Assay and Relationship of HT-2 Toxin and T-2 Toxin Formation in Liquid Culture

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Both T-2 toxin and HT-2 toxin can be conveniently quantitated in crude extracts by using a combination of thin-layer chromatography and fluorodensitometry. This technique was used to follow the production of these toxins by liquid cultures of Fusarium poae (NRRL 3287). T-2 toxin was produced prior to HT-2 toxin and hexadeutero-T-2 toxin was converted by the culture to trideutero-HT-2 toxin.

T-2 toxin (Fig. 1) is a well-characterized member of a family of sesquiterpene mycotoxins produced by fungi which develop well at low temperatures (In I. F. H. Purchase [ed.], Mycotoxins, in press). Of those fungi which produce T-2 toxin, Fusarium tricinctum has been recognized as particularly important with regard to moldy corn toxicosis in the midwestern United States (7). In pure culture, optimum production of the toxin was found at 8 C using corn as substrate (J. R. Bamberg, Ph.D. thesis, Univ. of Wisconsin, Madison, 1968). Recently, T-2 toxin was isolated from a field sample of toxic moldy corn, thus making more convincing its role as a natural toxin as opposed to being an artifact of pure culture (3). When grown at higher temperatures the pure culture produces a related compound, HT-2 toxin (Fig. 1). This toxin has been less well studied although preliminary work suggests that its toxicity is similar to that of T-2 toxin. Both compounds are of comparable toxicity in the rabbit reticulocyte assay and HT-2 toxin is approximately one-half as toxic when measured as mean lethal dose in 6-week-old male mice (10). Further, both toxins appear to have comparable emetic activity in test animals (2, 10).

By inspection it would appear that the two compounds are closely related in a biogenetic sense, i.e., by the addition or loss of one acyl group. Indeed, HT-2 toxin may be obtained by careful base-catalyzed hydrolysis of T-2 toxin (11); it has been recently shown that HT-2 toxin is produced from the latter by exposure to bovine and human liver cells in vitro (2). Although many interesting details of the biosynthesis of trichotheccenes have been elucidated, the detailed sequence of compound production remains only speculative. For example, it might be asked whether T-2 toxin is the result of partial acetylation of a polyhydroxylated precursor or, rather, hydrolysis of a fully esterified compound. As a preliminary step toward studying this question, we chose to examine the production of T-2 toxin and HT-2 toxin in a strain of F. tricinctum which we have found produces both of these compounds at room temperature in liquid culture. To assist this work, a convenient fluorodensitometric assay was developed.

MATERIALS AND METHODS

General. The organism used in this study was Fusarium poae (NRRL 3287, F. tricinctum according to Snyder and Hansen [8]). The medium was Richards solution using either 0.1 M potassium nitrate or 0.1 M ammonium chloride as a nitrogen source (5). Media were sterilized by autoclaving at 15 lb/in² and 120 C for 20 min. Liquid cultures were grown at room temperature on a gyratory shaker (New Brunswick Scientific Co.) at 300 rpm. pH measurements were made using a Beckman model H-T glass electrode pH meter. Packed cell volumes (milliliters) were measured by withdrawal of a 10-ml aliquot from the fermentation followed by centrifugation in graduated 120-mm tubes for 10 min using a model CL International clinical centrifuge. Ethyl acetate extracts of moldy corn were prepared according to previous procedures (1). T-2 toxin and HT-2 toxin were extracted with chloroform from 25-ml aliquots of liquid culture adjusted to pH 9.0 with saturated sodium bicarbonate. The chloroform layers were dried over magnesium sulfate and evaporated in vacuo. The toxins in this organic residue were quantitated as described below. Fluorodensitometric measurements were made on a G. K. Turner Associates model III fluorometer and thin-layer chromatography (TLC) scanner. The primary filter permitted a peak transmission of 365 nm and the secondary filter had a sharp cut-off below 485 nm. Pure T-2 and HT-2 toxin for comparison was available from previous work (2). Thin-layer plates were Silplate F-22 from Brinkmann Instruments, Inc. All solvents were fractionally dis-
tilled before use. Before spotting, plates were developed twice with 15% methanol in chloroform to displace interfering bands to the top of the plate. For analytical results ethyl acetate was the solvent of choice. All samples were applied to the plates using 2-μl Drummond microcaps. Silica for column chromatography was EM Silica Gel 60 (70 to 230 mesh) from Brinkmann Instruments, Inc. Nuclear magnetic resonance spectra were taken on a Brucker HX-90E instrument and mass spectra were taken with either a Finnegan 1015 or MS-9 spectrometer.

