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Enzymatic Detoxification of HC-toxin, the Host-Selective Cyclic Peptide from *Cochliobolus carbonum*[1]

Robert B. Meeley and Jonathan D. Walton*

Department of Energy Plant Research Laboratory (R.B.M., J.D.W.), Department of Biochemistry (R.B.M.), and Department of Botany and Plant Pathology (J.D.W.), Michigan State University, East Lansing, Michigan 48824

ABSTRACT

Resistance to the fungal plant pathogen *Cochliobolus carbonum* race 1 and to its host-selective toxin, HC-toxin, is determined by *Hm*, a single dominant gene in the host plant maize, (*Zea mays* L). Radiolabeled HC-toxin of specific activity 70 milliCuries per millimole, prepared by feeding tritiated D,L-alanine to the fungus, was used to study its fate in maize leaf tissues. HC-toxin was converted by resistant leaf segments to a single compound, identified by mass spectrometry and nuclear magnetic resonance as the 8-hydroxy derivative of HC-toxin formed by reduction of the 8-keto group of 2-amino-9,10-epoxy-8-oxodecanoic acid, one of the amino acids in HC-toxin. Reduction of HC-toxin occurred in cell-free preparations from etiolated (*Hm*/hm) maize shoots, and the activity was sensitive to heat and proteolytic digestion, dependent on NADPH, and inhibited by *p*-hydroxymercuribenzoate and disulfiram. The enzyme (from the Hm/hm genotype) was partially purified by ammonium sulfate precipitation and diethylaminoethyl-ion exchange chromatography. By gel filtration chromatography, the enzyme had a molecular weight of 42,000. NADH was approximately 30% as effective as NADPH as a hydride donor, and flavin-containing cofactors had no effect on activity. When HC-toxin was introduced to maize leaf segments through the transpiration stream, leaf segments from both resistant and susceptible maize inactivated toxin equally well over a time-course of 9 hours. Although these data suggest no relationship between toxin metabolism and host selectivity, we discuss findings in apparent conflict with the current data and describe why the relationship between enzymatic reduction of HC-toxin and *Hm* remains unresolved.

A number of phytopathogenic fungi, especially in the genera *Alternaria* and *Cochliobolus,* produce low mol wt compounds known as host-selective toxins that determine their host range and contribute to their virulence (16). Race 1 of *Cochliobolus carbonum* Nelson (*Helminthosporium carbonum* Ullstrup or *Bipolaris zeicola* [Stout] Shoem.) produces a cyclic tetrapeptide, called HC-toxin, that accounts for its exceptional virulence on maize (*Zea mays* L.) varieties that are homozygous recessive at the nuclear *hm* locus (12, 17). HC-toxin has the structure cyclo-[D-prolyl-L-alanyl-D-alanyl-L-Aeo[2]] (7, 8, 11, 13, 22). Both the terminal epoxide and vicinal ketone of Aeo are required for biological activity of HC-toxin and of its naturally-occurring analogs (1, 2, 9, 20).

Considerable research has been published on the differential effects of HC-toxin on resistant and susceptible maize (see ref. 16), but the mode of action of this compound remains unknown. As an alternative to studying the effect of HC-toxin on maize tissues, we have taken an approach that examines the effect of maize tissues on the biological activity of HC-toxin. Given the requirements for the epoxy-ketone moiety of Aeo, we sought to determine if the integrity of these groups is maintained *in vivo*. Plants, including maize, are known to contain enzymes capable of inactivating xenobiotic compounds (10). For example, atrazine tolerance in maize is due to elevated levels of glutathione-*S*-transferase enzymes that inactivate atrazine by conjugation (18). Knowledge of the biochemical fate of HC-toxin within maize tissues lays the groundwork to ultimately address the hypothesis that host selectivity in maize to race 1 of *C. carbonum* is due to a difference in ability to detoxify HC-toxin.

We introduce this topic by presenting a method to prepare tritiated HC-toxin for use in metabolic studies. The biochemical fate of HC-toxin is described as we report the conversion of HC-toxin by resistant maize leaves and cell-free extracts to a single nontoxic compound. The enzyme responsible for detoxification is described, and our initial comparison of HC-toxin metabolism between resistant and susceptible maize is critically presented.

MATERIALS AND METHODS

Growth of the Fungus

Maintenance of stock cultures of *Cochliobolus carbonum* Nelson and liquid growth conditions were as described (21). The toxin-producing isolate SB111 of *C. carbonum* was originally provided by S. P. Briggs, Pioneer Hi-Bred International (Johnston, IA).

