Viriditoxin Production by Aspergillus viridi-nutans and Related Species

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Bioproduction of viriditoxin on various substrates by strains of the Aspergillus fumigatus group was determined under several incubation conditions. Aspergillus viridi-nutans strains NRRL 4365 and 576 produced the largest quantities of toxin, A. brevipes gave reduced yields, and there was no detectable synthesis by isolates of four related species. After 30 days in static culture at 20 C on various autoclaved agricultural commodities, optimal yields of 440 and 380 mg of toxin were observed per kilogram of sorghum and rice. Toxin levels were reduced on corn, rye, and wheat (40–200 mg/kg); yields were low on cottonseed, barley, and oats. Incubation at 10 C restricted biosynthesis of viriditoxin, and no toxin accumulated on substrates maintained at 5 C for 120 days. In a liquid, yeast extract-sucrose medium, maximal mycotoxin production developed in shake flasks; after 156 h, 10 mg of toxin accumulated per gram of mycelium. Viriditoxin produced in submerged culture was associated with the mycelium; less than 1% was detected in the filtered broth after 156 h of incubation.

The pathogenicity of Aspergillus fumigatus has prompted extensive investigation of this group of fungi. Although original identification of this fungus was based on characteristics associated with an isolate from an avian mycosis, further observation demonstrated that members of the species were common as saprophytic forms in soil and that isolates of the organism could be obtained routinely from organic materials undergoing aerobic decomposition (4, 6). An unusual property of the A. fumigatus group is a wide temperature tolerance. Most Aspergillus strains grow best at 25 to 30 C, but some A. fumigatus isolates develop optimally at 37 C and continue to grow at temperatures up to 50 C (4, 6). Fungi in this group are common on moldy grain, and toxigenic strains have been identified as a source of mycotoxin production (1, 7).

Reportedly, fungi in the A. fumigatus series produce various biologically-active substances, including macromolecular endotoxins, quinones, polyphenols, alkaloids, steroids, fumagillin, tryptacardin, kojic acid, and gliotoxin (8, 11, 13). In previous studies, we isolated and characterized a unique toxic substance, viriditoxin, from mycelia of A. viridi-nutans Ducker and Thrower NRRL 4365 (5, 12). This fungus is a member of a group of closely related species in the A. fumigatus series (6).

Structural determination of viriditoxin demonstrated that the substance is a dimer in which a substituted isocoumarin is fused to an aryl group (Fig. 1) (12). Although aryl-fused isocoumarins are not commonly found as fungal products, Blank and co-workers (2) isolated from Trichophyton violaceum a pigment, vioxanthin, that is structurally similar to viriditoxin. Fungal biosynthesis of isocoumarins has been extensively studied, particularly since the structural elucidation of ochratoxin demonstrated that it consists of a 3,4-dihydro-3-methylisocoumarin moiety linked to L-phenylalanine (10).

Viriditoxin has a mean lethal dose (LD₅₀) of 2.8 mg/kg in 20-g mice and inhibits growth of several bacterial species (5). These observations prompted the current study of some of the critical conditions involved in bioproduction of the toxin by A. viridi-nutans and related fungi. (This paper was presented at the 73rd annual meeting of the American Society for Microbiology, Miami Beach, Florida, 6–11 May, 1973.)

MATERIALS AND METHODS

Organisms. Strains of Aspergillus were obtained from the ARS Culture Collection maintained at the Northern Regional Research Laboratory. Cultures were grown on potato dextrose agar slants, and
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production of spores for inocula was carried out on slants incubated at 28 C for 10 days.

Toxin production. Samples (50 g) of solid substrate were individually added to each flask (300-ml Erlenmeyer) and sterilized; autoclaving of corn involved a slightly different procedure than that used for other commodities. After corn was immersed in tap water, it was autoclaved for 15 min at 121 C; excess water was decanted, and the flasks were finally autoclaved for 20 min at 121 C. Samples of barley, oats, wheat, rice, rye, sorghum, and cottonseed were steeped in 25 ml of tap water for 2 h before autoclaving for 20 min at 121 C. Steep water was not decanted before autoclaving. Submerged fermentations were carried out in a medium containing 4% sucrose and 2% yeast extract. Each flask was inoculated with 1 ml of an aqueous spore suspension.

Toxin extraction and assay. Contents of a solid substrate fermentation were extracted with 250 ml of chloroform-water (90:10, vol/vol) in a Waring blender for 5 min. The extraction solvent was recovered by filtration with subsequent removal of water through addition of anhydrous sodium sulfate and evaporation of the chloroform to desired volume under vacuum. Submerged fermentations were adjusted to pH 2 before extracting with 250 ml of chloroform-methanol (90:10) in a Waring blender for 5 min; the chloroform solutions were dried with anhydrous sodium sulfate, filtered, and concentrated to 10 ml under vacuum. Fractions of test solutions were spotted on thin-layer chromatographic (TLC) plates coated with 0.5 mm Adsorbosil-1 (Applied Science Laboratories, Inc., State College, Pa.). The developing solvent was chloroform-acetonitrile-formic acid (97:15:1.5). Quantitative measurements were made on developed TLC plates by comparison of fluorescent intensity of viriditoxin from fermentation extracts with a reference standard of pure toxin. Fluorescent spectra were obtained with an Amino-Bowman spectrophotofluorometer and ultraviolet (UV) spectra in a Beckman DB spectrophotometer.

