J. Biochem. Mol. Biol. Vol. 28, No. 3 pp. 227~231 May 31, 1995

Relationships between Structural Features and Biological Activities of HC-toxin

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(Received November 11, 1994)

Abstract: In order to figure out the relationships between structural features and biological activity of the host-specific HC-toxin in maize, structurally related cyclic tetrapeptides, chlamydocin and CYL-2 were isolated, and their biological activities in maize were examined. Biological activities of preparations were determined by root growth inhibition and electrolyte leakage bioassays. Chlamydocin and CYL-2 showed toxicities to maize. However, the toxicities of these compounds were non-specific. Thus, the precise peptide ring structure of HC-toxin apparently does not play an important role in toxicity, while resistance of maize to HC-toxin is based on a precise ring conformation.
Key words: chlamydocin, CYL-2, HC-toxin, host-specificity, resistance.

The host-specific toxin produced by *Helminthosporium carbonum* causes a disease in certain types of maize and has been called HC-toxin. The structure of HC-toxin has been determined to be a cyclic tetrapeptide containing the residues each of D-alanine, L-alanine, D-proline and an unusual amino acid, 2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe) (Pope *et al.*, 1983).

The epoxide group of the Aoe residue in the HC-toxin is easily hydrolyzed in acidic solutions yielding a dihydroxy group in place of the epoxy function. This product was identified as cyclo-(Pro-Ala-Ala-Aod), where Aod stands for a residue of 2-amino-8-oxo-9,10-dihydroxydecanoic acid. The product was determined to be totally inactive in root growth inhibition and electrolyte leakage bioassays, leading to the conclusion that the epoxy group of HC-toxin is essential for HC-toxin's toxicity towards susceptible maize genotypes (Ciuffetti *et al.*, 1983).

Reduction of HC-toxin with sodium borohydride converted the Aoe residue to a 2-amino-8-hydroxy-9,10-epoxydecanoic acid (Ahe) residue. Two isomers were isolated and shown by NMR spectra to be diastereomers of cyclo-(Pro-Ala-Ala-Ahe) that differed by their configuration of the carbon atom number eight of the Ahe residue. Neither isomer alone nor a 1 : 1 mixture was toxic to maize genotype sensitive to HC-toxin (Kim *et al.*, 1987). Consequently, the ketone group of the Aoe residue in HC-toxin appeared to be just as necessary for the toxicity of this host-specific toxin as the epoxide group.

In continuing studies on structure-activity relationships for HC-toxin, I have investigated the roles that other structural features play in the toxicity and specificity of HC-toxin by comparing the biological activities in maize of structurally related cyclic tetrapeptides, chlamydocin and CYL-2. Chlamydocin and CYL-2 are cyclic tetrapeptides and each contains an Aoe residue. Chlamydocin has an amino acid sequence of cyclo-(Pro-Aoe-Aib-Phe) (Close and Hugenin, 1974) and CYL-2 has the sequence, cyclo-(Pip-Aoe-meTyr-Ile) (Hirota *et al.*, 1973). Aib, Pip and meTyr stand for α-aminoisobutyric acid, pipecolic acid and O-methyltyrosine, respectively (Fig. 1).

Materials and Methods

Preparation of HC-toxin

Single spore isolates of *Helminthosporium carbonum*, race 1 were obtained from Dr. Dunkle of Purdue University. For toxin production, cultures were started with small pieces of inoculum grown on potato-dextrose agar and were grown for 21 days on liquid modified Fries medium. HC-toxin was isolated as described by Ciuffetti *et al.* (1983).