Purification and Analysis of HC-Toxin

Methods of toxin purification by solvent extraction and reverse-phase HPLC were as given (22). HC-toxin was quantified by HPLC and a Spectra-Physics model 4270 automatic

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[2] Abbreviations: Aeo, 2-amino-9,10-epoxy-8-oxodecanoic acid; *p*-NBP, 4-(*p*-nitrobenzyl)-pyridine; HCTR, HC-toxin reductase; FAB, fast-atom bombardment.

integrator. HC-toxin identity was confirmed by root growth bioassay (22), fast-atom bombardment MS, and proton NMR. Mass spectra were collected on a JEOL model HX110-HF double focusing instrument (accelerating voltage, 10 kV; ionization, 6 keV xenon beam; mass resolution, 1000). NMR analyses were performed in $CDCl_3$ on a Varian VXR-5 500 MHz instrument.

The 8-ketone group of Aeo in HC-toxin was specifically reduced to the corresponding 8-alcohol with sodium borohydride (9). The 9,10-epoxide group of Aeo was hydrolyzed with TFA (20). Derivatives and metabolites of HC-toxin were separated either by reverse-phase HPLC (22), by TLC (silica gel 60, Merck) developed in CH_2Cl_2:acetone (1:1, v/v) (15), or by flash chromatography (15). Epoxides were detected after TLC by spraying plates with *p*-NBP (4).

In Vivo Production of Radiolabeled HC-Toxin

Static liquid cultures (125 mL) of *C. carbonum* were grown in 1-L Erlenmeyer flasks. For testing incorporation of various amino acid precursors, 15 μCi of D-[^{14}C]alanine (specific activity 30–60 mCi/mmol, ICN); 40 μCi of L-[^{3}H]alanine (30–50 Ci/mmol, ICN); or 40 μCi of L-[^{3}H]proline (60–100 Ci/mmol, ICN) were added per flask. In experiments conducted to maximize incorporation of radiolabeled alanine, the original growth medium from 8- to 14-d-old cultures was replaced under sterile conditions by fresh medium containing 5 μCi of [^{14}C]D-alanine (46 mCi/mmol, Amersham). The original medium, which contained any unlabeled HC-toxin, was discarded. The length of incubation in the presence of radioactivity was varied from 24 to 96 h. Once favorable conditions for label incorporation and toxin yield were established, the production of tritiated HC-toxin of high specific activity was initiated by adding 20 mCi of D,L-[2,3-^{3}H]alanine (59 Ci/mmol, Amersham) to a single flask. Radioactivity was monitored during HPLC purification with an in-line scintillation-flow detector (Radiomatic model CT) at a scintillant:column flow ratio of 3:1 (v/v). Radioactivity was quantified by scintillation counting, corrected for ^{3}H efficiency, and HC-toxin mass was quantified by HPLC with absorbance monitoring at 230 nm.

Growth of Plant Materials

Caryopses of near-isogenic resistant (Pr1 × K61 or K61 × Pr1, genotype *Hm/hm*) or susceptible (Pr × K61 or K61 × Pr, genotype *hm/hm*) maize (*Zea mays* L.) were surface sterilized with 0.5% hypochlorite plus 0.1% Tween-20 for 30 min, rinsed thoroughly with sterile distilled water, and allowed to imbibe water for 2 to 4 h. For the production of green leaves, imbibed seeds from both genotypes were sown in 8-inch diameter clay pots containing a perlite:fine-vermiculite:sphagnum (1:1:1 w/w) mixture. The pots were subirrigated with water. Plants were grown in a growth chamber under the following conditions: daylength, 16 h; light intensity, 126 $\mu E/m^2 \cdot s$ (PAR cool white fluorescent lights); day temperature, 21°C; relative humidity, 72%; night length, 8 h; temperature, 18°C; relative humidity, 80%.

For production of etiolated shoots for enzyme extraction, imbibed seeds of the resistant genotype were sown in flats of vermiculite that had been saturated with half-strength Hoagland's solution. The flats were covered with lids and placed in a dark cabinet for 5 to 6 d.

Metabolism of [^{3}H]HC-Toxin by Maize Leaf Segments

HC-toxin was administered to mature green leaves through the transpiration stream. Segments of green leaves, approximately 10 cm in length, were cut (approximately 10 cm from the leaf tip) from 3- to 5-week-old resistant and susceptible plants. The segments were submerged in a beaker of water, evacuated of intercellular air with a laboratory aspirator for 30 min, and then blotted dry with paper towels and placed in 18-mm test tubes containing 5 mL H_2O, with or without 0.25 μCi of [^{3}H]HC-toxin (0.3 μg/mL). The leaf segments were placed in a lighted laboratory fume hood during uptake of [^{3}H]HC-toxin.

