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# T-2 Toxin Production by *Fusarium tricinctum* on Solid Substrate[1]

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A method has been developed to produce and purify gram quantities of T-2 toxin [4$\beta$,15-diacetoxy-8$\alpha$-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-en-3$\alpha$-ol], a mycotoxin elaborated by a strain of *Fusarium tricinctum* isolated from toxic corn. After growing for 3 weeks at 15 C on 1,200 g of white corn grits, *F. tricinctum* NRRL 3299 elaborated at least 9.0 g of T-2 toxin, and 2.3 g of crystalline product was recovered. A lesser amount of toxin was produced on rice, but none was detected in wheat incubated at 20 C. The amount of toxin measured in white corn grits declined as the incubation temperature was raised to 20, 25, and 32 C.

Outbreaks of toxicosis in animals ingesting moldy corn are accompanied by several signs (E. B. Smalley et al., Proc. 1st U.S.-Japan Conf. Toxic Microorganisms, *in press*). Although many species of fungi are usually isolated from toxic corn samples, *Fusarium tricinctum* is one of the more common isolates, and extracts from cultures of this fungus are more potent than those of other fungi selected from molded corn (6). *F. tricinctum* NRRL 3299 (strain T-2 from E. B. Smalley, University of Wisconsin) is one of the more toxic strains and produces three characterized mycotoxins: T-2 toxin, 4-desacetoxy T-2 toxin (J. R. Bamburg, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1969), and a butenolide (9). The molecular structure of T-2 toxin, the principal component produced at low temperatures by the T-2 strain, is 4$\beta$,15-diacetoxy-8$\alpha$-(3-methylbutyryloxy) - 12,13 - epoxytrichothec - 9 - en-3$\alpha$-ol (2). Its oral $LD_{50}$ in rats is 4 mg/kg (3).

Albino rats fed diets containing 5 and 15 $\mu$g of T-2 toxin per ml for 3 weeks were severely stunted and developed inflammations of the skin around the nose and mouth. Microscopic examination of the liver of these animals showed small areas of focal change and cytoplasmic degradation. Rats receiving a diet containing 10 $\mu$g of T-2 toxin per ml for 8 months consumed approximately 20 times the single $LD_{50}$ without apparent ill effects and without any indication of hepatoma development (8). Signs of metabolic disorder in large animals consuming T-2 toxin have not been observed, but, in a preliminary study (7), a 650-pound steer receiving daily intramuscular injections of 30 mg of T-2 toxin lost weight during the study and died on the 65th day. Autopsy revealed evidence of general internal hemorrhage, a sign occasionally found in cattle after ingestion of moldy corn (5).

The production of T-2 toxin and a purification process by which 100 mg was routinely recovered from 1 liter of medium have been described, and it has been reported that 1 kg of corn fermented with the T-2 strain may contain up to 1 g of toxin (J. R. Bamburg, Ph.D. Thesis). Because this toxin may be involved in moldy corn toxicosis, a method was sought for producing it in amounts sufficient to determine its effect on livestock consuming chronic and acute dosages. A simple procedure for obtaining this toxin in gram quantities is presented here.

## MATERIALS AND METHODS

**Preparation of inoculum.** Conidia of *F. tricinctum* NRRL 3299 were produced by the fungus on yeast-malt (YM) agar incubated for 14 days at 25 C. The conidia were suspended in water by gently scraping the agar surface with a wire loop to give a turbid suspension.

**Production of T-2 toxin.** Fernbach flasks containing 300 g of white corn grits (WCG), pearled wheat, or polished rice were autoclaved for 30 min. After the flasks were autoclaved, 2 ml of the conidial suspension and 100 ml of sterile water were added to each flask. Four flasks of the inoculated WCG, wheat, and rice were kept for 3 weeks at an incubation temperature of 20 C. In addition, flasks of WCG were incubated at 15, 20, and 32 C, also for 3 weeks.

**Extraction and purification of T-2 toxin.** Each of four flasks of WCG fermented at 15 C was extracted with 1 liter of chloroform–acetone (85:15) by blending in a Waring Blendor for 2 to 3 min. The corn slurry was filtered on paper toweling, and the solvent was

[1] A preliminary report of this work was presented at the 70th Annual Meeting of the American Society for Microbiology, Boston, Mass., 26 April–1 May 1970 (Bacteriol. Proc., 1970, p. 14).

pressed out with a large spatula. Solids were returned to the blendor jar with a second liter of solvent, and the process was repeated for a total of three extractions. A portion of each extract was saved for quantitative analysis, and the combined extracts were reduced to about 75 ml of an oil-like residue (OLR) in a rotary evaporator. Two volumes of acetone was added to the OLR, and a gummy substance precipitated as the crude mixture dripped into the hexane.