Isolation of chlamydocin and CYL-2

Other cyclic tetrapeptides containing Aoe, chlamydocin and CYL-2 were isolated from culture filtrates of *Diheterospora chlamydosporia* and *Cylindrocladium macrosporum*, respectively. The starting cultures of *Diheterospora chlamydosporia* (ATCC No. 36384) and

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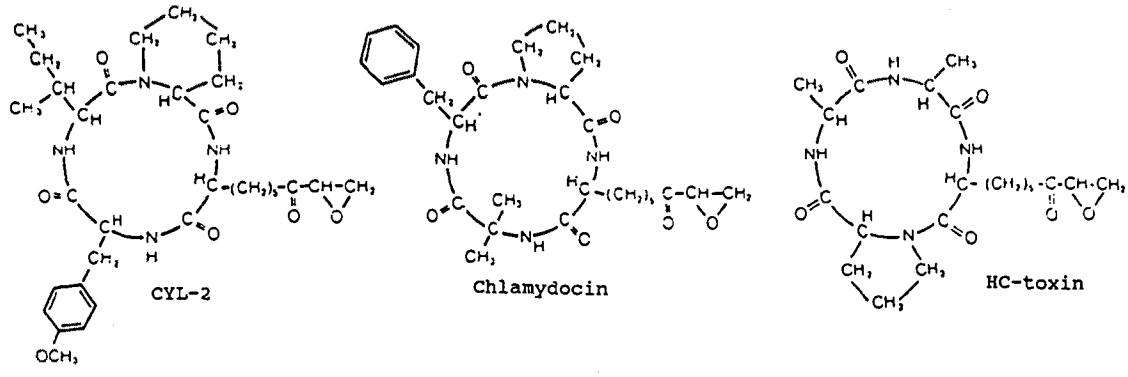

Fig. 1. The structures of HC-toxin, chlamydocin and CYL-2.

Cylindrocladium macrosporium (ATCC No. 34395) were purchased from American Type Culture Collection, and they were grown on petri plates containing maltose extract agar and potato-dextrose agar, respectively. For production of cyclic tetrapeptides, cultures were grown under the same conditions as for HC-toxin. Cultures were harvested at 21 days after inoculation. Concentrated culture filtrates were deproteinized with methanol and extracted with chloroform. Chloroform extracts were subjected to the preparative TLC. The cyclic peptides were detected by spraying the TLC plates with a reagent specific for epoxy groups (vide infra), and were further purified by HPLC.

Thin layer chromatography

TLC was performed on commercially prepared silica gel 60 G plates from Merck-EM reagents. HPLC-grade acetonitrile or methyl ethyl ketone : pyridine : water : acetic acid (70 : 5 : 5 : 2, v/v/v) was used as a solvent system. Bands were located by spraying either 0.05% bromcresol green in acetone or the epoxy detecting reagent.

Detection of epoxy-containing compounds on TLC plates

The 4-(p-nitrobenzyl)-pyridine reagent, NBP, was used for detection of epoxy-containing compounds on TLC plates (Hammock *et al.*, 1984). TLC plates were sprayed with a 2% (w/v) solution of NBP in acetone, heated at 110℃ for 5 min, allowed to cool, and sprayed with a 10% (v/v) solution of tetraethylenepentamine in acetone to yield blue spots on a light yellow background.

High performance liquid chromatography

High performance liquid chromatography (HPLC), the final purification step for HC-toxin and other cyclic tetrapeptides, was carried out with a Waters pump, model 510 and a Waters analytical or preparative μ-bondpak C-18 reverse phase column. Isocratic solvent systems (19 to 42% acetonitrile in water) were used. The effluents were monitored by absorbance at 206 nm.

Nuclear magnetic resonance spectrometry

Proton NMR spectra experiments were carried out with a 360 MHz, model 1080 nicolet instrument, with the HC-toxin and other cyclic tetrapeptides dissolved in deuterochloroform.

Mass spectrometry

Collision activated decomposition (CAD) spectra and fast atom bombardment (FAB) mass spectra were obtained using a kratos MS-50 triple analyzer.

Bioassays

All experiments were carried out with two maize hybrids that differ only at the nuclear Hm locus, which conditions reaction to *Heminthosporium carbonum*, race 1 and to HC-toxin. Pr 1×K 61 (genotype Hmhm) is resistant; Pr×K 61 (genotype hmhm) is susceptible. These hybrid corn seeds were graciously supplied by Dr. Dunkle of Purdue University.

