet was resuspended in distilled water and extracted three more times in this manner. The pellet then was extracted by homogenization in two changes of chloroform-methanol (1:1, 20 ml/g dry wt). The insoluble material was removed by filtration on a sintered glass funnel (M grade), resuspended, and homogenized in acetone (20 ml/g dry wt). The acetone was removed by filtration, and homogenization was repeated. The final wall preparation was rinsed by filtration with 100 ml of redistilled methanol and dried in a forced-air oven at 80 C. Microscopic examination indicated the wall preparation was composed of broken pieces of hyphae, most of which contained some remnants of cytoplasmic material. Therefore, we called the material "cell wall enriched fraction" rather than "cell wall preparation," the term used by Anderson-Prouty and Albersheim (2).

Extraction of toxin from cell wall enriched material. Toxin was extracted by autoclaving, a technique used by Anderson-Prouty and Albersheim (2) and originally described by Raschke and Ballou (23) for release of surface antigens of yeast. One gram of cell wall enriched material from race 1 or 12 was autoclaved in 100 ml of water, the preparation was centrifuged at 11,000 × g for 10–15 min, and the pellet was suspended in another 100 ml of water. This procedure was repeated until three autoclaved fractions were obtained. The separate supernatants—extracts 1, 2, and 3—were concentrated to 20 ml, and serial dilutions were bioassayed.

Polyacrylamide gel electrophoresis. Disc electrophoresis was performed on 6 cm of 7.5% acrylamide gels with glycine-tris buffer, pH 8.3 (9). Samples of Cf race 1 purified toxin (Table 1) containing 100 μg glucose equivalents of polysaccharide and 23 μg of protein were subjected to 4 mA per tube until the bromphenol blue marker was 0.5 cm from the end of the gel (about 3.5 hr). Gels were stained with either Coomassie Blue R250 protein stain (8) or Schiff's base reagent for glycoproteins (31). Gels were scanned on a Beckman Acta C III spectrophotometer with a Beckman 198402 gel scanner accessory using a wavelength of 540 mμ for Schiff's stain and 575 mμ for Coomassie Blue stain.

The SDS (sodium dodecyl sulfate) electrophoresis method of Weber and Osborn (30) was used with purified Cf race 1 toxin (25 μg glucose equivalents per tube) as an alternate method of estimating molecular weight range. Gels with toxin were stained by Schiff's stain and gels with protein markers were stained with Coomassie Blue as done by Weber and Osborn (30).

Identification of sugar components by GLC. For hydrolysis, toxin samples (about 1 mg) and 1 mg of the internal standard (inositol) were dissolved in 1–2 ml of water in 5-ml glass ampules, and an equivalent volume of 2 M H_2SO_4 was added. The sealed ampules were heated at 100 C for 10–12 hr. After hydrolysis, the acid was removed by ion exchange on AG 1 × 8 anion exchange resin (Bio-Rad Laboratories, 2200 Wright Ave., Richmond, CA 94804) (20–50 mesh OH^- form) that had been converted to the acetate form. The eluate (10 mM acetic acid) was dried in vacuo at 40 C.

Alditol acetate derivatives of the products of the hydrolysis were prepared by the method of Jones and Albersheim (14). In addition, 1-mg samples of glucose, galactose, and mannose, sugars initially indicated by paper chromatography to be in hydrolysates (16), were derivatized. For preparation of a standard curve, solutions containing 1 mg of inositol and 1, 0.75, 0.5, or 0.25 mg of mannose, glucose, and galactose were derivatized.

Gas-liquid chromatographic (GLC) separation of the alditol acetates was done on a Pye Unicam Series 105 Automatic Preparative chromatograph using a column similar to that described by Jones and Albersheim (14). The column (copper, 1.22 m × 0.32 cm i.d., prepacked by Chromatographic Specialties Ltd. Inc., 300 Laurier Blvd., Brockville, Ontario, Canada) had a liquid phase consisting of 0.2% (poly) ethylene glycol adipate, 0.2% (poly) ethylene glycol succinate, and 0.4% XF-1150 silicone oil coated on Gas-Chrom P (100–200 mesh). Before use, the column was conditioned overnight at 210 C. Samples were run at a column temperature of 157 C with a nitrogen carrier gas flow rate of 32.7 ml per minute. The area of each component peak was calculated by triangulation or by cutting out and weighing the peaks.