**Quantitation by dilution to extinction.** Quantities of standard T-2 toxin, standard HT-2 toxin, and organic residue (containing either or both toxins) extracted from moldy corn or liquid culture were dissolved in chloroform and serially diluted. Duplicate portions (2 μl) of each solution were spotted on a TLC plate at 1-cm intervals with a margin of 2 cm at plate edges. After development with ethyl acetate to 12 cm, the plate was uniformly sprayed with a fine mist of concentrated sulfuric acid-ethanol (3:7), and charred at 120 C for 15 min.

**Quantitation by fluorodensitometry.** The organic residue extracted from moldy corn or liquid culture was dissolved in chloroform (1 ml) and portions (2 μl) were spotted on a TLC plate alongside standard solutions of T-2 toxin and HT-2 toxin, which were spotted to obtain applied quantities of 0.25, 0.50, 0.75, and 1.0 μg. Spots were set at 2-cm intervals with 2-cm margins at plate edges. The plate was developed to 12 cm with ethyl acetate, uniformly sprayed with a fine mist of concentrated sulfuric acid-ethanol (3:7), and charred at 120 C for 20 min. After 45 min, the plate was scanned at a speed for 20 mm/min in the direction of chromatography. The 1.0 μg standard was scanned both at the beginning and the end of the run to assess the reduction in fluorescence intensity over the scanning period. The maximum reduction in these runs was 5%. Peak areas (square tentimeters) were calculated by multiplying peak height by the peak width at one-half peak height. Standard curves were generated for each plate and the corresponding unknown toxin concentrations, were interpolated directly. Results were obtained from duplicate runs. Standard curves for both toxins are shown in Fig. 2.

**Toxin production in Richards solution.** F. poae (NRRL 3287) was transferred from sterile soil to potato-dextrose agar slants and grown at room temperature for 1 month. The resulting mycelium was washed into Richards solution using either potassium nitrate or ammonium chloride as nitrogen source. This culture was shaken at room temperature for 1 week and then used to inoculate fresh Richards solution. Portions were periodically withdrawn and analyzed as described above for pH, cell volume, T-2 toxin, and HT-2 toxin. Both toxins were determined by dilution to extinction for the potassium nitrate culture (see Fig. 4) and by fluorodensitometry for the ammonium chloride culture (see Fig. 5).

**Stability of T-2 toxin to the medium.** Richards solution (500 ml) using potassium nitrate as nitrogen source was adjusted to pH 7.5 with 1 M sodium hydroxide and autoclaved. The pH was readjusted to 7.5 under sterile conditions due to a reduction in pH under the autoclaving conditions. T-2 toxin (5 mg) was added to the medium and the flask shaken at room temperature. Every 2 days for 2 weeks a portion was withdrawn, the pH was measured, and the concentration of T-2 toxin and HT-2 toxin was determined by dilution to extinction.

T-2 toxin (10 mg) in Richards solution using ammonium chloride as nitrogen source was similarly treated with the exception that the pH was adjusted to 2.0 with 1 M HCl.