Root growth inhibition : Seeds were soaked overnight in aerated water, washed with 1% (v/v) commercial bleach for 3 min and rinsed with sterilized distilled water. These seeds were rolled inside damp paper towels, and germinated at 24℃ for 1 day. Seedlings with primary roots of similar size were selected and were incubated for 3 days in 9 cm petri dishes containing 15 ml of water or dilutions of toxin preparations. Net elongation of the primary roots were measured.

Electrolyte leakage : Seedlings were grown in nutrient solution for 4 days and then transferred to water or test compounds in 0.5% DMSO. Conductivity of the solution surrounding roots was measured at hourly intervals using a flow-through pipette-type conductivity cell.

Results and Discussion

Preparation of HC-toxin

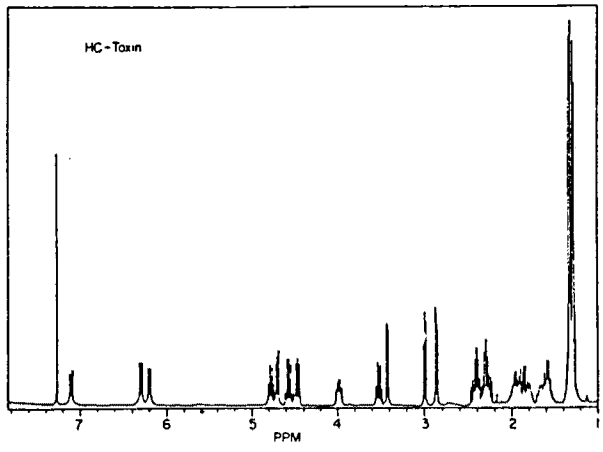

Fig. 2. NMR spectrum of HC-toxin.

Freshly purified HC-toxin gave a single band (R_f=0.47) on silica gel TLC plates when developed plates were sprayed with bromcresol green or 4-(p-nitro benzyl)-pyridine. HC-toxin was eluted from a reverse phase HPLC column as a single UV-absorbing peak at 23 min with 19% acetonitrile in water.

HC-toxin inhibited root growth of susceptible corn by 50% at about 250 ng/ml, but did not affect root growth of resistant corn at 50 μg/ml. The NMR spectrum of a HC-toxin preparation (Fig. 2) confirms that this is the same compound as the HC-toxin reported by Pope *et al.* (1983).

Isolation of chlamydocin

Chlamydocin from culture filtrates of *Diheterospora chlamydosporia* was detected by TLC with acetonitrile as a solvent, and by spraying plates with 4-(p-nitobenzyl)-pyridine. The compound also produced spots with bromcresol green. Spots were extracted from TLC absorbent with chloroform and subjected to HPLC. Chlamydocin was eluted at 23 min with 30% acetonitrile in water using a reverse phase column.

The preparation was identified as chlamydocin of Close and Hugenin (1974), by NMR and MS spectrometry (Fig. 3 and 4).

Preparation of CYL-2

The cyclic tetrapeptide called CYL-2 had been isolated from *Cylindrocladium scoporium* (Hirota *et al.*, 1973). However, cultures of that organism are restricted, since it is a plant pathogen. Therefore, I decided to see if a related fungus, *Cylindrocladium macrosporium*, also produced CYL-2 and if so, use that organism to obtain sufficient quantities of that cyclic tetrapeptide for biological testing.

