Isolation and Biological Activities of Four Selective Toxins from Helminthosporium carbonum

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ABSTRACT

A new and simpler purification procedure was developed for host selective toxins from Helminthosporium carbonum race 1. Four analogs or forms of toxin with the same selectivity as the fungus were isolated from culture fluids; two forms (HC toxins III and IV) have not been reported by other workers. Crystals of the major form of toxin (HC toxin I) were recovered in high yields (>80 milligrams per liter of culture fluid) without the use of high performance or preparative thin layer liquid chromatography. E\textsubscript{DM} values, based on inhibition of root growth of susceptible seedlings, for HC toxins I, II, III, and IV were 0.2, 0.4, 2.0, and 20 micrograms per milliliter, respectively. The specific activity of crystalline HC I matched the most active preparation reported previously; the preparation of HC toxin II was more active than that reported previously. Resistant seedlings tolerated 100-fold higher concentrations of each form of toxin than did susceptible seedlings. Hydrolysis of the epoxide group of HC toxin I to a diol destroyed toxicity to susceptible and resistant seedlings. The data suggest that the same mechanisms are affected in resistant and susceptible plants.

Helminthosporium carbonum (Ullstrup) race 1 (anamorph of Cochliobolus carbonum Nelson), the causal agent of a leaf spot disease of maize, produces a host-selective toxin that is required for pathogenicity (12). Leisch et al. (8) identified HC toxin as a cyclic tetrapeptide containing alanine (2 residues), proline (1 residue), and an unusual epoxide-containing amino acid, Aoe\textsuperscript{1} (1 residue). Subsequent papers confirmed the amino acid composition and cyclic nature of the compound, and indicated the amino acid sequence of the molecule (9, 15) (Fig. 1, toxin I). The toxin was first identified as cyclo-(2-amino-8-oxo-9,10-epoxydecanoyl-prolyl-alanyl-alanyl) by Walton et al. (15). This form of HC toxin has been synthesized (4), confirming the structure. Hydrolysis of the epoxide to a diol produced a compound that is nontoxic (1, 16). An analog of HC toxin was discovered in culture fluids of the fungus (5) (Fig. 1, toxin II). The compound differed structurally from the previously known HC toxin by the substitution of glycine for alanine adjacent to Aoe. The substitution did not alter specificity of HC toxin, but reduced its potency (5).

Existing purification schemes for HC toxin are tedious and time consuming, requiring TLC as a preparative step and analytical HPLC of relatively impure preparations. We describe here an improved purification scheme for HC toxin that is based on flash chromatography (13) on silica gel. The protocol allows for rapid and easy accumulation of large quantities of crystalline HC toxin I (Fig. 1), the most abundant and potent form of the toxin. At least three analogs of toxin I were separated with the new purification scheme. These include the glycine-containing compound (HC toxin II) (5), the hydroxylproline-containing compound (HC toxin III) (14) and an additional form, not reported previously (HC toxin IV). Chemical characterization of toxin IV is not yet complete.

MATERIALS AND METHODS

Toxin Production and Initial Purification. Procedures for production and initial purification of HC toxins were modified from those used previously (10). Pathogenic, single spore isolates of H. carbonum race 1 were maintained on potato dextrose agar slants at 4°C. For toxin production, the fungus was grown in a modified Fries solution containing yeast extract (10). Cultures (21 d old, 10–20 L) were filtered through cheesecloth, then through paper, and concentrated to approximately 0.1 volume under reduced pressure at 40°C. An equal volume of methanol was added to the concentrated filtrate and the solution was stored overnight at −20°C; a precipitate was removed by filtration. Methanol was then removed under reduced pressure, and the aqueous solution was extracted three times, each time with an equal volume of methylene chloride.

Steps to this point were completed as quickly as possible to avoid hydrolysis of the epoxide. If delays were encountered, the product of each additional purification step was dried under reduced pressure and stored under Ar or N\textsubscript{2} at −20°C. The combined methylene chloride extracts were concentrated under reduced pressure to a reddish oil (several ml) which was dissolved in methanol and placed on a column (4.1 × 35 cm) of Sephadex LH-20 (Sigma Chemical Co.) previously equilibrated with methanol. The column was developed with methanol and 5 ml fractions were collected. Fractions containing selective toxic activity were identified by TLC and by bioassay (10).

