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Chemical Constitution of the Host-Specific Toxin of *Helminthosporium carbonum*[1]

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ABSTRACT

The host-specific toxin of *Helminthosporium carbonum* Ullstrup has a molecular formula approximating $C_{32}H_{50}N_6O_{10}$. The compound has been crystallized and a crystalline hydrochloride derivative has been produced. The molecular weight, as determined by chromatography on Sephadex G-10, is slightly less than 700. The toxin appears to be a cyclic peptide, since, although it does not react with ninhydrin or dinitrofluorobenzene, it yields, on hydrolysis, compounds which react to these reagents. It is unstable in dilute acids, yielding ninhydrin-reacting products. Complete acid hydrolysis yields alanine, proline, and three other ninhydrin-reacting components. The infrared spectrum of the toxin reveals an ester band in addition to amide absorption. Its ultraviolet spectrum reveals the presence of unsaturation in the molecule. The toxin is relatively unstable and loses its specific toxicity. This loss of activity appears to be associated with loss of nitrogen and with decreased solubility in water.

The importance of host-specific toxins in the etiology of infectious plant diseases is becoming more evident as highly bred crops are being planted more widely (9, 16). Certain cultivars of cereals have been found to be highly susceptible to certain species of fungi that do not cause noticeable disease in nonselected populations of the crop. In each case that has been thoroughly investigated, it has been found that this abnormal susceptibility of the host was correlated with the production by the pathogen of a substance that is highly toxic to the susceptible plant and which is much less toxic to resistant plants. In these special cases, the toxic substances are true chemical determinants of pathogenicity. For convenience, they are generally referred to as host-specific toxins.

These compounds reproduce all the visible and all the known biochemical symptoms of the associated diseases. They are useful models for studying disease, since many complexities of the host-parasite interaction are avoided. Three examples of host-specific toxins have been studied intensively. They are the host-specific toxin of *Helminthosporium victoriae* Meehan and Murphy, which is toxic to susceptible oat cultivars but is harmless to resistant oat cultivars and to all other nonhost plants that have been tested (5, 6, 7, 14, 15). This fungus was responsible for the epiphytotic Victoria blight of oats which became serious and widespread in all oat-growing areas some years ago (4). A number of different host-specific toxins are produced by *Periconia circinata* (Mangin) Sacc., which are toxic to susceptible cultivars of grain sorghum (*Sorghum vulgare* Pers. var. subglabrescens [Steud.] A.F. Hill) (8, 10, 11). The most recent studies have been concerned with the host-specific toxin of *Helminthosporium carbonum* Ullstrup, which is toxic to susceptible inbred dent corn (*Zea mays* L.) (12, 13, 17).

Although each of the three host-specific toxins has been isolated and crystallized in milligram quantities, the difficulty of isolation and the great instability of the compounds have delayed chemical characterization. In 1958, HV[2] toxin was shown to be composed of a tricyclic secondary amine, victoxinine ($C_{17}H_{29}NO$), and a peptide of five amino acids: aspartic, glutamic, glycine, valine, and leucine. The PC toxins were also found to be peptides. One which was crystallized was found to contain a peptide of 6 moles of alanine, 4 moles of aspartic acid, and 2 moles each of glutamic acid and serine; at least to the extent of 80% of its molecule. Further work on these toxins has been delayed by the variability of toxin production. In 1967, HC toxin was purified and crystallized and was shown to have peptide characteristics. The purified toxin did not react with ninhydrin but was easily hydrolyzed to yield ninhydrin-reacting substances.

Although HC toxin does not exhibit the extreme specificity of HV or PC toxins (preparations that inhibit susceptible corn at 0.5 μg/ml also inhibit resistant corn at 50 μg/ml), it was produced in larger quantities, its production was consistent, and it appeared to be more stable than HV or PC toxins. For these reasons it seemed practical to start a detailed chemical characterization of HC toxin.