For extraction, the leaf segments were rinsed thoroughly in deionized water, frozen in liquid nitrogen, and ground to a powder in a mortar and pestle. Five milliliters of methanol were added and the leaves were ground again. The methanolic extracts were passed through glass fiber filters (Whatman GF/A), and the methanol was evaporated under vacuum. The aqueous residues were transferred to 1.5-mL polyethylene microfuge tubes and centrifuged at 15,000*g* for 5 min. The supernatants were transferred to fresh microfuge tubes and the pellets discarded. Radioactivity recovered from leaf extracts was analyzed by HPLC coupled to an in-line scintillation-flow detector or by TLC and a scanning β-detector (Bioscan). As a control, 0.25 μCi of [^{3}H]HC-toxin was added to leaf segments immediately prior to freezing and methanol extraction.

Preparation of Maize Crude Extracts

All steps were carried out at 4°C or on ice. Etiolated plumule tissue (from 5–25 g) from freshly germinated resistant (*Hm/−*) maize was cut 1 cm below the coleoptilar node and ground in extraction buffer (0.5 mL/g fresh weight) with a mortar and pestle. The extraction buffer contained 0.1 M Mops (pH 7.4), 0.3 M sucrose, 5% (w/v) PVP, 10% (v/v) glycerol, 5 mM DTT, 1 mM EDTA, 15 mM ascorbate, and 0.2 mM PMSF. The extract was filtered through four layers of cheesecloth, centrifuged at 3000*g* for 10 min, and the supernatant saved.

Enzyme Enrichment

The crude extract was initially fractionated with ammonium sulfate. Material precipitating between 30 and 55% saturation (30% saturation equals 17.6 g/100 mL) was collected by centrifugation (10,000*g*, 10 min) and desalted by gel filtration (PD-10 column, Pharmacia) in 25 mM potassium phosphate (pH 7.5), 2.5 mM DTT, 1 mM EDTA, and 1% (v/v) glycerol. The material was further fractionated on an anion exchange HPLC column (TSK-DEAE-5PW, 7.5 mm × 7.5 cm, Beckman), with a 25-min linear gradient from 0 to 0.5 M NaCl in the same buffer. The flow rate was 1 mL/min and 2-mL fractions were collected.

The mol wt of the enzyme was estimated by gel filtration HPLC on a TSK-4000 column (30 cm × 7.5 mm, Beckman)

equilibrated with 0.15 M potassium phosphate (pH 7.2), 1 mM EDTA, and 5 mM DTT. Approximately 1 mg of protein from an ion exchange fraction containing HCTR activity was loaded onto the column, and 2-mL fractions were collected. Immediately after elution, ascorbate to 10 mM was added to each fraction. The column was calibrated with the following proteins (Sigma): thyroglobulin (M_r 670,000), immunoglobulin G (M_r 158,000), ovalbumin (M_r 44,000), and myoglobin (M_r 17,000).

Enzyme Assay Conditions

Typical assay volumes were 125 μL and contained 115 μL enzyme solution, 4 mM NADPH (Sigma), and 0.25 μCi [^{3}H] HC-toxin (23 μM). Reactions were run at 30°C and were stopped by the addition of an equal volume of chloroform and rapid mixing. The reactions were extracted twice more with chloroform, and the organic phases were combined and concentrated under vacuum. Concentrated extracts were analyzed by TLC or HPLC. Tritiated substrates and products were quantified with a scanning β-detector or analyzed by spraying TLC plates with a fluorography enhancer (enHance, DuPont) and exposing the plates to x-ray film (Kodak XAR-5) for several days.

For production of large quantities of the HC-toxin metabolite, unlabeled HC-toxin was used, and the reaction was scaled up to a volume of 4 mL. The HC-toxin metabolite was purified by chloroform extraction, flash chromatography (15), and reverse-phase HPLC (22).

RESULTS

Production of Radiolabeled HC-Toxin

Several radiolabeled amino acids present in native HC-toxin were evaluated as precursors for *in vivo* production of radiolabeled HC-toxin. As expected from the fact that D-alanine is a substrate for HC-toxin synthetase *in vitro* (21), D-alanine was incorporated into HC-toxin *in vivo*. D-Alanine was incorporated 68-fold more effectively than L-alanine and sixfold more effectively than L-proline (Table I). Radiolabeled D-proline was not tested because it was not commercially available. The racemic mixture of tritiated alanine used in the final experiment was not evaluated in the manner presented in Table I.

Table I. *Incorporation of Radiolabeled Amino Acid Precursors into HC-Toxin in Vivo*

The indicated amino acids were added to *C. carbonum* race 1 after 10 d growth, and the culture filtrates were harvested after an additional 2 d.