Periodate oxidation. The effect of periodate oxidation on toxin activity was tested with partially purified Cf race 1 toxin at 400 μg glucose equivalents/ml. The treatments used were (i) 1 ml of toxin plus 1 ml of 30 mM $NaIO_4$, (ii) 1 ml of toxin plus 1 ml of 30 mM $NaIO_4$ previously exposed to 100 μl of ethylene glycol for 48 hr to inactivate the periodate, (iii) 1 ml of toxin plus 1 ml of distilled water, and (iv) 1 ml of distilled water plus 1 ml of $NaIO_4$. After 48 hr of incubation in sealed vials in darkness at about 25 C, 100 μl of ethylene glycol was added to the first, third, and fourth treatments to inactivate the periodate. After a further 48-hr incubation, the

TABLE 1. Purification of *Cladosporium fulvum* race 1 toxin preparation used in electrophoresis, dansylation, and enzyme inactivation experiments

Stage of purification	Glucose equivalents (mg)	Protein (mg)	Protein-carbohydrate ratio
Methanol precipitation	68.6	40.4	0.59
DEAE-cellulose[a]	41.0	14.8	0.35
ConA-Sepharose,[a] Sephadex G-100	...[b]	7.5	...
Sepharose 4B	15.8	3.3	0.21
Sephadex G-100[c]	15.9	3.7	0.23

[a] Preparations were filtered on Amicon PM-10 filters (cutoff at 10,000 mol wt) after these steps to remove small molecular weight molecules.
[b] Inaccurate carbohydrate analysis because of residual mannoside.
[c] Primarily to remove the NaCl in fractions after Sepharose 4B chromatography.

solutions were dialyzed, reduced to dryness, and redissolved in 1 ml of distilled water, and a dilution series was bioassayed.

Dansylation. Dansylation of the toxin was done by the procedure described by Johnson and Strobel (13) using 1-mg samples of Cf race 1 toxin purified as described in Table 1.

Enzyme inactivation. Tests for enzymatic inactivation of Cf toxin were made on various toxin preparations, but the data presented were obtained using Cf race 1 toxin purified as described in Table 1. Reaction mixtures contained the appropriate enzyme, 66 μg glucose equivalents of toxin per milliliter, 25 ppm of streptomycin sulfate, and 25 ppm of tetracycline in 50 mM citrate-phosphate buffer at pH 4.5 (or 50 mM phosphate buffer at pH 7.2 for β-galactosidase). Controls were (i) autoclaved enzyme plus toxin, (ii) active enzyme minus toxin, and (iii) toxin minus enzyme. After incubation for specified times at 25 C, reaction mixtures were tested for toxin activity by necrosis bioassays and ion leakage assays.

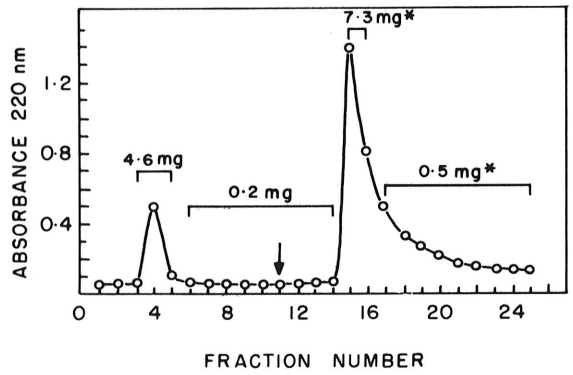

Fig. 1. Affinity chromatography of *Cladosporium fulvum* race 10 toxin on ConA-Sepharose 4B. Partially purified toxin (13.5 mg glucose equivalents) was layered on the column in 2 ml of ConA buffer and eluted first with 80 ml of ConA buffer, then (arrow) with 100 ml of 4% (w/v) of α-methyl-D-mannopyranoside in ConA buffer. Joined bars indicate fractions pooled for analysis. Carbohydrate content (mg glucose equivalents) is given over the bars. Asterisks signify toxin activity in pooled fractions. (Fraction volume, 7.5 ml; bed dimensions, 1.5 × 16.5 cm; flow rate, 15.9 ml/cm^2/hr.)

