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Amino Acid Composition of the Host-specific Toxin of *Helminthosporium carbonum*[1]

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ABSTRACT

The host-specific toxin of *Helminthosporium carbonum* ($C_{32}H_{50}N_6O_{10}$) was hydrolyzed by 6 N HCl to yield a number of α-amino acids. The common amino acids, proline and alanine, occurred in a ratio of 1:2. Two other unstable α-amino acids that produced lower color values with ninhydrin were also produced. One of these was tentatively identified as 2-amino-2,3-dehydro-3-methylpentanoic acid by electrolytic reduction to isoleucine. Additional ninhydrin-reacting substances were produced in low yield and probably represented secondary hydrolysis products of the unstable amino acids. The finding of an α,β-unsaturated linkage in *H. carbonum* toxin explains the instability of the compound and may also account for its specific toxicity.

The vulnerability of cereals to new diseases mediated by host-specific toxins is an alarming prospect for world food supply. The southern corn leaf blight which appeared suddenly in 1970 in the major corn-producing regions of North America is the latest example of this type of disaster. A new race of *Helminthosporium maydis* Nisikado and Miyake that is highly virulent for corn bearing the Texas or T-type cytoplasm for male sterility was responsible for 15% reduction in crop yield (21). Circumstantial evidence indicates that a host-specific toxin which damages only corn plants bearing T cytoplasm is responsible for the invasion and development of the fungus in susceptible corn. Some preliminary experiments have demonstrated the presence of this host-specific toxin (2).

The new corn blight appears to be analogous to other diseases mediated by host-specific toxins (11, 18). Three examples of host-specific toxins have been studied intensively. These include 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 non-host plants that have been tested (4, 5, 8, 9, 16, 17). This fungus was responsible for the epiphytotic Victoria blight of oats which became serious and widespread in all oat-growing areas some years ago (44). 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) (10, 12, 13). 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.) (3, 6, 7, 14, 15, 19).

Although each of these host-specific toxins has been isolated and crystallized in milligram quantities, because of the difficulty of isolation and their great instability only HC[2] toxin has been characterized to any extent (5, 6, 7). It was shown to have a molecular formula approximating $C_{32}H_{50}N_6O_{10}$ by analysis of crystalline material and by analysis of a crystalline hydrochloride derivative. The molecular weight was determined by bioassay of the eluate of a Sephadex G-10 chromatography column and shown to be slightly less than 700. The toxin appeared to be a cyclic peptide, because, although it did not react with ninhydrin or fluorodinitrobenzene, after acid hydrolysis it yielded compounds which reacted to these reagents. The ultraviolet absorption spectrum of freshly lyophilized toxin dissolved in freshly distilled ethanol showed a single, well defined maximum at 230 nm. The molar absorptivity of a 1.4×10^{-5} M solution (assuming a molecular weight of 679) was approximately 5000. The position of the maximum and its relatively high intensity suggested a double bond in the vicinity of a carbonyl group, possibly an α,β-unsaturated amide. Because the presence of such a function in HC toxin would explain the relative instability of the molecule, further work was done in characterizing the ninhydrin-reacting products of acid hydrolysis.

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, Michigan State University. Isolation and purification of the host-specific toxin was carried out by the methods described previously (6, 14).

Hydrolysis of HC Toxin. Complete acid hydrolysis was carried out by the method described previously (6). Partial acid hydrolysis was performed as follows. One hundred milligrams of freshly prepared HC toxin were dissolved in 5.0 ml of freshly boiled and cooled glass-distilled water to form a clear colorless solution. A stream of nitrogen was bubbled through this solution for a few minutes to remove traces of dissolved oxygen, and the solution was frozen in a Dry Ice-ethanol bath. Five milliliters of concentrated HCl were added to the frozen solution, which was then allowed to warm gradually to room temperature in an atmosphere of nitrogen. The resulting colorless solution was then refluxed in an atmosphere of nitrogen for 4 hr. After cooling, the solution, which had turned brown, was diluted to 50 ml with water and extracted 3 times with 50 ml of chloroform to remove unchanged toxin. The extracted

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[2] Abbreviation: HC: *H. carbonum* Ullstrup.

aqueous solution was concentrated under reduced pressure to remove HCl and chloroform and then lyophilized.

Amino Acid Analyses. A Beckman amino acid analyzer, model 120C, equipped with a Beckman integrator, model 125, was used. Amino acids used as standards were N.R.C. grade, supplied by Calbiochem.