Although it was quickly discovered that HC toxin was not as stable as had been hoped, it was possible to obtain enough data to outline its chemical constitution, which has unusual features. This report also suggests chemical changes that may account for the instability of the toxin and for its loss of specific toxicity.

MATERIAL AND METHODS

Preparation of HC Toxin. Concentrated culture filtrates of high toxin-yielding strains of race 1 of *H. carbonum* Ullstrup were very kindly supplied by Dr. R. P. Scheffer, Department of Botany and Plant Pathology, Michigan State University. These had been produced as previously described (12, 17) and represented 50 times the concentration of the original culture filtrate. Isolation of the host-specific toxin was carried out by the methods previously described (12). A total of 2.17 g of material active at 0.5 μg/ml was produced. Most of the material used in this report was lyophilized after purification by countercurrent distribution. As

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[2] Abbreviations: HC: *H. carbonum* Ullstrup; HV: *H. victoriae* Meehan and Murphy; PC: *P. circinata* (Margin) Sacc.

will be explained later, this material was more stable and more easily dissolved than crystalline material.

Bioassay of Toxin Derivatives. Quantitative bioassays of specific toxin activity were carried out following the previously described procedure (12, 17) with use of White's salts solution to make the dilution of the preparations being tested.

Chemicals. Sephadex G-10, particle size 40 to 120 μ, Pharmacia, Uppsala, Sweden, was used for gel filtration studies. This preparation has a fractionation range for solutes of molecular weight up to 700. All solvents were freshly distilled before use.

Spectrometry. Infrared spectra were recorded from films deposited on NaCl windows from chloroform solutions. A Perkin-Elmer model 700 double beam infrared spectrophotometer was used. Ultraviolet spectra were recorded from absolute ethanol solutions in a Beckman model DK-1 spectrometer.

RESULTS

Stability of Toxin. When freshly crystallized from ethanol-ether, HC toxin appeared as fine colorless, fluffy needles which were freely soluble in water. On standing a few days at room temperature, this crystalline material darkened, softened, and collapsed to a yellow-brown sticky mass. In storage under reduced pressure at −20 C, HC toxin was less stable than HV toxin stored under the same conditions (9). The crystalline material became yellow in color, developed a sour odor, and became less soluble in water. Aqueous solutions of various preparations used as standards in the isolation work (12) and stored frozen at −20 C for periods of 3 years were found to have lost their specific toxicity, while the toxicity to susceptible corn had fallen to one-fourth of its original value. These solutions, which were clear when frozen, contained a great deal of flocculent, insoluble material when thawed 3 years later. Lyophilized material stored over a year at −20 C developed a yellow tinge and a slightly sour odor. It was not completely soluble in water, forming a milky suspension. A clear solution resulted when the suspension was acidified to pH 4.0. This material was still active at 0.5 μg/ml, which was the average activity of freshly prepared, crystalline, or lyophilized preparations. However, when aliquots were assayed from solutions and were freshly prepared by countercurrent distribution or gel filtration without preliminary lyophilization, values indicating activity at 0.2 or 0.1 μg/ml were obtained.

Elemental Analysis of Toxin. Freshly crystallized material was dried under 10 μ of Hg pressure at room temperature over P_2O_5 and paraffin shavings for 8 hr and immediately analyzed for C, H, and N (Dumas).

Found: C = 55.75%, 55.96%; H = 7.44%, 7.43%; N = 12.43%

The preparation was ash-free.

One hundred milligrams of the same crystalline material were dissolved in 10 ml of chloroform and were shaken with 10 ml of 0.1 N NaOH (which had been previously extracted with chloroform). The chloroform layer was extracted with water, and the water layer was discarded. The chloroform layer was evaporated to dryness under reduced pressure. The residue was taken up in water, lyophilized, and analyzed immediately.