Commercial enzymes were tested under the conditions to be used in toxin inactivation tests. Each enzyme was tested for specified activity and for contamination by protease, mannosidase, galactosidase, and glucosidase by 24-hr incubation with the following substrates: Congocoll, 1 mg per milliliter (Calbiochem, Los Angeles, CA 90054), 0.2 mM *p*-nitrophenyl-α-D-mannoside (Sigma), 0.2 mM *p*-nitrophenyl-β-D-galactopyranoside (Sigma), and 0.2 mM *p*-nitrophenyl-β-D-glucopyranoside (Sigma). Proteinase K at 1 Anson unit per milliliter (EM Laboratories, Inc., 500 Executive Blvd., Elmsford, NY 10523) showed only protease activity, and α-mannosidase at 4.2 units per milliliter (EC. No. 3.2.1.24, Sigma) showed only mannosidase activity. β-Galactosidase at 0.5 units per milliliter (EC. No. 3.2.1.23, Sigma) and β-glucosidase at 17.5 units per milliliter (EC. No. 3.2.1.21, ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, OH 44128) showed activity on all three *p*-nitrophenyl substrates.

RESULTS

Partial purification of toxin from races 1, 10, and 12. Fries' medium (10 flasks per race) seeded with *C. fulvum* race 1, 10, or 12 or with killed spores of race 1 was incubated for 3 wk, then filtrates were harvested. Toxin was detectable in culture filtrates at all times, with maximum concentrations at about 3 wk (16). Culture filtrates were dialyzed, passed through a DEAE-cellulose column, and chromatographed on Sephadex G-100. Biologically inactive fractions were pooled, concentrated, and bioassayed again before being discarded.

Results of this purification procedure are summarized in Table 2, with activity as the lowest dilution tested at which all injections showed a response. Filtrates from races 1, 10, and 12 differed slightly in degree of activity but generally behaved similarly. Dialysis eliminated approximately 90–98% of the protein and carbohydrate (or compounds interfering with protein and sugar determinations of the filtrates). About 50% of the activity was lost during dialysis (Table 2), possibly because other toxic components contributing to total activity of crude filtrates were eliminated.

DEAE-cellulose chromatography with 20 mM phosphate buffer, pH 8.0, reduced the carbohydrate and protein components of the dialyzed filtrate (Table 2). No active material was recovered when the DEAE-cellulose column was further eluted with increasing concentrations (100, 200, 300, 400, and 500 mM) of sodium phosphate buffer, pH 8.0, but 15% of the carbohydrate and 8% of

TABLE 2. Partial purification of toxin from culture filtrates of races 1, 10, and 12 of *Cladosporium fulvum* on modified Fries' medium

Purification step	Race[a]	Protein (mg/L filtrate)	Carbohydrate (mg glucose equiv/L filtrate)	Lowest dilution at which activity detected[b]	
				μg glucose equiv/ml	Relative to original volume
None (crude)	Control[c]	784	3×10^5	···	None at 5 ×
	1	1,978	2,466	493	1/5
	10	1,825	3,880	388	1/10
	12	1,757	5,890	1,178	1/5
Dialysis	Control	36	25	···	None at 1 ×
	1	112	98	44	1/2
	10	207	229	46	1/5
	12	129	97	48	1/2
DEAE-cellulose	Control[d]	5.5	6.5	···	None at 5 ×
	1	18.7	26.6	53	2 ×
	10	67.0	145.0	36	1/4
	12	36.4	36.6	36	1 ×
Sephadex G-100[e]	1	2.3	24.4	30–40	···
	10	9.0	61.8	20–30	···
	12	3.8	16.2	20–30	···

[a] Mycelium dry weight (g/L medium): race 1, 10.3; race 10, 9.3; race 12, 8.1.
[b] All four injected sites had a rating of at least 1 on a scale from 0 (no necrosis) to 4 (total necrosis).
[c] Inoculated with dead spores of race 1.
[d] Material passing through DEAE-cellulose in 20 mM sodium posphate buffer, collected (100 ml), concentrated, and dialyzed.
[e] Column (2.6 × 100 cm, void volume 196) eluted with water after 2–5 ml sample applied; fractions with biological activity pooled.

the protein were in these fractions. The remaining carbohydrate and protein, also inactive, were recovered by elution of the column with 1.0 M NaCl (100 ml).

DEAE-cellulose chromatography was generally followed by Sephadex G-100 gel filtration. Activity was greatest in the Sephadex G-100 fractions corresponding to the void volume and initial fractionation range of the column. Activity occurred across a wide band corresponding to about one-half of the inclusion volume. When fractions with high activity were pooled, activity was found at 20–40 μg glucose equivalents/ml. At this stage of purification, the protein-carbohydrate ratios for races 1, 10, and 12 preparations were 0.09, 0.15, and 0.23, respectively (Table 2).