Electrolytic Reduction. A Research Specialties Co. electrolytic desalter, model 1930C, was used. The material to be reduced was dissolved in 10.0 ml of 0.1 N HCl to carry the current and placed in the cathode chamber of the apparatus. A current of 0.6 amp was passed through the solution until a current drop to 0.2 amp was obtained. This generally took about 10 min.

Paper Chromatography. One-dimensional descending chromatography and two-dimensional ascending paper chromatography were carried out in the solvent systems described below. Specific color tests on the paper chromatograms were carried out according to procedures described by Smith (20).

RESULTS

Amino Acid Analysis of Complete Acid Hydrolysate of HC Toxin. The five different ninhydrin-reacting products, previously found on paper chromatograms (7), could be recognized as distinct peaks on the recording from the amino acid analyzer, but only two of these, proline and alanine, could be identified with known amino acids. Analyses of different preparations, as shown in Table I, indicated that the molar ratio of proline to alanine was 1:2.

The three unknown peaks emerged from the column of the amino acid analyzer in the range from methionine to norleucine unaffected by different buffer elution programs or column temperatures. The first of the unknown peaks (compound no. 5) to emerge from the column came near the methionine position. However, neither the intact toxin nor the hydrolysis mixture gave positive tests for sulfur. An elemental microanalysis performed on 10 mg of HC toxin yielded no detectable sulfur.

The second unknown peak (compound 3) and the third unknown peak (compound 4) emerged in the vicinity of the leucines. However, by adding standard amino acids to the hydrolysis mixture it was found that neither of the unknown peaks corresponded to alloisoleucine, isoleucine, leucine, or norleucine.

When calculated on the basis of color yield with ninhydrin in the range of the common amino acids (leucine assumed to yield 100%), none of these unknown peaks reached a height approaching 0.1 molar equivalent to the amount of proline present in the hydrolysis mixture.

A very small amount of ammonia and glycine was found consistently in each of the samples tested, but in each case it was less than 0.01 mole compared to the amount of proline present. A small amount of material was also found which emerged in the region after ammonia but before the point where arginine would be expected. This peak was found to be coincident with that produced by α-aminoguanidinoacetic acid. However, neither the intact toxin nor the hydrolysis mixture gave a positive Sakaguchi test. Paper chromatograms tested for mono-, di-, or trisubstituted guanidines by Sakaguchi reagent (20), pentocyanoaquoferriate reagent (20), diacetyl reagent (20), or Jaffe reagent (20) gave completely negative results.

Partial Acid Hydrolysis of HC Toxin. The conditions described above for partial hydrolysis were determined to obtain an optimum yield of the two unknown amino acids (compounds no. 3 and no. 4) as indicated by quantitative amino acid analyses. Although yields of compounds 3 and 4 were appreciably higher than those from complete acid hydrolysis, they still did not approach equimolar equivalents to proline as judged by relative peak heights. Ammonia, glycine, and compound 5, which are apparently secondary products of hydrolysis, were found in much lower yields in the partial hydrolysates. Proline and alanine were completely released from peptide bonding by this mild treatment.

Table I. *Proline and Alanine Content of HC Toxin*

Weight of Sample	Proline	Proline Residues	Theoretical Proline Content (Mol. Wt. = 679)	Alanine	Alanine Residues	Theoretical Alanine Content (Mol. Wt. = 679)	Proline: Alanine Found
mg	*μmole*	*%*		*μmole*	*%*		*ratio*
0.70	0.963	13.3	14.3%	1.790	18.2	20.9%	1.00:1.86
0.26	0.324	12.1		0.638	17.4		1.00:1.97

The two unknown compounds (compounds 3 and 4) could be clearly separated from each other and from proline and alanine by two-dimensional paper chromatography, as shown in Table II.

Reaction of Products of Hydrolysis with Copper Ion. Each of the ninhydrin-reacting products of acid hydrolysis was shown to be an α-amino acid by its ability to chelate Cu^{2+}. A small amount of powdered basic cupric carbonate was mixed with a solution of the hydrolysis mixture and submitted to two-dimensional paper chromatography. The normal blue color produced by ninhydrin was not seen when these chromatograms were tested.

Reactions of Unknown Amino Acids. The following experiments were carried out to determine if one of these unknown amino acids (compounds 3 or 4) represented the α,β-unsaturated amino acid postulated from ultraviolet absorption studies of HC toxin (7).