$C_{32}H_{50}N_6O_{10}$
Found: C = 56.52%; H = 7.19%; N = 12.32%
Calculated: C = 56.61%; H = 7.44%; N = 12.38%

Molecular Weight of Toxin. Ten milligrams of lyophilized toxin, purified by countercurrent distribution, were dissolved in 0.25 ml of 10% (v/v) acetic acid and were applied to a 1.2- × 23-cm column of Sephadex G-10 that had been equilibrated with 10% acetic acid. One-half milliliter fractions of eluate were collected. One drop of effluent from every second tube was evaporated on a 7-cm circle of filter paper and was bioassayed as described previously (12). The void volume of the column as determined by Blue Dextran 2000, was 16.5 ml. The toxin first emerged in the fourth tube following the void volume (18.5 ml). The elution volume of the column was calculated to be 22.5 ml. The toxin appears to have a molecular weight less than 700, which is the exclusion limit of this gel. The calculated molecular weight for $C_{32}H_{50}N_6O_{10}$ is 679.

Table I. *Principal Absorption Maxima in the Infrared Spectrum of H. carbonum Toxin*

HC Toxin	HC Toxin Hydrochloride	Assignment
cm^{-1}	cm^{-1}	
3500 (shoulder)	3500 (shoulder)	Free —NH—
3320	3300	Bonded —NH—
2950	2950	—CH_3
1710	...	—C(=O)—NH_2
1650–1680 (broad)	1650–1690 (broad)	—C(=O)—NH—R
1525	1525	—C(=O)—NH—R
1440	1440	—CH_3
1375	1375	—CH_3
1230	1230	—O—C(=O)—CH_3
1045	1045	—CH_2—O—C(=O)—CH_3

Infrared Absorption Spectrum of Toxin. The infrared absorption spectrum from 3600 cm^{-1} to 800 cm^{-1} of freshly lyophilized toxin purified by countercurrent distribution revealed the major absorption maxima shown in Table I. Since the presence of ester absorption was unexpected, a number of crude preparations that had not been exposed to acetic acid were checked. Each showed strong absorption at 1230 cm^{-1} and 1045 cm^{-1}.

Ultraviolet Absorption Spectrum of Toxin. The ultraviolet absorption spectrum of freshly lyophilized toxin dissolved in freshly distilled absolute ethanol showed a single, well defined maximum at 230 nm. The molar absorptivity of 1.4×10^{-5} molar solution (assuming a molecular weight of 679) was approximately 5000.

Preparation of Toxin Hydrochloride. One hundred milligrams of toxin produced by countercurrent distribution and lyophilizing, which had been stored at −20 C for several months, were used to prepare the hydrochloride salt. On testing with 1 ml of water, the friable powder collapsed with release of air and formed

a fairly clear solution with a slightly milky appearance. On adding more water, the milkiness increased until at 5 ml a curdy precipitate began to separate. When 2 drops of N HCl were added, the precipitate dissolved leaving a light yellow solution, pH 4.0.

The volume of the solution was increased to 10 ml, and 10 ml of a freshly prepared, saturated solution of sodium bicarbonate were added. The mixture turned milky immediately, but no precipitate formed. This mixture was extracted six times with 10-ml portions of chloroform. Even after six extractions the aqueous layer remained milky, showing that the free base is much less easily extracted with chloroform despite its decreased water solubility.

The chloroform extracts containing the free base were washed with water, and the chloroform was removed by distillation under reduced pressure. The residue was treated with 4 drops of N HCl followed by 10 ml of water. The resulting colorless solution was lyophilized, yielding 92 mg of white powder.

The hydrochloride was crystallized by dissolving in 1 ml of absolute ethanol at 35 C. Diethyl ether was added in small portions to the first sign of cloudiness. On cooling in an ice bath, the toxin hydrochloride crystallized in small colorless needles. A few crystals were dissolved in a drop of water which had been acidified with nitric acid. The addition of a drop of 0.1 N silver nitrate solution caused a typical precipitate of silver chloride, indicating that the crystals contained the chloride ion.

A sample was dried at 10 μ of Hg pressure over P_2O_5 and paraffin chips for 8 hr at room temperature and immediately analyzed.