Characteristics of partially purified toxin. Because partially purified toxin from all three races behaved similarly during further chromatographic procedures, only the data for race 10 are presented. The elution profile of toxin from race 10, containing 13.5 mg glucose equivalents and 2.2 mg of protein, chromatographed on ConA-Sepharose 4B is shown in Fig. 1. The material retained by ConA and removed on elution of the column with mannoside contained all the toxin activity, with pooled fractions 15 and 16 (7.5 g glucose equivalents) showing greatest activity. Fractions 17–25 (0.5 mg glucose equivalents) were active when pooled and concentrated to the same degree as fractions 15 and 16 (about 10–20 μg glucose equivalents/ml. The material not bound to the column (ie, eluting with the buffer) contained no toxin activity even when concentrated to 2.3 mg equivalents/ml. Most of the inactive carbohydrate (4.6 mg glucose equivalents) was in fractions 3–5. If this inactive material contained toxin bound to ConA that came off the column (1), the bound toxin should have been released by dialysis against acetic acid (0.8%, v/v, pH 2.9), but this did not happen.

Active material from ConA-Sepharose chromatography was chromatographed on Sephadex G-100 (Fig. 2). As with less purified toxin, the active material eluted with the void volume and also was partially included in the column. Some low molecular weight material (fractions 14–20) eluting behind the toxin had strong absorbance but contained very little carbohydrate (0.3 mg glucose equivalents) or protein and was not active at 0.3 mg glucose equivalents/ml. A small amount of this material still appeared when the active fractions were rechromatographed on Sephadex G-100, indicating it was a minor breakdown product.

Toxin obtained after three passages through Sephadex G-100 was chromatographed on Sepharose 4B in 50 mM NaCl to obtain an estimate of the molecular weight (Fig. 3). All the activity was detected in fractions 13–21, which corresponded to the fractions with maximum absorbance and contained all the detectable carbohydrate (4.8 mg glucose equivalents, 0.6 mg protein). The approximate molecular weight of the toxin, as estimated by standard methods from the fractionation range of dextran markers, was 6×10^4. Compounds eluting at fractions 13 and 21 (Fig. 3) had approximate molecular weights of 2.5×10^5 and 3.0×10^4, respectively.

The protein-carbohydrate ratio of race 10 toxin after these steps was 0.13. This ratio remained basically the same from the initial Sephadex G-100 step (Table 2) through subsequent procedures, suggesting that the material removed by affinity chromatography was also a protein-carbohydrate complex of similar proportions. In subsequent purifications of several toxin preparations, the protein-carbohydrate ratio was generally about 0.2. Typical data are given

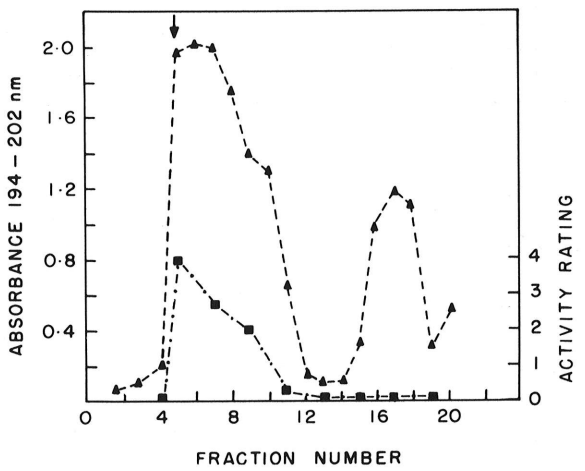

Fig. 2. Chromatography of *Cladosporium fulvum* race 10 toxin on Sephadex G-100. Affinity chromatography on ConA-Sepharose had been done on the partially purified toxin. Fractions were assayed for absorbance (▲) and for necrosis, given as the average activity rating (□). (Sample size, 1 ml; fraction volume, 2.6 ml; bed dimensions, 1.5 × 27.5 cm; void volume 12 ml; flow rate, 11.5 ml/cm^2/hr.)