RESULTS AND DISCUSSION

Toxin production on solid substrates. The ability of various autoclaved agricultural commodities to support development of viriditoxin at several temperatures was determined by inoculation of the substrates with A. viridi-nutans NRRL 4365 and subsequent static incubation (Table 1). Although toxin accumulation is only recorded for 10, 20, and 30 C, production flasks were also incubated at 5 and 35 C; no toxin was observed at the temperature extremes during 120 days of incubation. In the temperature range conducive to toxin biosynthesis, a distinct difference in toxin yield was noted between substrates. Rice and sorghum supported maximal production with 19.0 mg of toxin per 50 g (380 mg/kg) for the former and 22.0 mg of toxin per 50 g (440 mg/kg) for the latter after 30 days at 20 C. Under the same conditions, yellow corn supported production of about one-half the toxin observed on sorghum or rice; other commodities yielded significantly less. Quantities of toxin from a substrate incubated at 20 or 30 C were similar, but yields at 10 C were reduced dramatically.

Table 1. Production of viriditoxin on static solid substrates

| Agricultural commodity | Incubation (days) | Viriditoxin (mg/50 g of substrates) |
|------------------------|------------------|-----------------------------------|
|                        | 10               | 20                                | 30                                 |
| Barley                 | 0.3              | 0.8                               |                                     |
| White corn             | 0.9              | 1.7                               |                                     |
| Yellow corn            | 0.6              | 1.0                               |                                     |
| Cottonseed             | 0.7              | 1.0                               |                                     |
| Oats                   | 0.4              | 0.9                               |                                     |
| Wheat                  | 1.7              | 1.8                               |                                     |
| Rice                   | 5.5              | 5.0                               |                                     |
| Rye                    | 4.8              | 2.2                               |                                     |
| Sorghum                | 2.5              | 6.3                               |                                     |

* Each flask was inoculated with $6.0 \times 10^4$ spores of A. viridi-nutans.

* Values represent means of duplicate flasks assayed independently.
Earlier studies demonstrated that aflatoxin production on rice could be optimized by incubation on a rotary shaker with yields of up to 1,511 mg/kg of substrate (9). A similar test was conducted on the bioproduction of viriditoxin by *A. viridi-nutans* on rice; culture conditions were identical to those used for the static fermentations. A maximum yield of 31.0 mg of viriditoxin/50 g of rice (620 mg/kg) was obtained after 12 days at 30 C. The yield of toxin from shake culture was approximately five times greater than that from an analogous static incubation (10 days).

**Toxin production in liquid culture.** Elaboration of viriditoxin by *A. viridi-nutans* NRRL 4365 was examined in a static, yeast extract-sucrose (YES) medium. Toxin recovery after incubation at various times and temperatures in plotted in Fig. 2a. Rapid accumulation of the substance occurred during the first 8 days at 25 C and at 30 C with a subsequent decline during the remainder of the incubation at these temperatures. Although bioproduction of the compound was restricted during the initial 12 days at 20 C, this temperature supported maximal yield of the toxin after 28 days. No toxin accumulated in static, liquid medium at 10 C, but limited biosynthesis did occur at 35 C during the first 8 days.

Mycelial dry weights during static fermentation in YES are shown in Fig. 2b. The growth pattern resembled that of toxin accumulation (Fig. 2a); rapid synthesis occurred during the initial 8 days at 25 to 35 C and then subsequently declined. Mycelial yields at 20 C were initially retarded, but rapid growth began between 8 and 16 days after which there was a limited decline. The organism grew at 10 C but produced no viriditoxin.

The growth pattern of *A. viridi-nutans* NRRL 4365 was compared with toxin biosynthesis in shake culture at 30 C (Fig. 3). Viriditoxin is a typical secondary metabolite since the substance is not produced before the later stage of exponential growth (3). Toxin production in shake culture is about four times greater than the optimal yield observed in static incubation. Viriditoxin was exclusively in the mycelia of shake cultures during the first 108 h, with less than 0.2 mg of toxin per 50 ml of medium detected in the fermentation beer after 156 h.

**Toxin production by various Aspergillus strains.** Originally, viriditoxin was identified as a metabolite of *A. viridi-nutans* NRRL 4365. A limited screening was carried out to identify other strains of mold in the *A. fumigatus* series that were capable of producing viriditoxin. Fungi were grown on autoclaved rice in shake
culture at 30 °C with subsequent extraction of the fermentation contents and assay for the toxin (Table 2). The two strains of A. viridi-nutans in the ARS Culture Collection produced approximately equivalent quantities of toxin with a slightly greater yield in strain NRRL 576 after 2 weeks. The only other species that elaborated viriditoxin was A. brevipes. The A. brevipes metabolite was verified as viriditoxin by equivalent TLC mobilities of admixtures of the test compound with authentic toxin and identical ultraviolet and fluorescent spectra (5). The A. fumigatus isolates including those obtained by Tilden et al. (Bacteriol. Proc., p. 144, 1957) from aspergillosis lesions in birds, did not synthesize viriditoxin.

| Table 2. Production of viriditoxin by various Aspergillus species* |
|---------------------------|----------------|----------------|
| Culture                  | NRRL no. | Viriditoxin* |
| A. viridi-nutans         | 4365     | 3.8 35.0     |
| A. brevipes              | 576      | 3.6 40.0     |
| A. unilateralis          | 4078     | 0 0.3       |
| A. duricaulis            | 577      | 0 0         |
| A. fumigatus             | 4021     | 0 0         |
| A. fumigatus*            | 163, 5563| 0 0         |
| A. fumigatus var. ellipticus | A-6061, A-6063 | 0 0 |
|                          | A-6064, A-6065 | 0 0        |
|                          | A-6066, A-6069 | 0 0        |
|                          | 5109     | 0 0         |

*Each flask was inoculated with $6.0 \times 10^5$ spores and incubated on a shaker at 30 °C.
*Values represent means of replicated flasks assessed independently with toxin levels presented as milligrams per 50 g of rice.
*Isolates obtained from E. B. Tilden.

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