The gummy substance was separated from the toxin-containing solvent by decanting. (T-2 toxin precipitates in hexane but is quite soluble in acetone. To ensure that the toxin remains in solution, the volume of acetone in the solution should be 5 to 10%.) A greenish-yellow OLR continued to settle from the solution for several hours as the solvent was evaporated at room temperature. The solution was decanted at hourly intervals until it was almost free from OLR. The T-2 toxin crystallized and precipitated along with a small amount of OLR as the solvents evaporated from the clear solution standing in an open beaker. The toxin and OLR were dissolved in 10 ml of acetone to which 1 to 2 g of activated charcoal was added. After 30 min, the charcoal slurry on Whatman no. 1 filter paper was washed five times with 10-ml volumes of acetone; extraneous charcoal was removed with a second filtration before the acetone volume was reduced to 10 to 15 ml. Hexane was poured slowly into the toxic solution until it became slightly cloudy, and then a few drops of toluene were added to clear the solution. The T-2 toxin crystallized from the hexane-acetone-toluene left at room temperature for 18 hr. The toxin was dried at 80 C and weighed, and the purity was confirmed. A melting point determination (150 to 152 C), the presence of a single spot on thin-layer chromatograms with an $R_F$ corresponding to the standard T-2 toxin, and nuclear magnetic resonance spectroscopy were criteria for judging product purity.

The gummy residue from the initial hexane precipitation was dissolved in 100 ml of acetone, and the T-2 toxin recovery procedure was repeated. Hexane-insoluble residue remaining after the second precipitation was also dissolved in 100 ml of acetone, and the purification process was carried out for the third time. The residue remaining after the third precipitation and the oily substance present in the hexane-acetone-toluene solution from which crystalline toxin was recovered were kept for quantitative analysis.

An estimate of the T-2 toxin produced on the various substrates fermented at given temperatures was obtained from a single extraction by using 1 liter of solvent for each flask of substrate. Although less than 75% of the solvent was recovered from the blended corn slurries, the toxin estimates are based on the 4 liters of solvent used in the extraction. It was assumed that each milliliter of solvent held the same quantity of toxin whether recovered or absorbed by the substrate. Crystalline toxin was obtained from the extracts by the described procedure, but only one precipitation of the OLR in hexane was carried out.

**Estimation of T-2 toxin by microbiological assay.** The minimum amount of T-2 toxin required to inhibit *Rhodotorula rubra* NRRL Y-7222, a yeast sensitive to this mycotoxin (4), was 4 μg when applied on a filter paper disc (Schleicher and Schuell, no. 740E). Cells of *R. rubra* growing on YM agar at 25 C were suspended in YM broth and diluted to a reading of 50% transmittance at a wavelength of 600 nm. One-tenth milliliter of the yeast suspension was added to 6 ml of YM agar at 45 C before the agar was poured into a standard petri dish. Decimal dilutions of the chloroform–acetone (85:15, v/v) extracts were added to antibiotic assay discs at the rate of 0.1 ml per disc. Discs made from test extracts and standard discs containing 5 and 10 μg of crystalline toxin were placed on the same petri dish. After a 30-hr incubation at 25 C, the inhibition zones around the discs containing the extracts and the standard discs were compared. Extracts from samples with the smallest amounts of toxin were concentrated before their fungistatic effects were compared with the standards. An estimate of the total amount of toxin in the fermented grain was based on the dilution, or concentration, of a test extract required to give an inhibition zone comparable to that of the standard and on the volume of solvent used in the extraction procedure.

**Estimation of T-2 toxin by thin-layer chromatography.** Culture extracts were spotted on Silica Gel G thin-layer chromatographic plates along with 5, 10, 15, and 20 μg of T-2 toxin. The chromatograms were developed with ethyl acetate–toluene (3:1). After the development, the plates were air-dried, sprayed with

TABLE 1. *Estimate of T-2 toxin recovered and lost from 1,200 g of substrate during toxin purification*

| Fraction examined | Solvent recovered (ml) | Thin-layer chromatography (g) | Yeast assay (g) | Crystalline product (g) |
|---|---|---|---|---|
| Extract no. 1 | 2,810[a] | 5.96 | 4.87 | |
| Extract no. 2 | 3,520 | 4.20 | 3.35 | |
| Extract no. 3 | 3,860 | 0.53 | 0.64 | |
| Combined extracts (evaporated) | 75 | 10.69 | 8.86 | |
| Hexane precipitation no. 1 | | | | 1.21 |
| Hexane precipitation no. 2 | | | | 1.11 |
| Hexane precipitation no. 3 | | | | 0.45 |
| Total product | | | | 2.77[b] |
| Hexane precipitation (residue) | | 5.32 | 4.65 | |
| Hexane soluble oil | | 0.83 | 0.67 | |
| Losses not accounted for | | 1.77 | 0.86 | |

[a] Each extraction used 4 liters of chloroform-acetone (85:15).