An epoxy-containing compound from culture filtrates of *Cylindrocladium macrosporium* was detected by TLC when plates were sprayed with 4-(p-nitrobenzyl)-

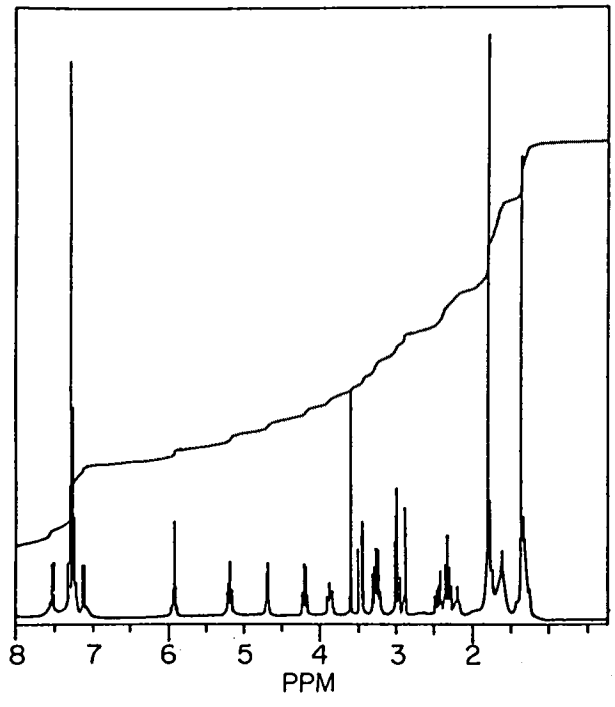

Fig. 3. NMR spectrum of Chlamydocin.

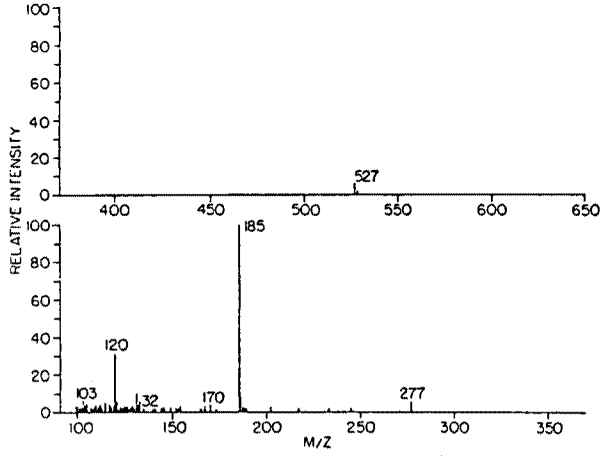

Fig. 4. Fast atom bombardment mass spectrum of Chlamydocin.

pyridine. When subjected to HPLC, the compound was eluted at 31 min with 42% acetonitrile in water as the solvent and a reverse phase column.

In the mass spectrum (Fig. 5) of this epoxy-containing compound using FAB, a protonated molecular ion peak $(M+H)^+$ was observed at m/z 599, which corresponds to a molecular formula of $C_{32}H_{46}N_4O_7$. The ions at m/z 621, 753 and 775 are due to $(M+Na)^+$, $(M+matrix+H)^+$, and $(M+matrix+Na)^+$, respectively. Signals at m/z 402, 291, 263 and 225 in the CAD mass spectrum of $(M+H)^+$ (Fig. 6) confirm that the structure of this compound is cyclo (me-Tyr-Ile-Pip-Aoe), which is equivalent to CYL-2, a cyclic tetrapeptide originally isolated from *Cylindrocladium scoparium*.