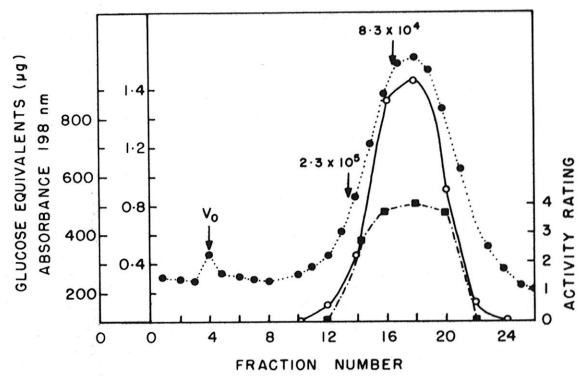

Fig. 3. Chromatography of *Cladosporium fulvum* race 10 toxin on Sepharose 4B-C. Partially purified toxin was chromatographed on ConA-Sepharose and Sephadex G-100 before chromatography on Sepharose 4B-Cl in 50 mM NaCl. Fractions were assayed for absorbance (•), carbohydrate as glucose equivalents (o), and necrosis-producing activity (■), for which the average rating of four leaves is given. Dextran standards of average molecular weights of 8.3×10^4 and 2.3×10^5 were chromatographed under the same conditions, and elution maxima were determined by carbohydrate tests. (Fraction volume, 1.4 ml; bed dimensions, 1.5 × 26.5 cm; void volume, 15 ml; flow rate, 7.7 ml/cm^2/hr.)

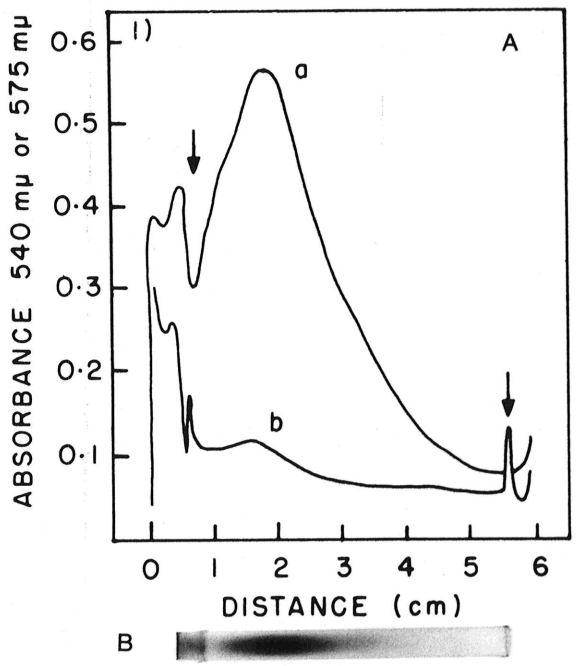

Fig. 4. Acrylamide gel electrophoresis of purified *Cladosporium fulvum* race 1 (CfR1) toxin. **A,** scanner tracings of gels after staining with (a) Schiff's base reagent for carbohydrate (540 nm) and (b) Coomassie Blue protein stain (575 nm). Control gels (no toxin in sample buffer) stained for carbohydrates or protein showed minimal background absorbance. Arrows indicate beginning and end of the separation gel. **B,** Photograph of gel stained with Schiff's reagent.

in Table 1 for the race 1 toxin used for electrophoresis and inactivation experiments and for the leakage experiments described previously (19).

Acrylamide gel electrophoresis. After polyacrylamide gel electrophoresis by the method of Davis (9), purified toxin gave a broad band that showed a positive Schiff reaction (31) (Fig. 4). The central (peak) area of this band sometimes showed a faint reaction with Coomassie Blue (Fig. 4). Purified toxin also gave a broad band after SDS electrophoresis, indicating molecules of varying molecular weights. Precise interpretation of the results of SDS electrophoresis of glycoproteins is difficult (24). The average molecular weight of the toxin was estimated to be 7×10^4, a value in reasonably close agreement with the 6×10^4 obtained by Sepharose chromatography.

Isolation of *C. fulvum* toxin from cell wall enriched preparations. The extracts obtained after the first autoclaving of races 1 and 12 cell wall enriched material were active at dilutions with 22 and 17 μg glucose equivalents/ml, respectively (Table 3). Extracts obtained by the second and third autoclaving contained progressively less protein and carbohydrate, but toxin activity was still detected at dilutions with about 18 μg glucose equivalents/ml. The total carbohydrate released in the three extracts was 8.6 and 8% of the weight of the cell wall enriched material for races 1 and 12, respectively.

The three cell wall extracts for each race were pooled and purified by chromatography on DEAE-cellulose and Sepharose 4B (Table 4). Purificaton did not significantly improve activity of the toxin on a μg glucose equivalents/ml basis. The levels of activity shown in Tables 3 and 4 are probably not significantly different because of the variation between plants and because of the concentration gaps in the end-point dilution technique. Both DEAE-cellulose and Sepharose 4B chromatography reduced protein content more than carbohydrate content, with final protein-carbohydrate ratios for races 1 and 12 of 0.17 and 0.35, respectively. In additional experiments, toxin from cell wall enriched material behaved similarly to toxin obtained from filtrates when chromatographed on ConA-Sepharose 4B (27) and on Sephadex G-100.