[b] Represents a recovery of 25.8% based on thin-layer chromatography estimate.

TABLE 2. *Effect of temperature and substrate on the production of T-2 toxin by Fusarium tricinctum NRRL 3299*

| Substrate | Temp (C) | Toxin recovered (g/1.2 kg) | Estimated thin-layer chromatography (g/1.2 kg) | Estimated yeast assay (g/1.2 kg) |
|---|---|---|---|---|
| White corn grits | 15 | 1.44 | 9.96 | 9.00 |
| | 20 | 0.50 | 5.40 | 6.24 |
| | 25 | 0 | 0.67 | 1.40 |
| | 32 | 0 | ND[a] | 0.01 |
| Wheat | 15 | 1.21 | ND-P[b] | ND-P |
| | 20 | 0 | ND | ND |
| Rice | 20 | 0 | 0.19 | 0.32 |

[a] Not detected.
[b] No determination; preliminary study.

concentrated sulfuric acid, and placed in an oven at 125 C for 15 min. Amounts of toxin in the extract dilutions were determined by comparing the intensity of the charred spots with those of the standard. A sample was judged to contain no toxin when 20 μliters of an extract concentrated 10-fold did not give a visible spot at the $R_F$ of T-2 toxin.

## RESULTS AND DISCUSSION

An estimate of the quantity of T-2 toxin extracted from WCG fermented with *F. tricinctum* NRRL 3299 incubated at 15 C and the amounts recovered or lost at various stages in the purification process are given in Table 1. The solvent recovered from 1,200 g of substrate after blending three successive times with 4-liter volumes of chloroform-acetone contained about 10 g of toxin, and more than one-fourth of it (2.77 g) was recovered as crystalline product. Nearly 95% of the toxin was in the first two extracts and only 5% was in the third extract. The relatively large quantity of toxin (40%) in the second extract was due to the absorption of nearly 30% of the solvent by the substrate during the first extraction. The quantity of crystalline toxin obtained from hexane-acetone decanted from the first, second, and third precipitations of the OLR-acetone in hexane was 1.2, 1.1, and 0.45 g, respectively. Most of the toxin not reclaimed (68%) was retained by the gummy OLR remaining after the third hexane precipitation. A lesser amount of the unclaimed toxin (10%) stayed in the oily substance dissolved in the hexane-acetone-toluene from which the T-2 toxin crystallized and in hexane used to wash the product. Toxin not accounted for (22%) was probably lost during filtering and decanting or may be due to variations inherent in the assay methods.

In Table 2 are given estimated quantities of T-2 toxin elaborated by the fungus growing on WCG, pearled wheat, and polished rice at 20 C and those produced on WCG incubated at 15, 20, 25, and 32 C.

Apparently the amount of T-2 toxin produced by this fungus is influenced by temperature and by the nature of the substrate. Most toxin, about 9.0 g/1,200 g, was produced on WCG incubated at 15 C. At incubation temperatures of 20 and 25 C, the amount of toxin produced declined 50 and 85%, respectively. No toxin was detected in WCG incubated at 32 C as determined by thin-layer chromatography, and the yeast assay suggested the presence of only a small quantity of a fungistatic substance. In this study, T-2 toxin was not detected in the pearled wheat, but, in a preliminary investigation, 1.3 g of toxin was recovered from 1,200 g of pearled wheat when the incubation temperature was 15 C. Polished rice was a comparatively poor substrate for toxin production, and only a small amount was present in the extract.

Incubation of WCG inoculated with the fungus at 15 C yielded gram quantities of T-2 toxin. Bamburg et al. (1) report 8 C as the best temperature for toxin production in a cornsteep liquor-soybean meal based (Gregory's) medium. The twofold increase in toxin quantities when the incubation temperature of the WCG was reduced from 20 to 15 C suggest that larger amounts may be produced at a lower temperature on WCG as in Gregory's medium.

Strict precautions are advised when handling the toxin or culture extracts because severe inflammation occurs if either comes in contact with the skin.

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