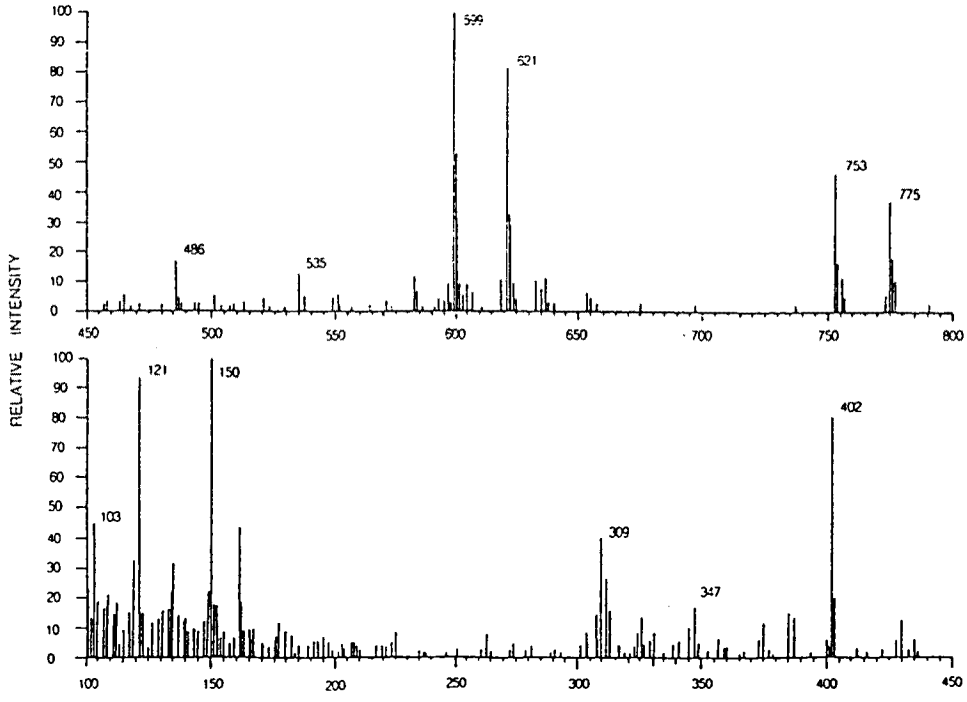

Fig. 5. Fast atom bombardment mass spectrum of CYL-2.

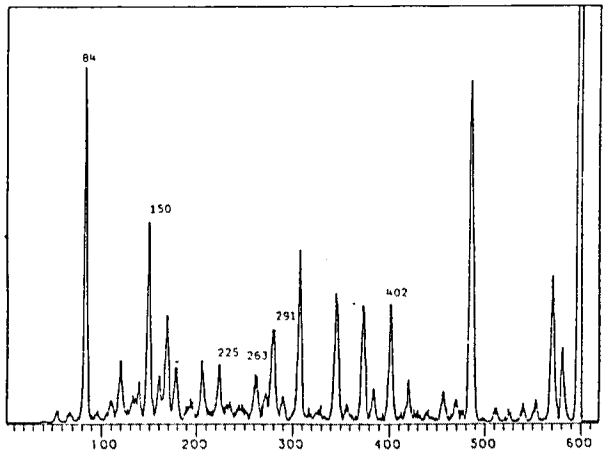

Fig. 6. Collision activated decomposition mass spectrum of the $(M+H)^+$ ion of CYL-2.

Table 1. Effects of the cyclic tetrapeptides, HC-toxin, Chlamydocin and CYL-2 on electrolyte leakage from maize seedlings susceptible and resistant to *Helminthosporium carbonum*

Treatment	Concentration (μg/ml)	Conductivity (μS) at 48 h		
		Susceptible genotype	Resistant genotype	S/R
HO control	–	1.8	1.8	–
0.5% DMSO control	–	0.1	3.0	–
HC-toxin	2	267	1.3	205
	5	319	1.6	200
Chlamydocin	2	233	80.3	2.9
	5	351	88.0	4.0
CYL-2	2	96.6	28.4	3.4
	5	179	88.8	2.0
	10	229	97.5	2.4

S/R: The ratio of the response of susceptible and resistant seedlings.

Biological activities

In addition to the Aoe residue, all of these cyclic tetrapeptides, HC-toxin, Chlamydocin and CYL-2 contain an imino acid moiety, either proline or pipecolic acid. Thus, chemical differences among these tetrapeptides reside largely in the nature and the sequence of the two remaining amino acid residues, which may have a significant effect on their biologial activities and specificities. Therefore, a comparison of biological activities in maize was thought to offer possible information about the structural features need for toxicity and specificity of HC-toxin.

