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Note

Bioautographic detection of T-2 and HT-2 toxins

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The trichothecenes are a group of mycotoxins produced by numerous fungi that contaminate many agricultural products worldwide. The salient features of these molecules are the 9, 10 double bond and the 12, 13 epoxide ring. Without the presence of these two groups the molecules lose their toxicity[1]. The trichothecene mycotoxins are the causative agents of a large number of animal and human diseases[2]. T-2 toxin is one of the most toxic trichothecenes. In humans[3], cultured chinese hamster ovary cells[4], bovine rumen bacteria[5,6] and soil bacteria[7] the major metabolite resulting from the breakdown of T-2 toxin is HT-2 toxin.

A simple method is described for the detection and quantitative determination of T-2 toxin and its separation from HT-2 toxin on silica gel layers based on growth inhibition of *Kluyveromyces fragilis* and *Saccharomyces cerevisiae* by these mycotoxins. The detection limit for T-2 toxin is 0.2 nmol per spot. The area of growth inhibition corresponds logarithmically to the toxin concentration. T-2 toxin could be quantitatively detected from 0.2 to 160 nmol per spot.

EXPERIMENTAL

Test organism

Cultures of *S. cerevisiae* strain GR 262, *ade 2 ura 1* [ERY^S OLI^R] and *K. fragilis* strain GK 1005 were grown in YPD which consisted of, on a weight per volume basis, 1% yeast extract, 1% peptone and 2% dextrose[8,9]. To keep the number of cells constant in all procedures a standardized culture was prepared by growing cells in YPD at 30°C/200 rpm (3.3 Hz) for *S. cerevisiae* and at 35°C/200 rpm (3.3 Hz) for *K. fragilis* until the optical density at 610 nm was 0.30, as determined with a colorimeter (Chemtrix, Hillsboro, OR, U.S.A.).

Mycotoxins

T-2 toxin (99% pure as supplied by the manufacturer) was purchased from Myco-lab (Chesterfield, MO, U.S.A.). HT-2 toxin was purchased from Sigma (St. Louis, MO, U.S.A.). Tritium-labelled T-2 toxin (1.35 mCi/ml) was from Nuclear Research Centre-Negev (Beer Sheva, Israel). Stock standard solutions of T-2 toxin were prepared at a concentration of 20 nmol/μl in 95% ethanol and stored at −20°C until used. All other chemicals were of analytical-reagent grade.

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Thin-layer chromatography

A 3-μl volume of the stock standard solution was spotted 2 cm from the lower edge of 20 cm glass or plastic-backed pre-coated silica gel 60 thin-layer chromatography (TLC) plates (Merck $60F_{254}$, Merck, Darmstadt, F.R.G.). To equilibriate, without contacting the liquid, dry spotted plates were placed horizontally in a chamber containing chloroform–methanol (9:1, v/v) for 1 h[10]. Following this, the chamber was raised and the TLC plate developed by the ascending technique for approximately 2.5 h until the front reached a height 3 cm from the top of the plate. Plates were removed and dried overnight at 60°C.

Detection

After evaporation of the solvent, divisions of TLC plates containing [^{3}H]T-2 toxin were scraped into fractions which were collected in scintillation vials containing 5 ml of Beckman Ready-Solv GP scintillation cocktail (Beckman Instruments, Irvine, CA, U.S.A.), and counted for radioactivity in a Beckman LS-7500 scintillation counter. For bioautographic detection both sides of TLC plates were sterilized by a 60-s exposure to ultraviolet light (General Electric, germicidal 30-W bulb, G30T8) from 60 cm with a fluence of 24 ergs/s/cm^2. The plastic-backed plates were secured to pieces of glass with double-sided tape. To contain the agar a 3-mm ridge was formed with Tygon® tubing running around the plate. Both glass and plastic-backed plates were placed on a horizontal bench and evenly layered with growth media, either sterile molten (80°C) YPD (1.75% agar) or YPG agar (1% yeast extract, 1% peptone, 4% glycerol, and 1.75% agar). After solidification, the surface of the agar growth medium was swabbed with an inoculum made of a standardized culture of *K. fragilis* or *S. cerevisiae*. These plates were placed in a sterile, tightly sealed plastic container and incubated at 35°C and 24 h for *K. fragilis* and 30°C and 48 h for *S. cerevisiae*. After incubation, the TLC plates swabbed with *K. fragilis* were layered with a 10% (w/v) triphenyltetrazolium chloride agar overlay[11]. Presence of the trichothecenes could be observed as areas where there was no growth. Use of tetrazolium with *K. fragilis* aids the visualization of the zone of growth inhibition as it stains the colonies red. Zones of growth inhibition were measured to the nearest 0.2 mm using calipers.