Reaction with Hydrogen Sulfide (1). A solution of 8 mg of the partial hydrolysate of HC toxin in 0.2 ml of 10% isopropanol in water was divided into two equal parts. Hydrogen sulfide was bubbled slowly through one part for 30 min, and samples of each solution were compared by descending paper chromatography in solvent A. Spot 4 (R_F 0.58) seen in the original hydrolysate was missing from the chromatogram of the material treated with H_2S and was replaced by a new ninhydrin-reacting spot (R_F 0.39). This new spot gave positive tests for a sulfhydryl function when tested with nitroprusside reagent (20), iodine-azide reagent (20), and iodo-platinate reagent (20). Chromatograms of the original hydrolysate on the

Table II. *Paper Chromatography of Partial Acid Hydrolysate of HC Toxin*

Compound	R_F				Assignment
	Solvent system[1]				
	A	B	C	D	
1	0.30	0.34	0.52	0.58	Alanine
2	0.34	0.41	0.82	0.90	Proline
3	0.45	0.62	0.83	0.46	Unknown amino acid
4	0.58	0.58	0.71	0.52	2-Amino-2,3-dehydro-3-methylpentanoic acid

[1] Solvent systems: A: 1-butanol-acetic acid-water (12:3:5); B: 1-butanol-pyridine-water (1:1:1); C: liquefied phenol; D: liquefied phenol-NH_4OH (200:1).

same sheet of paper were negative to these last three reagents. The conversion of compound 4 to a sulfhydryl compound by H_2S was confirmed by two-dimensional chromatography (solvent A followed by solvent D, see Table II) testing with these three reagents.

Reaction with Hydrogen Peroxide (1). A solution of 8 mg of the partial hydrolysate of HC toxin in 0.2 ml of 10% isopropanol in water was divided into two equal parts. One part was placed in a 50-ml round-bottomed flask, cooled in an ice bath, and treated with 1.0 ml of 30% H_2O_2. The temperature of the mixture was allowed to rise to room temperature. After 15 min at room temperature, the reaction mixture was diluted with 5 ml of water, and the solution was concentrated to dryness under reduced pressure at 30 C. The residue was dissolved in 0.1 ml of 10% isopropanol, and the solution was compared with the untreated hydrolysate by descending paper chromatography in solvent A. Spot 4 (R_F 0.58), seen in the original hydrolysate, was missing from the chromatogram of the material treated with H_2O_2, indicating cleavage at an activated double bond. The remaining ninhydrin spots appeared in their usual locations, and no new ninhydrin-reacting spots were seen.

Electrolytic Reduction. A solution of 8 mg of the partial hydrolysate of HC toxin in 0.2 ml of 10% isopropanol in water was divided into two equal parts. One part was mixed with 5.0 ml of 0.1 N HCl and reduced as described above. The reduced solution was concentrated to dryness under reduced pressure, and the residue was dissolved in 0.1 ml of 10% isopropanol. This solution was compared with the untreated hydrolysate by descending paper chromatography in solvent A. Spot 4 (R_F 0.58) seen in the original hydrolysate was missing from the chromatogram of the reduced material and was replaced by a new, strongly ninhydrin-positive spot (R_F 0.62). This new spot migrated at the same rate as leucine or isoleucine when these amino acids were run on the same chromatogram.

Identification of the New Amino Acid Produced by Reduction. A solution of 10 mg of the partial hydrolysate of HC toxin in 5.0 ml of 0.1 N HCl was reduced electrolytically as described above. The reduced solution was concentrated to dryness under reduced pressure, and the residue (8.98 mg) was dissolved in 5.0 ml of buffer. An aliquot of 100 μl was analyzed by the amino acid analyzer. The maximum corresponding to compound 4 was absent and was replaced by a maximum which emerged slightly earlier from the column, forming a double peak with compound 3. When a second 100-μl aliquot was mixed with 100 μl of a leucine solution containing 50 nmoles and analyzed, it was found that leucine displayed a separate maximum, emerging later than the new unknown peak. When a third 100-μl aliquot was mixed with 100 μl of an isoleucine solution containing 50 nmoles and analyzed, it was found that isoleucine emerged coincidently with the new unknown peak.

