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New Mycotoxin, Trichotoxin A, from *Trichoderma viride* Isolated from Southern Leaf Blight-Infected Corn

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A new mycotoxin, trichotoxin A, was found in a solvent extract of the mycelium of *Trichoderma viride* isolated from corn infected with southern leaf blight. Trichotoxin A is a cyclic peptide with the following amino acid composition: $(gluN)_2$, $(glu)_1$, $(pro)_2$, $(gly)_1$, $(ala)_3$, $(leu)_3$, (2-methyl alanine)$_1$.

During 1970, field corn produced in southern and midwestern United States was heavily infected with *Helminthosporium maydis* Nisikado and Miyake race T, the causative agent of southern corn leaf blight. The main secondary invaders of the blighted corn were *Trichoderma*, *Fusarium*, and *Alternaria* (J. J. Ellis, *personal communication*). Several toxic compounds have been isolated from *Trichoderma viride* Pers. ex Fr., such as gliotoxin (6), frequentin (3), and viridin (1).

We have isolated and purified a new mycotoxin from *T. viride* NRRL 5242. For convenience, we assigned the trivial name, trichotoxin A, to this new toxin. Another new mycotoxin, trichotoxin B, similar in chemical properties to trichotoxin A, was isolated from *T. viride* NRRL 5243; however, the yield was insufficient to determine its characteristics. This paper describes the isolation, purification, and some properties of trichotoxin A.

The *T. viride* strains used in this investigation were isolated from various ears of Texas (T) male-sterile corn; this corn was also heavily infected with *H. maydis* race T. Ten different isolates of *T. viride* grown on sterile corn for 3 weeks all produced trichotoxin A. Further studies of trichotoxin A production were conducted by growing these strains of *Trichoderma* in Fernbach flasks each containing 500 ml of Raulin-Thom medium and incubated statically at 25 C for 14 to 16 days. Of these, *T. viride* NRRL 5242 was the best trichotoxin A producer.

A chloroform-methanol (1:1, v/v) extract of the mycelium was evaporated to dryness, and petroleum ether was added to the residual solid to remove nonpolar compounds. After the residual solid was extracted with methanol, the methanol-soluble fraction was evaporated to dryness. The residue of methanol-soluble fraction from 10 Fernbach flasks was suspended in chloroform and applied to a Florisil column (2 by 40 cm) presaturated with chloroform. In addition, the column was washed with 300 ml of chloroform, and then trichotoxin A was eluted with 600 ml of chloroform-methanol (2:1, v/v). The crude trichotoxin A fraction was evaporated to dryness. The residue was dissolved in 5 ml of methanol and applied to a Sephadex LH_{20} column (2.4 by 200 cm) presaturated with methanol. The column was eluted with methanol and 5-ml fractions were collected. Trichotoxin A was collected in the tubes between 58 and 66 and these fractions were evaporated to a small volume. White trichotoxin A crystals were obtained by slow addition of an equal volume of diethyl ether to the trichotoxin A concentrate. From 10 Fernbach flasks, 450 mg of pure trichotoxin A (Fig. 1) was obtained.

Trichotoxin A decomposes at 187 C. Its infrared (IR) spectrum (Fig. 2) suggests the presence of an amide. A negative anthrone test denotes no carbohydrate in the molecule. Trichotoxin A exhibits a single pK_a value at 4.8 which, together with the fact that it does not react with ninhydrin, indicates that it is a cyclic peptide. The substance has only one ultraviolet absorption (191 nm) and contains no sulfur.

Analysis of a hydrolysate of trichotoxin A in a Phoenix automatic amino acid analyzer

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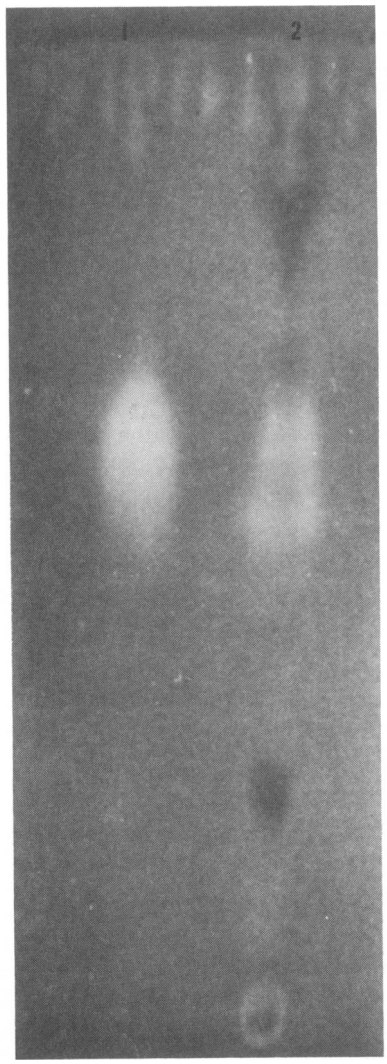