Densitometry

Following incubation the diameter of the area of no growth was determined with a densitometric scan at 610 nm and a beam slit of 0.5 × 5.0 mm (E-C Apparatus Corp., St. Petersburg, FL, U.S.A.). Scanning was carried out from the origin to the solvent front.

RESULTS AND DISCUSSION

Current TLC methods for trichothecene detection lack sensitivity mainly because type A and B trichothecenes have no absorption bands or fluorescence under ultraviolet or visible light[10]. Visualization of trichothecenes requires the TLC plates to be developed with suitable solvents so that the spots can be detected subsequently by color or fluorescence[12]. Different reagents work best with the different types of trichothecene mycotoxins. Aluminium chloride is relatively specific for type B trichothecenes while type A trichothecenes can be visualized with chromotrophic acid

(CA)[13]. Both these compounds have a poor structural affinity for the 12,13-epoxy group in the trichothecene nucleus[14]. 4-(*p*-Nitrobenzyl)pyridine (NBP) is reported to interact with the trichothecene nucleus and has been used for the detection of types A, B and D. These reagents react with a wide range of extraneous compounds. Unless the samples are put through several cleanup steps these reactions can obscure the toxins[14–16].

Unlike chemical detection bioautography is based on the biological effects of the substance to be detected[17]. As TLC procedures would not alter the chemical structure and hence the biological activity of trichothecenes, bioautography overcomes the visualization problems found when using chemical detection methods and is a suitable alternative. An earlier report[18] of the bioautography of T-2 toxin based on growth inhibition of *Prototheca wickerhamii* indicated a sensitivity range of 0.2 to 5.0 nmol per spot. While satisfactory, the detection range is narrow and there are numerous problems involved in the growth and maintenance of algae.

The study of the toxicity of trichothecene mycotoxins, has shown that several genera of yeast are sensitive[19]. Two of these yeast, *S. cerevisiae and K. fragilis* can be used to bioautographically detect T-2 toxin. Coincident with a single zone of growth