Amino Acid	Amount Added	Radioactivity Recovered		
		Crude culture filtrate[a]	Chloroform phase[b]	Final recovery[c]
	μCi	% of original		
D-[^{14}C]Alanine	15	10.5	4.4	2.7
L-[^{3}H]Alanine	40	51.0	0.06	0.04
L-[^{3}H]Proline	40	22.0	0.7	0.45

[a] % of total radioactivity present in crude culture filtrate. [b] % of total radioactivity extracted from culture filtrate with chloroform. [c] % of total radioactivity remaining after chloroform was evaporated and sample was redissolved in water.

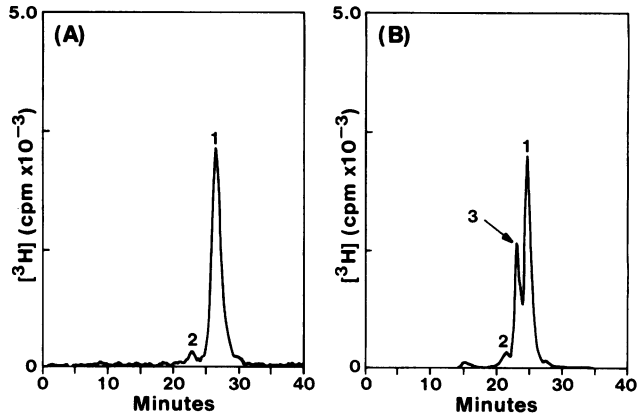

Figure 1. Formation of a metabolite of HC-toxin by green maize leaf segments. Following uptake of 0.25 μCi [^{3}H]HC-toxin by transpiration, the leaves were ground and analyzed by reverse-phase HPLC. Radioactivity (shown) eluting from the HPLC column was detected with an in-line scintillation counter. A, Control: [^{3}H]HC-toxin added to leaf segments immediately prior to extraction; B, extract of resistant leaves. Peak 1, native HC-toxin; peak 2, 9,10-diol-HC-toxin; peak 3, HC-toxin metabolite.

Although production of HC-toxin can vary from culture to culture, we attempted to optimize the conditions for *in vivo* incorporation of alanine into HC-toxin by evaluating the incorporation of D-[^{14}C]alanine with respect to the time added to culture and the duration of incubation in the presence of the radioactivity. In our hands, 8 d of fungal growth prior to media exchange followed by 48 h incubation with radiolabeled D-[^{14}C]alanine gave the best balance between specific activity and toxin yield (data not shown). Following this protocol, 20 mCi of D,L-[2,3-^{3}H]alanine were added to a single flask, and approximately 2 mg of pure, tritiated HC-toxin were recovered. The chromatographic behavior of tritiated HC-toxin was identical to that of unlabeled toxin during purification. Its biological activity in the root growth bioassay was the same as that of unlabeled HC-toxin (effective dose for 50% inhibition = 0.3 μg/mL). The specific activity of the tritiated HC-toxin was 70.1 ± 0.2 mCi/mmol.

Alteration of Toxin Structure within Maize Leaf Segments

Native HC-toxin was altered after its uptake by transpiration into resistant leaf segments (Fig. 1). An apparent metabolite (peak 3) eluted with a polarity intermediate to native HC-toxin (peak 1) and its 9,10-diol form (peak 2) (Fig. 1B). The diol of HC-toxin is formed by hydrolysis of the epoxide of Aeo, and was present as a minor contaminant in all chromatograms, including controls (Fig. 1A). Beyond a minor increase in the amount of diol produced during extraction, no metabolites of HC-toxin apart from 3 were detected. In addition, 3 was the only altered form of HC-toxin observed when leaf extractions were performed in aqueous solvents (not shown) or when leaf extracts were analyzed by TLC. Feeding partially purified 3 (0.05 μCi/mL) back to maize

leaves did not result in the formation of any additional tritium-containing compounds (data not shown), suggesting that 3 is an end product of HC-toxin metabolism.

Metabolite Identification

Figure 2 shows TLC analysis of native HC-toxin, $NaBH_4$-reduced toxin, and the HC-toxin metabolite recovered from resistant leaf segments (peak 3). The HC-toxin metabolite (lane 3) had the same R_F as $NaBH_4$-reduced HC-toxin (lane 2). The metabolite reacted with the epoxide indicator *p*-NBP (lane 7), indicating that the epoxide was still intact. The FAB-mass spectra of chemically reduced HC-toxin and purified toxin metabolite had molecular ions of m/e = 439, consistent with the addition of two atomic mass units to HC-toxin (Fig. 3). An NMR spectrum (data not shown) confirmed the structure of the metabolite, as it was identical to those of $NaBH_4$-reduced HC-toxin (9) and HC-toxin IV, the name given to the 8-hydroxy derivative of native HC-toxin (HC-toxin I) by Rasmussen (14, 15). Based on these results, we conclude that the metabolite formed in maize leaves is HC-toxin in which the 8-carbonyl group of the Aeo side chain has been reduced to the 8-alcohol. We have confirmed the results of Kim *et al.* (9) that showed this form of HC-toxin to be nontoxic.