Thin-Layer Chromatography, NMR, and FAB-MS. TLC was on 0.25 mm plates (Merck silica gel-60, 20 × 20 cm) developed with acetone:methylene chloride (1:1, v/v). HC toxins were detected by spraying the plates with the epoxide indicator NBP (3). NMR and FAB-MS spectral methods were described elsewhere (14).

Flash Chromatography. The toxin-containing fractions from the LH-20 column were combined and concentrated under reduced pressure, giving an orange oil that was fractionated by flash chromatography, as described by Still et al. (13). Diameter

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3 Abbreviations: Aoe, 2-amino-8-oxo-9,10-epoxydecanoic acid; NBP, 4-(p-nitrobenzyl)-pyridine; HC toxin, the host-selective toxin from H. carbonum race 1; FAB-MS, fast atom bombardment mass spectroscopy.
of the silica gel (Whatman LPS-2) column was determined by size (dry weight) of the sample, but the length of the packed silica bed was approximately 15 cm regardless of column diameter. The column was developed with hexane:methylene chloride:acetone (1:1:1, v/v/v; solvent A) and 50 ml fractions were collected. Each fraction was tested for epoxide, by TLC and NBP; a positive test was indicated by a reactive spot at Rf 0.55, which was shown to be HC toxin I. After toxin I was eluted in solvent A (first fraction), the mobile phase was changed to methylene chloride: acetone (1:1, v/v; solvent B). The column was then developed with 1000 ml of solvent B. TLC of aliquots of the solvent B eluate showed epoxide-containing spots (Rf 0.35–0.25) with host-selective toxicity. These were shown by NMR and FAB-MS to be toxin analogs (data not shown). The total solvent B eluate was then refractionated by flash chromatography on silica gel using a smaller column (20 mm diameter) with both solvents A (300 ml) and B (500 ml) as described above; 20 ml fractions were collected. The solvent A eluate was pooled with the solvent A eluate from the first flash chromatography step. HC toxin I was crystallized from diethyl ether solutions (8). The eluate analogs were located in the solvent B eluates by TLC; the fractions were pooled to form the minor toxin preparation.

In preparation for HPLC, the solvent B eluate was concentrated, dissolved in water (25 ml), and placed on a flash chromatography column (2.2 × 15 cm) of octadecyl C18 (40 µm, J. T. Baker Chemical Co.). Some nontoxic materials were removed by passing 100 ml of water through the column. Minor toxins were eluted with 30% ethanol in water (100 ml) and dried under reduced pressure. The residue was dissolved in water (200 µl or more).

HPLC. Aliquots of the solvent B preparation, from the octadecyl C18 column, were loaded onto a Waters µBondapac C18 HPLC column (0.78 × 30 cm) and chromatographed with a Varian 5000 instrument. The initial solvent of 7% ethanol in water was changed linearly over a 30 min period to 20% ethanol in water; this concentration was then held constant for the next 15 min. The flow rate was constant at 2.0 ml/min, and A was monitored at 215 nm. Final purification of the selectively toxic analogs was on a 0.4 × 25 cm column of Whatman Partisil 5. Elution was with an isocratic mixture of hexane:ethanol (95:5 or 93:7, v/v) at 3.0 ml/min, and absorbance was monitored at 215 nm.

Hydrolysis of Epoxide. The epoxide of HC toxin I was hydrolyzed to a diol with 0.1% (v/v) trifluoroacetic acid in water (1). The diol was purified on the octadecyl C18 column (2.2 × 15 cm), as described above for the minor toxins. Fractions from the column were tested for diol by the use of TLC with bromocresol green as indicator (1). The diol-containing eluate from the column was subjected to HPLC, using the Partisil 5 column and hexane:ethanol (85:15, v/v) as described above.

RESULTS

Isolation, Identification, and Activity of Major HC Toxin. Flash chromatography with solvent A gave a relatively pure toxin preparation that was dried, dissolved in diethyl ether, and crystallized from the ether solution. A liter of culture filtrate yielded >80 mg of crystalline HC toxin. A solution of the crystals gave a single peak (retention time, 11 min) on a Partisil 5 HPLC column that was eluted with hexane:ethanol (95:5, v/v) at a flow rate of 3.0 ml/min. Mass spectral and NMR data (not shown) were identical to those of the previously characterized HC toxin I (9, 15), confirming the chemical identity of the compound. ED50 for solutions of the crystals was 180–230 ng/ml (about 0.4 µm) in three assays with susceptible seedlings; resistant seedlings were not affected at concentrations up to 5.0 µg/ml. This specific activity matched the most active preparation reported for HC toxin, and the dose-response curve was almost identical to that reported by Ciuiffetti et al. (1). The ED50 value of HC toxin I against resistant seedlings was about 20 µg/ml, or about 100-fold higher than that for susceptible seedlings (Fig. 2).