Identification of Isoleucine in the Acid Hydrolysate of Reduced HC Toxin. A solution of 10 mg of freshly isolated biologically active HC toxin was dissolved in 5.0 ml of 0.1 N HCl and reduced electrolytically as described above. The reduced solution was extracted three times with an equal quantity of chloroform. The residue after evaporation of the chloroform extracts was dried at 10 μm over P_2O_5, and an aliquot of 3.65 mg was hydrolyzed with 6 N HCl for 24 hr at 110 C under the conditions described previously (6). After removal of HCl in a vacuum desiccator, the hydrolysate was dissolved in 5.0 ml of buffer for amino acid analyses. Aliquots of 100 μl of this solution were analyzed alone and with added leucine, isoleucine, and alloisoleucine as described above. The presence of isoleucine in the hydrolysate of reduced toxin was confirmed by the finding that a compound of the hydrolysate emerged with added isoleucine and not with leucine or alloisoleucine. The yield of isoleucine was not quantitative under these conditions. Most remained in a peptide which was particularly resistant to acid hydrolysis. This peptide emerged from the column after the amino acids and is possibly isoleucylisoleucine.

DISCUSSION

The presence of a chemically active double bond in the molecule of HC toxin has been demonstrated by finding an α,β-unsaturated amino acid among the products of acid hydrolysis of the toxin. The location of the double bond in this hydrolysis product has been confirmed by having the compound react with H_2S to form a sulfhydryl derivative and by finding that the ninhydrin reaction of the compound was destroyed by H_2O_2. Both these reactions took place with high yield under mild conditions. The skeletal structure of the compound was revealed by converting it to isoleucine through hydrogenation.

Because of the difficulties of production and isolation, together with the inherent instability of the purified material, it has been difficult to obtain precise information on the chemical nature of host-specific toxins. The identification of the very unstable α,β-unsaturated amide residue, 2-amino-2,3-dehydro-3-methylpentanoic acid (dehydroisoleucine), in the structure of the host-specific toxin of *H. carbonum,* reveals one reason for these difficulties, at least in the case of this particular toxin. In aqueous solution, or perhaps even in the presence of water of crystallization, the activated double bond may equilibrate with its hydrated form, an α-hydroxy amide, which can dissociate, opening the chain of the cyclic molecule. If the reverse reaction occurs intermolecularly rather than intramolecularly, dimers and higher polymers will be formed This would explain the decrease in solubility and biological activity which occurred rapidly in solution and the finding that lyophilized toxin was more stable than crystallized toxin (6).

Preliminary results from experiments which will be described in a later publication indicate that reduction destroys the specific activity of HC toxin. This indicates that the chemically reactive double bond also plays a role in the biological activity of the compound. The toxin molecule must have the *erythro*-configuration about this double bond, because isoleucine rather than alloisoleucine was produced after hydrogenation. This may have some importance in determining the biological specificity of the toxin.

The presence of 2 moles of alanine, 1 mole of proline, and, presumably, 1 mole of 2-amino-2,3-dehydro-3-methylpentanoic acid in the toxin molecule accounts for $C_{17}H_{26}N_4O_4$ of the presumed empirical formula $C_{32}H_{50}N_6O_{10}$ for HC toxin. The residual $C_{15}H_{24}N_2O_6$ must include the remaining amino acid residue (compound 3) and the ester residues indicated by infrared spectroscopy (7). This would indicate that the unknown residue, compound 3, is a hydroxyamino acid. It is an α-amino acid because it forms a chelate compound with Cu^{2+}. If 2 moles of an acetylated hydroxyamino acid are assumed, the empirical formula would be satisfied and the hydroxyamino acid would be about the size of hydroxyisoleucine. This, of course, is only speculation at this stage. Such a residue might be formed by the equilibration with water of the activated double bond of a dehydroisoleucine (2-amino-2,3-dehydro-3-methylpentanoic acid) residue in the toxin. The other ninhydrin-reacting substances, ammonia, glycine, and unknown products, found in low yield in hydrolysates of HC toxin are probably secondary products formed by the break-

down of the unstable unsaturated and hydroxyamino acids. Because of these problems associated with the unstability of the molecule, it appears that a positive identification of compound 3 and further characterization of the HC toxin can be more easily achieved by a study of a stabilized derivative of the toxin such as the compound produced by hydrogenation.

Although it would be premature to attempt to present a tentative structural formula for HC toxin, its basic outline is beginning to take form. It now appears that the toxin is, fundamentally, a cyclical molecule of proline, alanine, and dehydroisoleucine residues. The order in which these fragments are joined remains to be determined, and the question of the presence of other still unrecognized fragments is not yet completely resolved.

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