The effects of HC-toxin, CYL-2 and chlamydocin on electrolyte leakage from maize seedlings were determined (Table 1). Chlamydocin caused as much, if not more, ion leakage from susceptible seedling, than HC-toxin. However, Chlamydocin was not nearly as specific in its action as HC-toxin, shown by the ratios (S/R) of responses for susceptible and resistant maize seedlings. CYL-2 was slightly less active than chlamydocin and HC-toxin. Specificity of CYL-2 was similar to Chlamydocin, not to HC-toxin.

HC-toxin, Chlamydocin and CYL-2 differentially inhibited root growth of two maize hybrids, susceptible and

Table 2. Comparisons of the effects of HC-toxin, Chlamydocin, and CYL-2 on primary root growth of susceptible and resistant maize

Treatment	Concentration causing 50% inhibition of root growth (μg/ml)		
	Susceptible hybrid	Resistant hybrid	R/S ratio
HC-toxin	0.25	50	200
Chlamydocin	0.81	2.52	3.1
CYL-2	1.12	2.34	2.1

resistant genotypes (Table 2). Chlamydocin and CYL-2 inhibited root growth both of susceptible and resistant maize hybrids, showing no specificity to the susceptible genotype. Chlamydocin and CYL-2 required about more than twice the concentration of HC-toxin for 50% inhibition of a susceptible maize genotype.

One or both of two structural differences must be responsible for the biological differences reported here between HC-toxin and the structurally related cyclic tetrapeptides. Whereas chlamydocin and CYL-2 contain the amino acid sequence, Pro-Aoe or Pip-Aoe, HC-toxin has the reverse sequence Aoe-Pro (Fig. 1). Chlamydocin and CYL-2 also contain an aromatic amino acid instead of the alanine occurring in HC-toxin, and are thus larger and more hydrophobic. Kim *et al.* (1985) isolated an analogue of HC-toxin that contained a glycine residue in place of the D-alanine residue of HC-toxin. The analogue was only about 1/35 as toxic as HC-toxin and the analog (a methyl group) causes a large difference in toxicity on susceptible maize genotypes.

While glycine containing HC-toxin, a more polar compound than HC-toxin, showed low biological activity, the more hydrophobic analogs, Chlamydocin and CYL-2, were active although non-specific. Apparently, the biological activities of these analogs depend upon their polarity, suggesting that membrane permeabilities may be a crucial factor in toxicity.

It has been reported that non-specific alkylation of bionucleophiles is involved in HC-toxin's mode of action (Kim, 1993). Certainly, an epoxyketone function in Aoe is critical for the toxicity of HC-toxin. A precise peptide ring structure apparently does not play an important role in toxicity as long as the molecule is permeable to membrane. On the other hand resistance of maize to HC-toxin requires a precise ring conformation.

The HC-toxin's mode of action on susceptible plants, and the molecular level of specificity of the toxin could be due to either the same mechanism or quite different ones. However, because non-specific alkylation of bionucleophiles is involved in the toxin's mode of action (Kim, 1993), the specificity must be explained by a different mechanism. Perhaps, there might be a toxin receptor in the resistant genotype, whose function could be to detoxify the toxin or to induce a repair system. This is one possible explanation of why resistance of maize to HC-toxin may require a precise peptide ring conformation.

Because epoxides are highly reactive electrophiles, and can readily form Covalent bonds with cellular protein and DNA, they are common ultimate toxins, mutagens and carcinogens. Epoxides can be metabolized by epoxide hydrolase yielding dihydrodiols, or by glutathione transferase catalyzing the nucleophilic attack of the sulfur anion of glutathione on the epoxide, leading to the corresponding conjugate. Because dihydrodiols are less reactive than their epoxide precursors, epoxide hydrolases protect organisms from alkylation. Epoxide hydrolase activities responding to pesticides have been found in extracts from several plant species (Earl and Kennedy, 1975). It is possible to speculate that the susceptibilities of maize to HC-toxin may be due to lack of this enzyme.

Consequently, many more studies are needed. For example, tetrapeptides with fewer differences in their amino acid residues compared to HC-toxin need to be obtained for biological testing. Chemical synthesis or isolation of other peptides from other microorganisms are possible sources of the necessary compounds.

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