Identification of sugar components. Alditol acetates of the monosaccharides in hydrolysates of toxin obtained from three races at several different stages of purification were separated and identified by gas chromatography (Fig. 5). In addition, the quantity of each sugar (mg sugar/mg glucose equivalents of toxin) was estimated (Table 5). The predominant sugar in all highly purified samples was mannose. Galactose was measurable in toxin from races 1 and 12 but was barely detectable in highly purified race 10 toxin. Partially purified race 10 toxin contained considerably more

TABLE 3. Protein, carbohydrate, and biological activity extracted from 1 g of fungal wall enriched material from *Cladosporium fulvum* after repeated autoclaving in 100 ml of distilled water for 1 hr

Race	Extract[a]	Protein (mg/100 ml)	Carbohydrate (mg glucose equiv/ml)	Lowest dilution at which activity detected[b]: μg glucose equiv/ml	Lowest dilution at which activity detected[b]: Relative to original volume
1	1	73	54.1	22	1/25
	2	21	17.8	17	1/10
	3	19	14.5	18	1/8
12	1	56	34.3	17	1/20
	2	44	27.4	18	1/15
	3	25	18.1	18	1/10

[a] Mycelial wall enriched material (1 g), autoclaved in 100 ml of distilled water for 1 hr at 121 C, 15 lb pressure, was removed by centrifugation and the supernatant termed extract 1. The procedure was repeated twice on the same material to yield extracts 2 and 3.

[b] All four injected sites had a rating of at least 1 on a scale from 0 (no necrosis) to 4 (total necrosis).

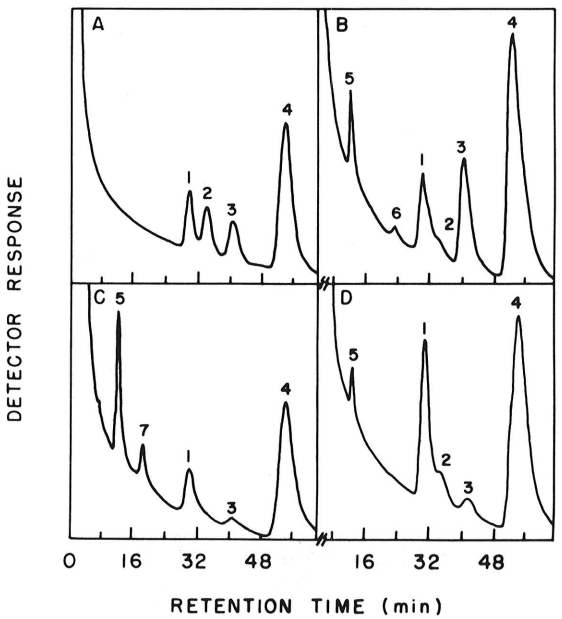

Fig. 5. Gas liquid chromatography of alditol acetate derivatives of sugars from hydrolyzed *Cladosporium fulvum* toxin preparations. **A,** Sugar standards = 1, mannose; 2, galactose; 3, glucose; 4, internal standard (inositol). Relative retention times ($R_{inositol}$) were 0.54, 0.64, and 0.76 for mannose, galactose, and glucose, respectively. **B,** Race 10 toxin partially purified by dialysis and chromatography on DEAE-cellulose and Sephadex G-100. **C,** Race 10 toxin further purified by affinity chromatography on ConA-Sepharose 4B. **D,** Race 12 toxin purified in the same manner as race 10 toxin in **C.** Peaks 5, 6, and 7 with $R_{inositol}$ values of 0.21, 0.44, and 0.33, respectively, were produced by unidentified compounds.

TABLE 4. Partial purification of toxin from extracts of mycelial wall enriched material from *Cladosporium fulvum* by DEAE-cellulose ion-exchange chromatography and Sepharose 4B-Cl gel chromatography

Purification step	Race	Protein (mg/g mycelial wall)	Carbohydrate (mg glucose equiv/g mycelial wall)	Lowest dilution at which activity detected[a] (μg glucose equiv/ml)
Crude[b]	1	113.0	86.4	40–50
	12	125.0	79.9	40–50
DEAE-cellulose[c]	1	28.0	45.4	30–40
	12	18.5	20.7	20–30
Sepharose 4B-Cl[d]	1	3.9	23.5	20–30
	12	4.2	11.9	20–30

[a] All four injected sites had a rating of at least 1 on a scale from 0 (no necrosis) to 4 (total necrosis); because glucose equivalents/dilution varied, results are given as a range.