HC-Toxin Metabolism *in Vitro*

In the presence of NADPH, extracts prepared from the etiolated shoots of resistant maize catalyzed the same metabolic conversion of HC-toxin as intact leaves (Fig. 2). The

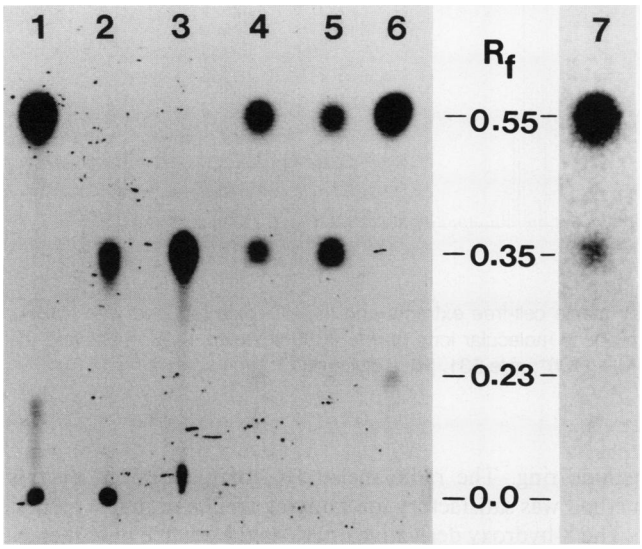

Figure 2. TLC analysis of HC-toxin metabolites produced by green leaf segments and by cell-free extracts of resistant maize. Native [^{3}H] HC-toxin (1); $NaBH_4$-reduced [^{3}H]HC-toxin (2); partially purified toxin metabolite recovered from maize leaves (3). Metabolites produced by 10 min (4) and 20 min (5) incubation of cell-free extracts with [^{3}H]HC-toxin, and extract boiled before incubation (6). Lanes 1 through 6 were detected by fluorography. Metabolites produced in cell-free extracts detected with the epoxide indicator *p*-NBP (7). Both native HC-toxin (R_F 0.55) and the toxin metabolite (R_F 0.35) react with the epoxide indicator.

amount of reduced HC-toxin recovered from incubations with cell-free extracts increased with time (Fig. 2, lanes 4 and 5), while activity was completely abolished by boiling the extract for 10 min prior to incubation (lane 6).

This enzymatic activity, which we call HCTR, was partially purified from etiolated resistant shoots. Ammonium sulfate fractionation and anion exchange HPLC resulted in a fivefold enrichment of reductase activity with an 18% recovery. TLC and fluorography of the products formed from [^{3}H]HC-toxin by individual fractions from an anion exchange separation (Fig. 4, bottom) is shown below the UV trace (Fig. 4, top). HCTR was eluted from the anion exchange column in a single fraction, No. 10. 8-Hydroxy HC-toxin is indicated by (a). A second product of greater polarity (labeled b) was formed by fraction 11. Product b reacted with *p*-NBP, indicating an intact epoxide, but its formation was partially resistant to boiling and did not require a hydride donor (data not shown). Its formation was never observed in crude HCTR preparations (see Fig. 2). When we purged fraction 11 with nitrogen or included oxygen-scavenging compounds such as GSH, ascorbate, or DTT in the reaction mixture, the formation of product b was reduced substantially, but no formation of 8-hydroxy HC-toxin was observed (not shown). We conclude that product b is a form of HC-toxin produced by a side reaction, perhaps oxygen-dependent, that occurs in solution in fraction 11. Importantly, this reaction is unrelated to HCTR activity.

Characterization of HCTR Activity

Table II shows the effects of various treatments and cofactors on HCTR activity. Activity of partially purified HCTR was completely abolished by boiling or by pretreatment with proteinase K. NADPH was a better cosubstrate than NADH. A 10-fold excess of $NADP^+$ over NADPH inhibited HCTR activity by approximately 30%, and a 10-fold excess of NAD^+ had no effect. Approximately 35% of the HCTR activity was lost by simply incubating the partially purified preparation for 60 min at 30°C. Incubation for 60 min in the presence of Zn^{2+} or Fe^{2+}, and to a lesser extent Cu^{2+}, further inhibited (or destabilized) HCTR activity (Table II). Two known inhibitors of carbonyl reductases, *p*-hydroxymercuribenzoate and disulfiram (3, 6), inhibited HCTR by 50 and 70%, respectively (Table II).

On gel filtration HPLC, HCTR was eluted as a single, symmetrical peak with an M_r of 42,000 (data not shown).