Found: C = 55.94%; H = 7.08%; N = 10.37% and 10.44%; Cl^- = 4.98%

$C_{32}H_{50}N_6O_{10}HCl$

Calculated: C = 53.73%; H = 7.20%; N = 11.75%; Cl^- = 4.96%

$C_{32}H_{48}N_5O_{10}HCl$

Calculated: C = 54.96%; H = 7.08%; N = 10.02%; Cl^- = 5.07%

The toxin hydrochloride did not react with ninhydrin by the Stein and Moore procedure or on paper chromatograms in the dark at room temperature. After heating for 30 min at 105 C, a faint gray spot could be seen on paper chromatograms treated with ninhydrin. The hydrochloride had R_F values similar to the parent toxin in various paper chromatography systems (12). The ultraviolet absorption spectrum was also similar to that of the parent toxin with a well defined maximum at 230 nm, ϵ_{max} = 5000 (assuming a molecular weight of 699). The assignment of the major peaks in the infrared absorption spectrum is shown in Table I. With the exception of the absence of a peak of 1710 cm^{-1} (unsubstituted amide), the spectrum was similar to that of the parent toxin.

For bioassay, 5.0 mg of toxin hydrochloride were treated with 0.01 ml of glacial acetic acid, and the solution was made up to a final volume of 50 ml with White's salts solution. A serial dilution of this solution was assayed against susceptible and resistant corn and the results were noted after 48 hr. Half-maximal inhibition of growth of roots of susceptible corn was produced at 25 μg/ml and the same degree of inhibition of roots of resistant corn occurred at 50 μg/ml.

Action of Dilute Acid on HC Toxin Hydrochloride. Three milligrams of recrystallized toxin hydrochloride were dissolved in 10.0 ml of 0.1 N HCl and refluxed for 3 hr. Samples (1.0 ml) equivalent to 300 μg were taken every hour and concentrated to dryness under reduced pressure at 35 C. The residues were taken up in 0.1 ml of 10% isopropanol and chromatographed (descending) in butanol-acetic acid-water (12:3:5).

The chromatograms were developed by double dipping through a solution of 0.2% ninhydrin in acetone containing 2% pyridine (w/v/v). Color development was allowed to proceed in the dark at room temperature.

The toxin hydrochloride, which did not react with ninhydrin, was broken down to show two ninhydrin-reacting spots after a 1-hr treatment with 0.1 N HCl. One spot (R_F = 0.38) corresponded to alanine. The other spot (R_F = 0.70), presumably a peptide, was much fainter in the 2-hr sample and had disappeared by 3-hr refluxing. A new spot (R_F = 0.53) appeared after 2-hr refluxing and became fainter by 3 hr. After 3-hr refluxing at least seven spots appeared after treatment with ninhydrin.

Action of Concentrated Hydrochloric Acid on HC Toxin. Ten milligrams of crystalline HC toxin hydrochloride were dissolved in 1.0 ml of ice cold concentrated hydrochloric acid and the solution was held at 38 C for 5 hr. The hydrochloric acid was then removed by placing a vacuum desiccator over KOH and evacuating continuously for 2 hr. The residue was left overnight in the desiccator and then dissolved in 10 ml of ice water to form a light brown opalescent solution. This solution was extracted three times with an equal volume of chloroform to remove 4.8 mg of unreacted material. The aqueous layer was concentrated under reduced pressure, dissolved in 0.2 ml of 10% isopropanol, and chromatographed. A number of poorly resolved ninhydrin-reacting products were revealed in the region R_F = 0.50 to R_F = 0.80 in the descending butanol system.

The remainder of the product was dissolved in 5.0 ml of 10% (w/v) freshly prepared $NaHCO_3$ solution. Three hundred milligrams of 2,4-dinitrofluorobenzene dissolved in 10 ml of ethanol were added, and the material was shaken in the dark for 3 hr. The mixture was then acidified with concentrated HCl and extracted three times with an equal volume of ether to remove dinitrophenol, and the dinitrophenyl peptides were extracted with butanol. Thin layer chromatography on silica gel with chloroform-methanol (7:3) revealed a number of poorly resolved yellow spots.