[b] Mycelial wall enriched material (1 g) was autoclaved successively in three separate 100-ml volumes of distilled water, and the three extracts were combined.

[c] Material eluted with 100 ml of 20 mM phosphate buffer was collected, dialyzed, and concentrated before tests.

[d] Fractions collected from Sepharose 4B-Cl column were bioassayed by injection, and active fractions were pooled and concentrated before analysis.

glucose than more purified race 10 toxin (Table 5, Fig. 5). The low total recovery of sugars in some samples probably can be explained by incomplete hydrolysis of the toxin. The peaks with $R_{inositol}$ values of 0.21, 0.33, and 0.44 (Fig. 5) represented unidentified compounds.

Periodate oxidation. Toxin incubated with sodium periodate was completely inactivated, whereas toxin incubated with inactivated periodate or with ethylene glycol retained activity. The control containing sodium periodate and ethylene glycol without toxin had no activity.

Dansylation. In bioassays for necrosis, the dansylated toxin treatment was as active as the toxin control (no dansyl chloride added). The dansyl chloride control (no toxin added) had no visible effect on tomato leaves. In the ion leakage assay, the net conductivities of the bathing solution after 4.5 hr of incubation of the leaf disks were: dansylated toxin, 31.6 ± 2.9; toxin control, 28.2 ± 1.7; dansyl chloride control, 15.9 ± 1.7.

Effect of heat on biological activity of toxin. A dilution series of partially purified toxin was autoclaved for 30 min, and an identical series was kept at room temperature. Both series had comparable activity when injected into tomato leaves.

Enzyme inactivation. Toxin incubated with either proteinase K or α-mannosidase for 20 hr or longer consistently caused less necrosis than did toxin incubated with autoclaved enzymes (Table 6). Incubation of reaction mixtures for shorter periods did not result in detectable reductions in toxin activity as judged by bioassays for necrosis. Neither β-galactosidase nor β-glucosidase consistently reduced toxin activity. Controls containing active enzyme but no toxin did not cause necrosis, and toxin without enzyme caused necrosis similar to that caused by autoclaved enzyme controls. Ion leakage assays in which leaves were injected with toxin treated with α-mannosidase or proteinase K confirmed that both enzymes partially or completely inactivated the toxin (Table 6).

TABLE 5. Estimates of mannose, galactose, and glucose content of different toxin preparations by gas liquid chromatography

Toxin preparation	Sugars (mg/1 mg glucose equivalents of toxin)[a]			
	Mannose	Galactose	Glucose	Total
Race 10, partially pure[b]	0.31	+[e]	0.56	0.87
Race 10, most pure[c]	0.31	±[e]	0.10	0.41
Race 12, most pure[c]	0.60	0.19	0.05	0.84
Race 1, partially pure[b]	0.50	0.13	0.08	0.71
Race 1, most pure[d]	0.48	0.18	0.10	0.76

[a] Estimated after separation in hydrolyzed toxin by gas liquid chromatography. Quantitation was by means of an internal standard (inositol).
[b] Purification by dialysis and DEAE-cellulose chromatography followed by chromatography on Sephadex G-100.
[c] Further purification by ConA-Sepharose 4B affinity chromatography followed by chromatography on Sephadex G-100 and Sepharose 4B-Cl.
[d] Purification by methanol precipitation, ConA-Sepharose 4B affinity chromatography, and chromatography on Sephadex G-100 and Sepharose 4B-Cl.
[e] + = too little to quantitate, ± = presence questionable.

DISCUSSION

The toxin isolated from *C. fulvum* culture filtrates appears to be a heat-stable polydisperse glycoprotein. The most purified preparation had a molecular size distribution on Sepharose 4B corresponding to a dextran with a molecular weight range of 3.0×10^4 to 2.5×10^5 daltons. This heterogeneity in molecular weight was confirmed by polyacrylamide gel electrophoresis. The heterogeneity of the toxin makes judging the purity of the toxin preparations difficult, but the relatively uniform behavior of the most purified preparations on Sepharose columns and during electrophoresis indicated no major contaminants.