**Preparation of hexadeutero-T-2 toxin.** T-2 triol [3,4,15 - trihydroxy - 8 -(3 - methylbutyryl oxy)- 12,13 - epoxy-Δ^9-trichothecene; 34 mg, 0.088 mmol] (11) was added to a solution of hexadeutero acetic anhydride (0.5 g, 4.6 mmol) (R. Ellison and F. Kotsoris, unpublished data) containing 5 drops of pyridine. The solution was stirred in a stoppered flask overnight at room temperature and then was evaporated in vacuo. A TLC of the product (36 mg) on silica gel, developed with ethyl acetate-acetone-methanol (50:50:1) and charred at 120 C for 15 min after spraying with concentrated sulfuric acid-ethanol (3:7), showed only one spot. This was characterized as acyl T-2 toxin by comparison of the mass spectrum and mixed TLC with a previously prepared pure sample. This material was dissolved in a solution (3.0 ml) of 1 M ammonia in methanol-water (4:1) and stirred in a stoppered flask for 20.5 h at room temperature. The evaporated residue was chromato-
graphed on a column of silica gel (25 g; 1.2 by 59 cm) with ethyl acetate-Skelly B (5:7:1) as solvent. Fraction volumes (1 ml) were collected and pure hexadeuterio-T-2 toxin was recovered from fractions 42 to 56 (29% yield). This derivative was characterized by mixed TLC and by its mass spectrum which was identical to that of T-2 toxin, with the exception that the high-mass fragments including the parent were 6 mass units higher than the corresponding non-deuterated compound (m/e 472/466 [M⁺], 388/382, 370/364, 307/304, 281/278, 206).

Incorporation of hexadeuterio-T-2 toxin. F. poae (NRRL 3287) was grown on potato-dextrose agar slants for 1 week and the mycelium from two slants was used to inoculate 500 ml of Richards solution (ammonium chloride as nitrogen source). After 3 days, a 20-ml aliquot of this fermentation was used to inoculate 500 ml of Richards solution. Duplicate fermentations were used. When maximum production of T-2 toxin was indicated in the control flask, a solution of hexadeuterio-T-2 toxin (12 mg) in sterile dimethylsulfoxide (5 ml) was added to the second flask. After 25 days the enriched culture was adjusted to pH 9.0 with 5% aqueous sodium carbonate and extracted with three portions (500 ml) of chloroform. The solvent was dried over anhydrous sodium sulfate and evaporated in vacuo to yield an organic residue (1.80 g) which was chromatographed on a column of silica gel (100 g; 2.3 by 58.5 cm) with ethyl acetate using 5-ml fractions. T-2 toxin (1.0 mg) was isolated from fractions 40 to 100. HT-2 toxin (6 mg) was isolated from fractions 110 to 190 and further purified by preparative TLC (2 mm) using ethyl acetate-acetone (50:50) as eluant. The amount of trideuterio-HT-2 toxin in this sample was found to be 48.4% based on an average of the ratio of peaks at m/e 368/365, 343/340, and 325/322 in the mass spectrum. The parent ion at m/e 427/424 was not sufficiently intense to be useful.

RESULTS

Quantitation of T-2 and HT-2 toxin. Using serial dilutions of a chloroform solution of T-2 toxin, the smallest amount that could be detected under typical experimental conditions was found to be 0.125 (0.012) µg when the developed and charred plate was examined directly with the eye. This and subsequent values are the means of several determinations with ranges given in parentheses. Upon visualization of the charred plate under ultraviolet irradiation at 365 nm, this value was lowered to 0.054 (0.004) µg. Although estimates of this type are known to be fraught with difficulties in precision and observer bias (9), application of this technique to an ethyl acetate extract of moldy corn resulted in reasonable agreement between the two visualization techniques. Thus, the percent concentration of T-2 toxin in a sample of corn inoculated with F. poae was found to be 0.029 (0.008) and 0.023 (0.006) using direct and 365 nm visualization, respectively.