HC-Toxin Metabolism by Resistant and Susceptible Leaf Segments

When green leaf segments of equal weight were evacuated of intercellular air and allowed to transpire water containing [^{3}H]HC-toxin, significant production of 8-hydroxy-HC-toxin occurred in both resistant and susceptible leaves (Fig. 5). The toxin concentration used (0.3 μg/mL) in these experiments was equivalent to the effective dose for 50% inhibition in the root growth bioassay. Under these conditions, HC-toxin uptake and metabolism occurred at the same rate in both resistant and susceptible leaves over a 9-h transpiration period (Fig. 5). A time course extended over 48 h showed a similar lack of host-selective detoxification (data not shown).

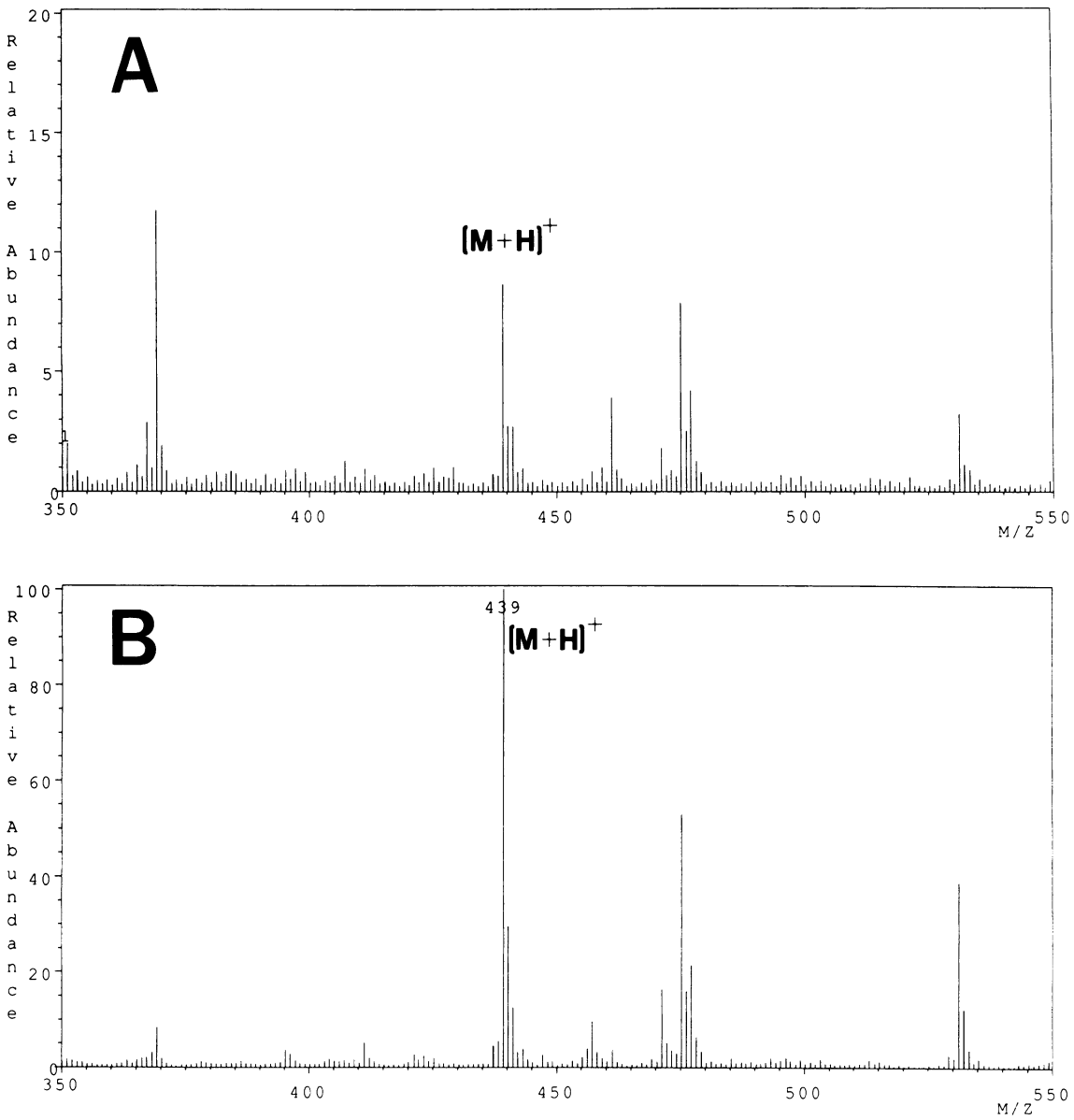

Figure 3. FAB mass spectra of (A) the metabolite of HC-toxin produced by maize cell-free extracts and (B) HC-toxin reduced with $NaBH_4$. Samples were dissolved in a matrix of glycerol and HCl. Both compounds produce molecular ions of m/e 439 (HC-toxin + 2H + H)$^+$ and the following: m/e 369, [(glycerol)$_4$ + H]$^+$; m/e 461, [(glycerol)$_5$ + H]$^+$; m/e 475, [M + HCl]$^+$; m/e 531, [M + glycerol]$^+$.