The glycoprotein nature of the toxin was initially inferred from chemical analysis for carbohydrate and protein and was supported by both electrophoresis and inactivation experiments. Also, Dow and Callow (10) recently reported that components from *C. fulvum* culture filtrates causing permeability changes in isolated mesophyll cells were glycopeptides. Inactivation of toxin by periodate oxidation and by α-mannosidase treatment indicates that the glycosyl component is essential for biological activity and that mannose may form an essential portion of that component. Because of the long incubation period required for enzymatic inactivation, the possibility that small amounts of contaminating enzymes may be involved in the inactivation cannot be eliminated. A glycosyl component also was confirmed by the binding of the toxin to concanavalin A, a lectin with a high affinity for molecules containing terminal α-D-glucosyl or α-D-mannosyl or sterically similar groups (12). GLC analysis of toxin hydrolysates showed that mannose was the major sugar component and that glucose and galactose were also present. Less is known about the protein portion of the toxin, but the relatively consistent ratio of protein and carbohydrate and the protein's electrophoretic behavior suggest it is part of a glycopeptide. After electrophoresis, an area staining weakly for protein was sometimes associated with the area of highest carbohydrate staining. The failure to consistently localize protein on the gels was attributed to its dilution during distribution throughout the broad carbohydrate band. Dansylation did not inactivate the toxin, but lengthy incubation with proteinase K resulted in detectable losses in activity, suggesting that the protein component may also be necessary for activity.

Comparisons of toxin from culture filtrates of races 1, 10, and 12 and from cell wall enriched material of races 1 and 12 indicated some differences in protein and carbohydrate compositions among

TABLE 6. Effect of α-mannosidase and proteinase K treatment on *Cladosporium fulvum* race 1 toxin activity as assayed by necrosis and ion leakage

Enzyme[a]	Treatment	Average necrosis rating[b]		Net ion leakage (μMHOS) at 4.5 hr[c]	
		21 hr	35 hr	Experiment 1	Experiment 2
α-Mannosidase	Active	0	0.8	24.4 ± 0.5 (188%)	24.9 ± 1.1 (151%)
	Autoclaved	3.3	2.8	37.6 ± 2.5 (289%)	34.6 ± 2.3 (210%)
	Control[d]	...	...	13.0 ± 0.2	16.5 ± 1.9
Proteinase K	Active	2.0	1.1	13.9 ± 0.9 (87%)	33.6 ± 3.4 (128%)
	Autoclaved	4.0	3.6	24.8 ± 3.8 (155%)	52.3 ± 11.1 (200%)
	Control[d]	...	...	16.0 ± 1.2	26.2 ± 2.8

[a] Reaction mixtures as described in text with toxin concentration of 66 μg glucose equivalents/ml except for proteinase-K ion leakage experiment 2, in which it was 19 μg glucose equivalents/ml.
[b] Reaction mixtures were injected into tomato leaves at the end of 21 or 35 hr of incubation at 25 C. Necrosis was rated on a scale from 0 (no necrosis) to 4 (total necrosis).
[c] Values are average of three or four replicates ± standard deviation, with conductivity listed as percentage of control treatment.
[d] Leaflets injected with distilled water, except for proteinase-K ion leakage experiment 2, in which control was active proteinase K and buffer instead of toxin.

the races. These comparisons were preliminary, however, and did not eliminate variation due to differences in development of each race in the culture medium. The degree of variation among batches of toxin from one race also was not adequately investigated. Detailed comparisons of toxins from different races are warranted because variations in fungal glycoproteins have been found to be biologically important in other systems. Yeast surface antigens, involved in recognition of mating types, were highly branched mannan-containing glycoproteins (6). These molecules are responsible for species-specific variation even though their glycosyl linkage composition varies by less than 5% among species (6).

Mannose-containing glycoprotein phytotoxins have been isolated from culture filtrates of *Ceratocystis ulmi* (26) and *Phoma tracheiphila* (7) and from other plant pathogens (20,25). Glycoprotein toxins have been implicated primarily in causing wilt symptoms (25), but a few, such as that from *P. tracheiphila* (7), reproduce necrotic symptoms of the disease. The role of the *C. fulvum* toxin in leaf mold disease is less clear, since it is not a necrogenic pathogen, but possibilities are discussed elsewhere (19).

Some of the chemical and biological properties (19) of the toxin invite comparison with the phytoalexin elecitors described by Anderson-Prouty and Albersheim (2) and Ayers et al (3–5) and, to a greater degree, with the mannose-containing, wall-associated glycoprotein elicitor described by Keen (15). The main difference between necrosis-inducing "phytotoxins" of this type and the phytoalexin "elicitors" may prove to be the choice of assay.

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