The concentrated hydrochloric acid treatment was repeated with a sample of HC toxin freshly prepared by countercurrent distribution. Although a great number of substances which reacted with ninhydrin and dinitrofluorobenzene were formed, no single substance could readily be separated from the mixture.

Complete Acid Hydrolysis of HC Toxin. Five milligrams of recrystallized HC toxin hydrochloride were weighed directly into a 1-ml glass ampule and were dissolved in 0.6 ml of constant-boiling hydrochloric acid. After nitrogen was bubbled through the solution for 15 min, the solution was frozen in a Dry Ice-ethanol bath, evacuated to 50 μ of Hg pressure, and the neck of the ampule was sealed with an oxygen-gas flame. The sealed, evacuated tube was heated at 110 $\pm$ 1 C for 22 hr. After hydrolysis, the solution had darkened to a relatively bright red-brown color. It was dried under reduced pressure over KOH to remove HCl, and the residue was dissolved in 0.2 ml of isopropanol for paper chromatography.

At least five distinct ninhydrin-reacting substances were formed, as shown in Table II. Alanine and proline were confirmed by standard solutions chromatographed with the unknowns. In addition, proline was confirmed by the yellow color it gave with ninhydrin and by the characteristic blue color with isatin.

The other three spots gave relatively fainter purple spots with ninhydrin, but still reacted at room temperature in less than 2 hr. They did not react with *p*-nitrobenzoyl chloride reagent.

In two-dimensional paper chromatography spots 3, 4, and 5 appeared at locations different from amino acids or other common ninhydrin-reacting substances. Spot 3 gave more color when pyridine was incorporated in the developing solution. The intensity of color produced by the other spots was not affected by pyridine. No spots of other acids or bases were detected when a chromatogram of the hydrolysate was dipped through a solution of bromocresol green in acetone.

The complete hydrolysis was repeated with a sample of HC toxin freshly prepared by countercurrent distribution. The same pattern of fragments as above was obtained with no evidence for any additional degradation products. Five milligrams of toxin

Table II. *Products of Complete Acid Hydrolysis of H. carbonum Toxin*

Spot	R_F		Assignment
	Butanol-acetic acid-water (12:3:5)	Liquified phenol-NH_4OH (200:1)	
1	0.30	0.58	Alanine
2	0.34	0.90	Proline
3	0.45	0.46	Unknown
4	0.58	0.52	Unknown
5	0.21	0.25	Unknown

hydrochloride hydrolyzed in the same manner produced a similar pattern of products.

Additional Attempts to Obtain a High Yield of a Single Degradation Product of HC Toxin. Five milligrams of HC toxin prepared by countercurrent distribution were dissolved in 4.5 ml of freshly redistilled absolute ethanol. The solution was chilled in an ice bath and 0.5 ml of concentrated hydrochloric acid was added by the drop into the solution. The solution was brought to room temperature and was allowed to stand for 24 hr. After this time, paper chromatography revealed the material to be largely unchanged but with a streak of ninhydrin-reacting material from $R_F = 0.70$ to $R_F = 0.80$.

Five milligrams of recrystallized HC toxin hydrochloride were dissolved in 10 ml of glacial acetic acid made 0.1 N with respect to hydrochloric acid. This solution was refluxed for 10 min and then cooled quickly and concentrated to dryness under reduced pressure at 30 C. On paper chromatography no original material remained, and, although a number of ninhydrin-reacting products were formed, judging by the streak produced on paper chromatography, the strongest reaction corresponded to a spot of $R_F = 0.70$. This appeared to be very similar to the initial peptide spot produced by refluxing with aqueous 0.1 N HCl (see above).

DISCUSSION

Because of the difficulties of production and isolation, together with the inherent instability of the purified material, it has been impossible to obtain a precise molecular formula for any host-specific toxin. Although it is evident that the host-specific toxin of *H. carbonum* is a complicated chemical substance and that much more work must be done on it, some insight into its structure is possible from the present data.