DISCUSSION

Previous work from this laboratory has described the purification and characterization of two enzymes involved in HC-toxin biosynthesis (19, 21). One of these enzymes, HTS-2, activates both D- and L-alanine for incorporation into HC-toxin. Perhaps because D-alanine, unlike L-alanine, is not diverted into cellular primary metabolism, its efficiency of incorporation into HC-toxin was relatively high (Table I). Once favorable conditions for radiolabel incorporation and toxin yield were determined, alanine incorporation was exploited to make tritiated HC-toxin *in vivo*. The chromatographic behavior and biological activity of HC-toxin were not affected by the incorporation of tritiated alanine into the peptide ring. The radiolabeled HC-toxin produced by this method was satisfactory for studying its fate in maize tissues.

The 8-hydroxy derivative of HC-toxin was the only metabolite recovered from resistant maize leaves after uptake of [^{3}H]HC-toxin by transpiration (Fig. 1). Importantly, 8-hydroxy-HC-toxin is biologically inactive and was the only toxin metabolite recovered from any of the tissues tested, including cut leaves, cell-free preparations from etiolated shoots, excised roots (not shown), and whole leaves (not shown). Notable is the observation that the other critical functional group, the epoxide of Aeo, was not altered *in vivo* or *in vitro*.

Reduction of the 8-keto group of HC-toxin is an enzymatic process. An enzyme, referred to as HCTR, that catalyzes this reduction was partially purified from extracts prepared from

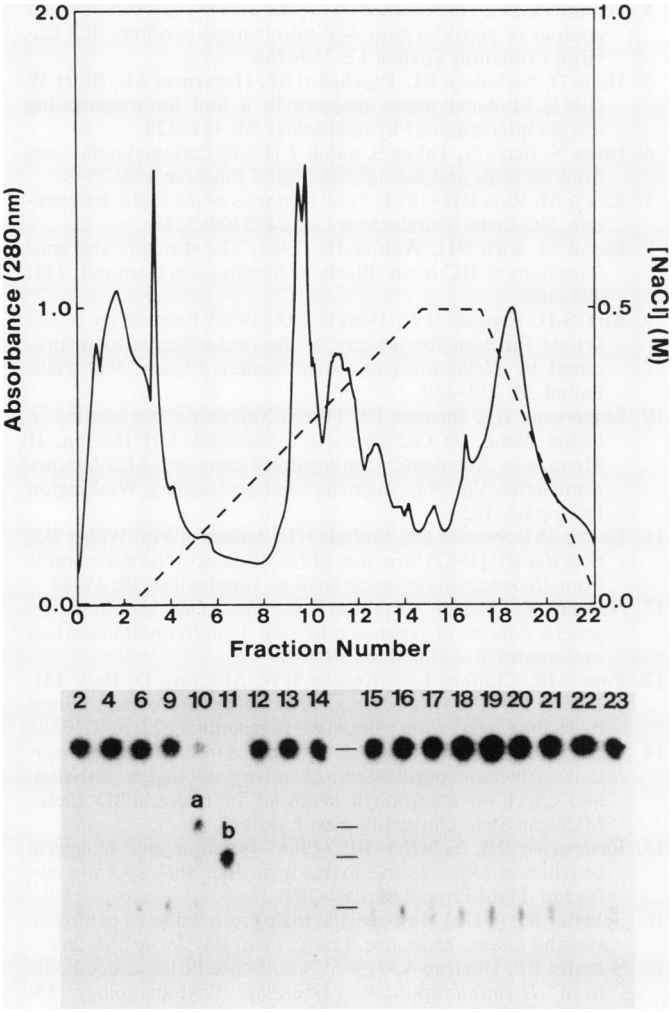

Figure 4. Anion exchange fractionation of HCTR activity. The top panel represents the elution of protein (A_{280}). Each fraction was assayed for HCTR activity, and the resulting products were separated by TLC and detected by fluorography (bottom panel). a, The 8-hydroxy derivative of HC-toxin. b, Unknown compound, formed as an artifact of the ion-exchange process (see text).

Table II. *Characterization of HCTR Activity*

Concentration of NADPH was 4 mM unless otherwise indicated. All ions were 2 mM sulfate salts. The concentration of [^{3}H]HC-toxin in each case was 23 μM and assays were run for 15 min at 30°C.