FIG. 1. *Thin-layer chromatogram of trichotoxin A. Precoated plastic sheets of Silica Gel N-HR (0.2 mm, Macherey-Nagel) were used, and chloroform-methanol-water (65:20:2, v/v/v) was the developing solvent. Trichotoxin A was located on the dried chromatogram by spraying with anisaldehyde-sulfuric acid reagent (8) followed by heating the plate to 100 to 110 C for 5 min. 1, Trichotoxin A; 2, chloroform-methanol extract of mycelia of Trichoderma viride NRRL 5242.*

model K1000 demonstrated the following composition: $(glu)_3$, $(pro)_2$, $(gly)_1$, $(ala)_3$, $(leu)_3$, and an unknown amino acid that emerged at the position ordinarily occupied by cysteine. Free amide nitrogen analysis indicated that two of the glutamic acid residues were present in the molecule as glutamine.

Sufficient material (2.0 g) was hydrolyzed to

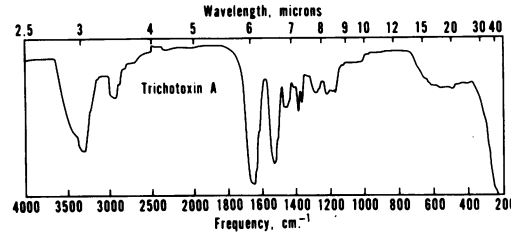

FIG. 2. *Infrared spectrum of trichotoxin A. Trichotoxin A at 2 mg/2 g of KBr was studied with a Perkin-Elmer model 621 infrared spectrophotometer.*

permit isolation of the individual amino acids in larger quantities. The procedure of Hirs et al. (5) was used for this purpose. Each of the amino acids was obtained in crystalline form and in approximately 70% yield based upon the total quantitative ninhydrin method used to locate the various fractions (7). The unknown constituent amino acid was identical in IR and co-chromatography on two-dimensional thin-layer chromatograms (TLC) to 2-methyl alanine. One mole is present per mole of peptide. Other amino acids were positively identified by comparison of their IR and co-chromatography on two-dimensional TLC with authentic samples.

A cyclic polypeptide with the composition $(glu\ N)_2$, $(glu)_1$, $(pro)_2$, $(gly)_1$, $(ala)_3$, $(leu)_3$, and $(2\text{-methyl alanine})_1$ has a theoretical elementary formula of $C_{58}H_{95}O_{17}N_{15}$: C, 54.67; H, 7.46; N, 16.49; found values were C, 54.64; H, 7.59; N, 15.58. The direct amide nitrogen analysis yield was 1.74%; the theory for two glutamine residues was 2.19%.

Animal toxicity was studied on male mice ranging in weight from 18 to 24 g by intraperitoneal (ip) injection of 0.05 ml of propylene glycol containing trichotoxin A. After injection, the animals became lethargic, and death ensued without any trembling within 1 hr to 3 days, depending upon the dosage. The single median lethal dose (LD_{50}) for mice was 4.36 mg/kg of mouse, and the 95% confidence interval of LD_{50} was 2.52 to 7.56 mg/kg of mouse as calculated from the formula of Weil (9), where according to the notations used by Weil: K = 3; n = 5; γ = 0, 3, 4, 4; d = 0.030103; Da = 2 mg/kg.

Trichotoxin A is much less toxic to mice by oral administration. Three mice, previously starved for 24 hr, were fed 600 mg of toxin per kg of body weight mixed with a commercial mouse ration. The toxin-contaminated material was ingested in a single feeding. Subsequently, the treated animals were maintained for 4 months under standard conditions with

no discernible effects. This lack of oral toxicity is most probably due to either detoxification in the digestive tract or failure of the toxin to be transported across the intestinal wall. Similar findings have been reported by Christensen et al. (2) and Harland et al. (4).

Some antimicrobial activity was noted. Trichotoxin A in a concentration of 1,000 μg/ml of methanol inhibits *Brucella bronchiseptica*, *Mycobacterium phlei*, *Staphylococcus aureus*, and *Mucor ramannianus* but shows no activity against *Candida albicans*.

Trichotoxin A exhibits a weak toxicity against Eagle KB cells in tissue culture. The toxin shows about one-fifth of the in vitro toxicity of penicillic acid to tumor cells.

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