The epoxide group on HC toxin I was hydrolyzed to a diol (1), and the preparation was purified by column chromatography and HPLC. Proton NMR and FAB-MS data matched those previously published for HC toxin I diol (1), confirming the chemical identity of the compound. The diol form was toxic to susceptible seedlings (data not shown), also confirming the earlier report (1). Toxicity against resistant seedlings was abolished by hydrolysis of the epoxide group to a diol; root growth by resistant seedlings was not inhibited by the diol form at concentrations up to 250 µg/ml (Fig. 2).

Isolation, Identification, and Activities of HC Toxin Analogs.
FIG. 3. HPLC of a preparation containing minor HC toxins. Chromatography was on a reverse phase C_{18} column (2.2 \times 30 \text{ cm}, Waters Bondapak). Elution was with a gradient; the initial solvent of 7% ethanol in water was increased linearly to 20% ethanol in water over 30 min. The solvent composition was then held constant at 20% ethanol for the next 15 min. The flow rate was constant at 2.0 ml/min. RP1 contains toxins II and III (Fig. 4); RP2 is toxin IV; and RP3 is nontoxic.

FIG. 4. HPLC of peak RP1 (from Fig. 3), separating HC toxins II and III. A Partisil 5 column (0.4 \times 25 \text{ cm}) was used; the solvent was hexane:ethanol (95:5, v/v). Flow rate was 3.0 ml/min and \( A \) was monitored at 215 nm.

The solvent B eluate from flash chromatography contained selectively toxic materials that were purified further by HPLC. Complete resolution of toxins was never achieved with the C_{18} sorbent by HPLC. Three major peaks, designated RP1, RP2, and RP3 in order of elution, were obtained in a water and ethanol gradient, using the C_{18} column (Fig. 3). RP1 was difficult to purify further by HPLC on C_{18} but was easily separated into two major components by use of the Partisil 5 HPLC column (Fig. 4).

Both peaks from the HPLC Partisil 5 column had host-selective toxicity; they were designated HC toxins II and III in order of elution (Fig. 4). The identities of toxins II and III were confirmed by spectral data (14) (Fig. 1). Toxin II had an \( E_{D_50} \) value of 370 to 390 ng/ml (about 0.8 \( \mu \text{m} \)) in three assays against susceptible seedlings (Fig. 5). Thus, our HC toxin II preparation was more active than that reported by Kim et al. (5), who used a slightly different assay (96 h incubation in 15 ml of solution) and obtained an \( E_{D_50} \) value of 7.0 \( \mu \text{g}/\text{ml} \). Our HC toxin II was assayed simultaneously by the Kim et al. method (5) and by our method. HC toxin II gave an \( E_{D_50} \) value of 0.4 \( \mu \text{g}/\text{ml} \) in our assay, using 72 h and 10 ml solutions; an \( E_{D_50} \) of about 0.3 \( \mu \text{g}/\text{ml} \) was observed with the assay conditions of Kim et al. (5) (Table 1). Thus, the two assays gave similar results for HC toxin II. The \( E_{D_50} \) for HC toxin III was 1800 to 2100 ng/ml (about 4.4 \( \mu \text{m} \)) in three assays (Fig. 6). Resistant seedlings were not inhibited in their growth by either toxin at the concentrations used (Figs. 5, 6).

Yields of HC toxin II were generally higher (1.6–1.7 times)

*Ten seedlings (5 per duplicate Petri plate) per treatment were used. Percent inhibition is relative to root growth in the appropriate water control. *The routine assay used in this study; seedlings were incubated in water or in toxin solutions (10 ml per plate) for 72 h. *Assay method of Kim et al. (5).
HC toxin I > toxin II > toxin III > toxin IV. Based on results of chromatography with octadecyl C₁₈ and silica gel, the polarity of the HC toxin forms were as follows: III > II > IV > I.