Treatment	HCTR Activity
	% of control
Experiment 1	
+ NADPH	100[a]
Boiled (10 min)	0
− NADPH	0
+ Proteinase K[b]	0
+ NADH (4 mM)	31
+ NADPH, FAD[c] (0.1 mM)	97
+ NADPH, FMN[d] (0.1 mM)	71
+ NADPH (1 mM), $NADP^+$ (10 mM)	70
+ NADPH (1 mM), NAD^+ (10 mM)	112
+ NADPH + disulfiram (10 μM)	52
+ NADPH + *p*-HMB[e] (10 μM)	30
Experiment 2	
Preincubation for 60 min with	
No divalent cations	100[f]
Fe^{2+}	0
Mg^{2+}	95
Zn^{2+}	0
Mn^{2+}	91
Co^{2+}	77
Cu^{2+}	42

[a] 100% activity = 659 pmol/min·mg protein. [b] 80 μg/mL, 10 min, 30°C. [c] FAD = flavin adenine dinucleotide. [d] FMN = flavin mononucleotide. [e] *p*-HMB = *p*-hydroxymercuribenzoate. [f] 100% activity = 427 pmol/min·mg protein.

etiolated resistant shoot tissue. HCTR appears to be similar to other NADPH-dependent carbonyl reductases found in plants and animals; it is soluble, uses NADPH more effectively than NADH as a hydride donor, and has an M_r in the range of 32,000 to 45,000. Characterized NADPH-dependent carbonyl reductases have biosynthetic functions in anthocyanidin production in plants (3, 23) and the interconversion of steroids in mammalian tissues (6, 24). Carbonyl reductases often contain metal ions for catalytic stability and are highly specific for their substrates. In the case of partially-purified HCTR, metal ions did not contribute to enzyme stability, and certain divalent cations inhibited activity (Table II). Whether HCTR has an endogenous substrate is not known at this time.

With the knowledge that HC-toxin is enzymatically metabolized to a single inactive compound in resistant maize tissues, we have sought to determine if this phenomenon is related to the *hm* locus of maize that governs host selectivity of *C. carbonum*. Our first comparative experiments are summa-

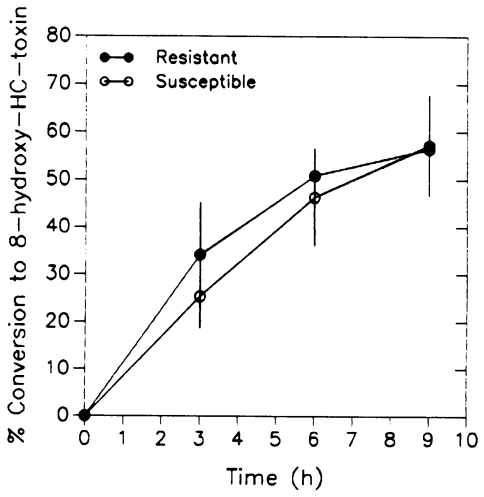

Figure 5. Time course of [^{3}H]HC-toxin metabolism by maize leaf segments during uptake by transpiration. Results are the average of duplicate samples from three independent experiments. Error bars represent ± 1 SD.

rized by Figure 5, which indicated that both resistant and susceptible maize were capable of reducing and thereby inactivating HC-toxin when toxin was delivered to green excised leaves through the transpiration stream. The similar kinetics of metabolism shown in Figure 5 suggest that reduction of HC-toxin is not the basis of host-selectivity and hence resistance to race 1 of *C. carbonum*. However, we feel it necessary to caution that delivery of toxin via transpiration is an artificial technique devised to deliver toxin to leaf tissues as quickly as possible.

As an alternative means to address whether kinetic aspects of toxin metabolism are related to host selectivity, we initiated a comparative study on HCTR from etiolated resistant and susceptible maize. In maize, host-selective reaction to *C. carbonum* is expressed in etiolated mesocotyls (5). Interestingly, these experiments have failed because repeated attempts to detect HCTR activity in extracts from etiolated susceptible maize have been unsuccessful (our unpublished results). This result is significant not only because it is in conflict with the results of our transpiration experiments, but because it points to a difference in toxin metabolism detected in near-isogenic resistant and susceptible maize. Thus, we feel that until this paradox is resolved, we cannot base our conclusions solely on data from transpiration experiments. It is possible that HC-toxin encounters a reductase during transpiration that is different from HCTR. Therefore, a relationship between HCTR and the *hm* locus remains an open question. However, whether or not such a relationship exists, the discovery that maize tissues contain an enzyme capable of inactivating HC-toxin establishes a novel facet of the interaction between *C. carbonum* and maize. Because toxin production is so critical to the infection process, a completely characterized enzymatic mechanism affecting the biological activity of HC-toxin may in time be considered as an integral part of this host/pathogen interaction.

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