T-2 Toxin Production by *Fusarium tricinctum* on Solid Substrate

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A method has been developed to produce and purify gram quantities of T-2 toxin \([4\beta,15\text{-dioxetoxy-8a-}(3\text{-methylbutyryloxy})-12,13\text{-epoxytrichothec-9-en-3a-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 (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 butenolid (9). The molecular structure of T-2 toxin, the principal component produced at low temperatures by the T-2 strain, is \(4\beta,15\text{-dioxetoxy-8a-}(3\text{-methylbutyryloxy})-12,13\text{-epoxytrichothec-9-en-3a-ol}\) (2). Its oral LD<sub>50</sub> in rats is 4 mg/kg (3).

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 moldy 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 butenolid (9). The molecular structure of T-2 toxin, the principal component produced at low temperatures by the T-2 strain, is \(4\beta,15\text{-dioxetoxy-8a-}(3\text{-methylbutyryloxy})-12,13\text{-epoxytrichothec-9-en-3a-ol}\) (2). Its oral LD<sub>50</sub> in rats is 4 mg/kg (3).

Albino rats fed diets containing 5 and 15 µg 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 µg of T-2 toxin per ml for 8 months consumed approximately 20 times the single LD<sub>50</sub> 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 (1:1) by blending in a Waring Blender for 2 to 3 min. The corn slurry was filtered on paper toweling, and the solvent was...
pressed out with a large spatula. Solids were returned to the blender 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 and precipitated from the acetone-toluene (85:15) solution with 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

```latex
\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Fraction examined & Solvent recovered & Thin-layer chromato- & Yeast assay & Crystalline product
\hline
Extract no. 1 & 2.810 & 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
Hexane precipitation (residue) & 5.32 & 4.65
Hexane soluble oil & 0.83 & 0.67
Losses not accounted for & 1.77 & 0.86
\hline
\end{tabular}
\caption{Estimate of T-2 toxin recovered and lost from 1,200 g of substrate during toxin purification.}
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\(^{a}\) Each extraction used 4 liters of chloroform-acetone (85:15).

\(^{b}\) Represents a recovery of 25.8%, based on thin-layer chromatography estimate.
PRODUCTION OF T-2 TOXIN

1. EXTRACTS FROM PRODUCTION MEDIUM. V. CEREALS.

The production of T-2 toxin by Fusarium tricinctum NRRL 3299 was studied. T-2 toxin production was influenced by temperature and the nature of the substrate. The highest amounts of T-2 toxin were produced at 15, 20, and 25°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Temp (°C)</th>
<th>Toxin recovered (g/1.2 kg)</th>
<th>Estimated thin-layer chromatography (g/1.2 kg)</th>
<th>Estimated yeast assay (g/1.2 kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White corn</td>
<td>15</td>
<td>1.44</td>
<td>9.96</td>
<td>9.00</td>
</tr>
<tr>
<td>grits</td>
<td>20</td>
<td>0.50</td>
<td>5.40</td>
<td>6.24</td>
</tr>
<tr>
<td>Wheat</td>
<td>15</td>
<td>1.21</td>
<td>ND-P</td>
<td>0.01</td>
</tr>
<tr>
<td>Rice</td>
<td>20</td>
<td>0.19</td>
<td>ND</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*Not detected.

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 through 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.

Apparenty 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.

ACKNOWLEDGMENT
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LITERATURE CITED