Application of fluorodensitometry presented several problems which were not evident during dilution to extinction. Thus, increased charring time at 120 C or an increased interval between charring and scanning resulted in a reduced peak intensity reflecting a diminished fluorescence intensity. These effects were not large but served to increase the error. For example, the peak intensity of 1 µg after development and charring at 120 C for 20 min was reduced by approximately 3.5% after a delay of 1 h before scanning. To reduce errors of this type, standard solutions were spotted on all plates alongside unknowns which were then interpolated from the corresponding standard curve. In spite of this, errors became larger when plates were scanned more than one time. Thus, mean relative standard deviations of 4.1 and 7.3% were obtained for T-2 and HT-2 toxins, respectively, for five repetitive scans over a period of 2 h for a range of 0.25 to 1.0 µg. The linearity of response for both toxins is shown in Fig. 2 and the loss of fluorescence intensity with time in Fig. 3. Regression analysis gave slopes of 5.64 and 4.11 with correlation coefficients of 0.995 and 0.962 for T-2 and HT-2 toxins, respectively.

Synthesis of T-2 and HT-2 toxin by F. poae. In a preliminary experiment, toxin production was monitored by fluorodensitometry. Concurrently, culture growth was characterized by changes in pH and cell volume. The results are shown in Fig. 4 for the culture using potassium nitrate as nitrogen source and in Fig. 5 for the ammonium chloride culture. As expected, T-2 toxin production was initiated shortly before the stationary phase was fully established. The subsequent decline in the concentration of T-2 toxin is accompanied by a corresponding ap-
production and the concentration of T-2 toxin was chosen to approximate that of the maximum amount produced by the culture. After 7 days under these conditions the potassium nitrate medium showed no conversion of T-2 toxin to HT-2 toxin upon TLC analysis. In the case of the ammonium chloride medium, approximately 10% conversion occurred over the same period. Examinations of actural culture conditions (Fig. 4 and 5) show that, in the potassium nitrate culture, 50% of the T-2 toxin is lost in 4.5 days and a similar decrease was observed after 5.5 days in the ammonium chloride culture.

For the incorporation study, a sterile solution of hexadeutero-T-2 toxin in dimethylsulfoxide was added to a growing culture (ammonium chloride) at the point of maximum T-2 toxin production. After 25 days both T-2 and HT-2 toxin were isolated and the extent of deuterium content in the latter was determined by mass spectrometry to be 48.4%, giving an incorporation of 96.8% based on the relative amounts of deuterated and undeuterated T-2 toxin available.

**DISCUSSION**

For the purpose of this type of work or, indeed, extensive screening, quantitation from TLC plates as described here appears to be more convenient than the presently employed gas-liquid chromatography assay (4). In the case of T-2 toxin and HT-2 toxin, solvent systems can be chosen so as to clearly separate these compounds from interfering substances in crude extracts such as corn oil. Consequently, elaborate clean-up procedures and derivatization would appear to be unnecessary.

Fluorodensitometry has now been applied to a wide range of compounds and has proved to be a valuable and rapid technique (9). The problem of quantitation when visualization must be accomplished by chemical reaction has been discussed in the context of lipid analysis (6) and the experience with the trichothecenes has proved similar. These difficulties were partially removed upon incorporating standards on the same plate as unknowns. During runs using the fluorodensitometer, background was routinely obtained which interfered with quantitation. This was substantially reduced by development of fresh plates with 15% methanol-chloroform before spotting the samples.

That T-2 toxin is a precursor of HT-2 toxin is clear from both the product development time course as well as the deuterium incorporation study. In the case of the ammonium chloride culture it is not unequivocal that T-2 toxin is
the immediate precursor since quantitatively the loss of T-2 toxin in culture is not matched by a correspondingly equal increase in HT-2 production, even though the maximum concentration ultimately becomes equal. This does, however, appear to corroborate the evidence that the deacylation may well be an enzymatic or related process, since a strictly chemical conversion would be expected to conform to the above condition.

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