Elemental analysis of the crystalline toxin and its hydrochloride derivative suggests an empirical formula in the neighborhood of $C_{32}H_{50}N_6O_{10}$. This gives a molecular weight of 679, consistent with the molecular weight determined by molecular sieving. At the present stage of the work, it is not possible to be very precise about this. The exact empirical formula will become evident in the light of structural evidence from degradative experiments. Carbon analyses of the toxin suggest that C_{32} may be a high value, while similar analyses of the hydrochloride suggest that C_{32} may be low.

The value of six nitrogen atoms is consistent with the finding of five different ninhydrin-reacting compounds formed on hydrolysis, plus one additional labile nitrogen, possibly in the form of an unsubstituted amide. The sixth nitrogen appears to be lost when the hydrochloride derivative is produced. This is seen in the difference in infrared spectra and by the lower nitrogen content of the hydrochloride. It does not appear that simple hydrolysis of an amide to form a carboxylic acid is sufficient explanation for this change.

The value of 10 oxygen atoms is calculated by difference. This implies an excess of 4 oxygen atoms over nitrogen, which is unusual for a peptide. The finding of ester absorption in the infrared region of the spectrum suggests that these extra oxygen atoms are present in two ester linkages. The most plausible assignment seems to be acetates of primary alcohols. The methyl absorptions at 1440 cm^{-1} and 1375 cm^{-1} are consistent with acetate. This acetate absorption must be an integral part of the toxin molecule since it appears in all preparations tested, regardless of whether they had been exposed to acetic acid or not.

The absorption of light in the ultraviolet region is also unusual for a peptide. The position of the maximum (230 nm) and its relatively high intensity suggests a double bond in the vicinity of a carbonyl group, possibly an α,β-unsaturated amide. Such dehydroamino acid units are appearing with increasing frequency in microbial peptides (2).

The loss of biological activity of HC toxin may be due to polymerization, since insoluble material seems to be formed as the material loses activity. This implies one or more chemically reactive sites in the molecule. An α,β-unsaturated double bond would be such a reactive site.

Since the toxin does not react with ninhydrin or 2,4-dinitrofluorobenzene, no free amino groups can be present. These must be acylated or the compound must be cyclic. The latter suggestion is favored, since the compound is basic to bromcresol green, therefore lacking a free acid group. The solubility in chloroform is consistent with the molecule being a cyclic peptide.

Two of the constituent amino acids can be identified with certainty as proline and alanine. This is the first time proline has been found in a host-specific toxin, although this amino acid is found in many peptide antibiotics (1). It is unlikely that any of the other three ninhydrin-reacting degradation products is a common amino acid, since they all fall in areas on the conventional two-dimensional paper chromatogram which are blank when common amino acids are chromatographed. Each yields considerably less color than alanine or proline with ninhydrin on paper chromatograms of hydrolysates. However, since all ninhydrin reactions take place at room temperature in the dark, the color must be due to amino groups on aliphatic carbons. None of these appear to be methylamino groups since none of the compounds react on paper with *p*-nitrobenzoyl chloride.

The relative instability of the toxin to dilute hydrochloric acid also indicates a reactive site or point of weakness in the molecule. Although this reaction is not quantitative and progresses through many side reactions, it seems that the ring of the cyclic peptide is first broken to yield alanine and a peptide with a free amino group which reacts with ninhydrin. One possible source of this reactivity could be an α,β-unsaturated amino acid such as those found recently in nisin, subtilin, and several other peptide antibiotics (3). The finding of absorption in the ultraviolet region at 230 nm supports this hypothesis. There is also some indication of absorption in the region around 1700 cm^{-1} in the infrared, but this is largely masked by amide I absorption.

This appreciation of the chemical nature of *H. carbonum* toxin should provide a rational basis for studying physiological mechanisms responsible for the selective toxicity of host-specific toxins.

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