At least three other constituents of peak RP2 contained an epoxide group, as determined by TLC and the epoxide indicator (NBP), and therefore may be toxin analogs. None of these additional constituents was characterized further either chemically or biologically. Peak RP3 (Fig. 3) appeared to be relatively pure, but it had no selective toxicity. Spectral data (not shown) indicated that it had no epoxide group but otherwise was chemically related to the HC toxins.

We have examined one additional isolate of _H. carbonum_ race 1; it produced HC toxins I, II, and III in ratios similar to those reported above. Production of HC toxin IV was not determined for the second isolate.

**DISCUSSION**

Our improved purification scheme is a rapid, easy, and efficient way to obtain large quantities of crystalline HC toxin I. Limiting steps in the previously used procedures were the use of TLC plates as a preparative step, use of analytical HPLC, and destruction of the epoxide by the lengthy procedure. The TLC and analytical HPLC steps were eliminated by crystallization of toxin from preparations obtained by flash chromatography. The crystals, which were collected in high yields, gave a single peak on analytical HPLC; in bioassays, the preparation had specific activity equal to the highest reported for HC toxin (1). Large quantities of highly purified toxin were useful in our studies on action of HC toxin (11); readily available toxins should facilitate future research. Flash chromatography on silica gel may simplify the purification of other relatively nonpolar selective toxins, such as those from _H. maydis_ race T, _Phyllosticta maydis_, and _Alternaria alternata_ (12).

Spectral data were collected on HC toxins II and III and reported elsewhere (14). Toxin II is the glycine-containing analog first reported by Kim _et al._ (5). HC toxin III contains trans-3-hydroxyproline rather than proline as its only chemical difference from toxin I (Fig. 1) (14). Complete chemical characterization of HC toxin IV is in progress; it appears to be another toxin analog. An important consideration for toxin IV is that it is the least active of all host-selective toxins reported to date; previously known selective toxins have EDₙ values well below 1.0 µg/ml. We must now consider the possibility that new selective toxins from other plant pathogens may have selective activity much lower than those of known toxins. This will affect the selection of methods used to detect unknown toxins.

The discovery of analogs of HC toxin, each of which shows quantitative differences in levels of activity, permits a more detailed examination of structure/activity relationships. HC toxin I contains alanine and proline adjacent to Aoe. HC toxins II and III have slight variations in the residues adjacent to Aoe; these differences do not alter selectivity but change the relative toxicities of the compounds. HC toxin II differs from toxin I by the substitution of a hydrogen atom for a methyl group, giving glycine rather than alanine in the molecule (Fig. 1). Biological data indicate that, on a weight or molar basis, HC toxin II is about 50% as potent as toxin I, as determined by EDₙ values in root growth assays (Fig. 5). HC toxin III differs from toxin I by the substitution of a hydroxyl group for a hydrogen atom at carbon 3 in the proline ring (Fig. 1), giving a molecule with about 10% of the activity of HC toxin I in root growth assays. Such differences in toxicity might be based on affinity for receptors or on permeation of plant cells.

Root growth of resistant seedlings was inhibited by HC toxin I at concentrations 100-fold higher than were required to inhibit susceptible seedlings (Fig. 2). This confirms earlier observations based on root growth inhibition with less pure toxin preparations.
(7) and suggests that HC toxin I and its analogs have similar target sites in susceptible and resistant maize. The epoxide group on the toxin is known to be essential for uptake or activity against susceptible seedlings (1, 16). Our data indicate that hydrolysis of the epoxide to a diol also destroys toxic activity against resistant seedlings (Fig. 2). Root growth by resistant seedlings was not affected by concentrations of the diol (inactive) form up to 250 µg/ml. Both susceptible and resistant maize plants respond to HC toxin with increases in respiration and CO₂ fixation in the dark (6), and uptake of certain amino acids and ions (18). Toxin-induced increases in the reduction of nitrate by susceptible and resistant maize tissues resulted from an increased uptake of nitrate within a few hours of HC toxin treatment (17). HC toxin also causes an increase in negative electropotential across the plasma membrane (2). The inhibition of Chl synthesis in susceptible and resistant maize is the most rapid inhibitory effect of HC toxin observed to date (11). In all cases, the activities against resistant tissues require 100-fold higher concentrations of HC toxin. The data support but do not prove the hypothesis that resistant maize cells have a toxin sensitive site similar to that in susceptible cells.

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LITERATURE CITED