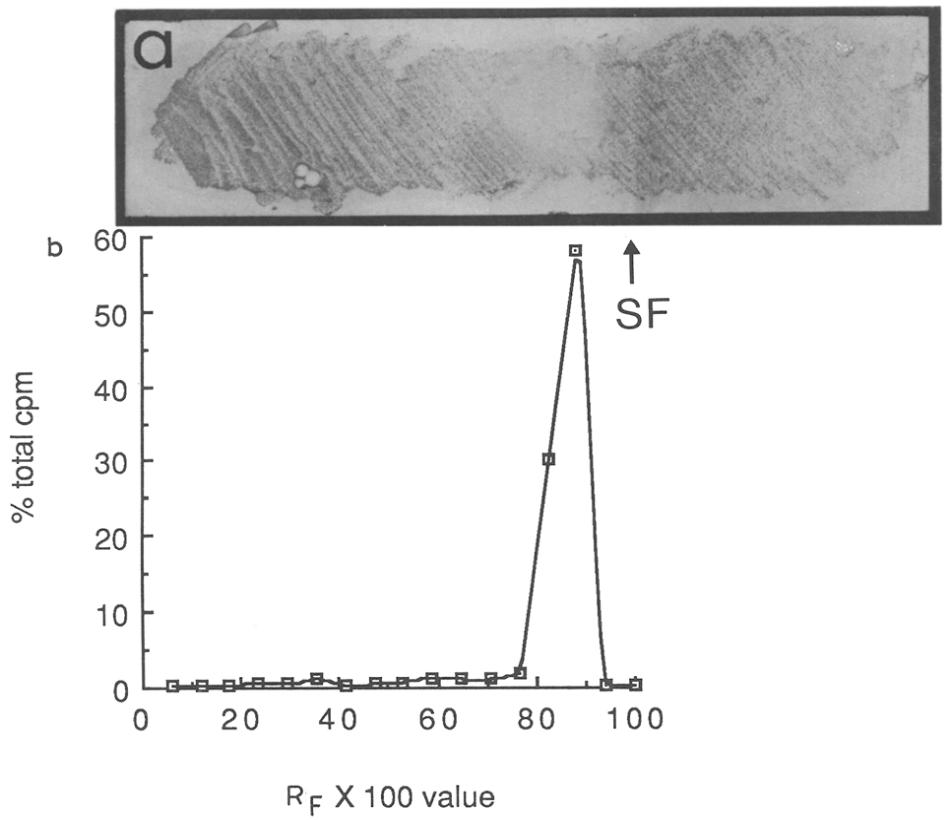

Fig. 1. Thin layer chromatography of T-2 toxin. (a) Detection by bioautography with *S. cerevisiae* on YPG agar (SF = solvent front). Spot contained 20 nmol T-2 toxin. (b) Detection with radioactivity of [^{3}H]T-2 toxin (100% cpm = 8070).

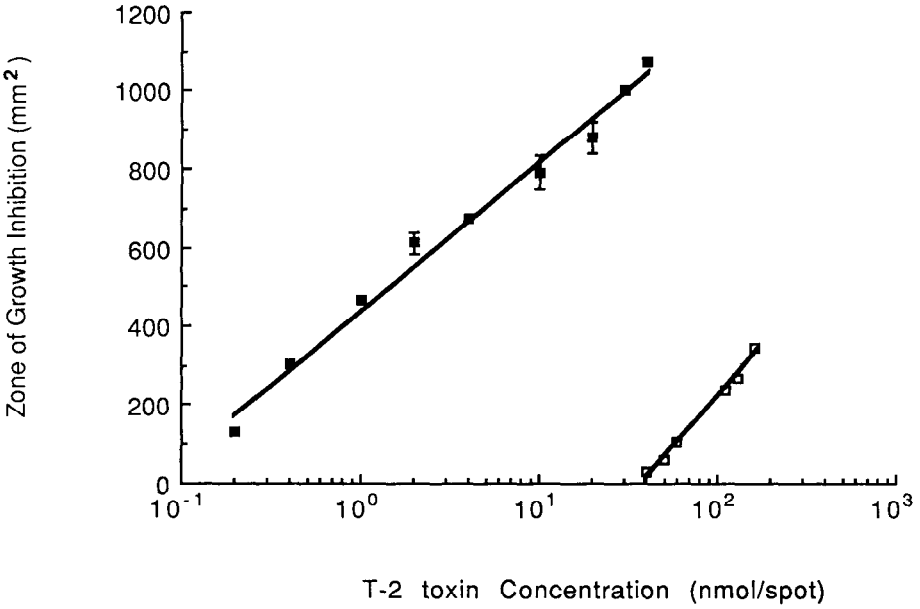

Fig. 2. Calibration graph for T-2 toxin. Bioautographic detection with (□) *S. cerevisiae* or (■) *K. fragilis* on YPD agar. Bars indicate the standard error. Lines represent fitted regression lines: (□) $y = -814.05 + 518.47x$; $r = 1.00$; (■) $y = 442.10 + 377.39x$; $r = 0.99$.

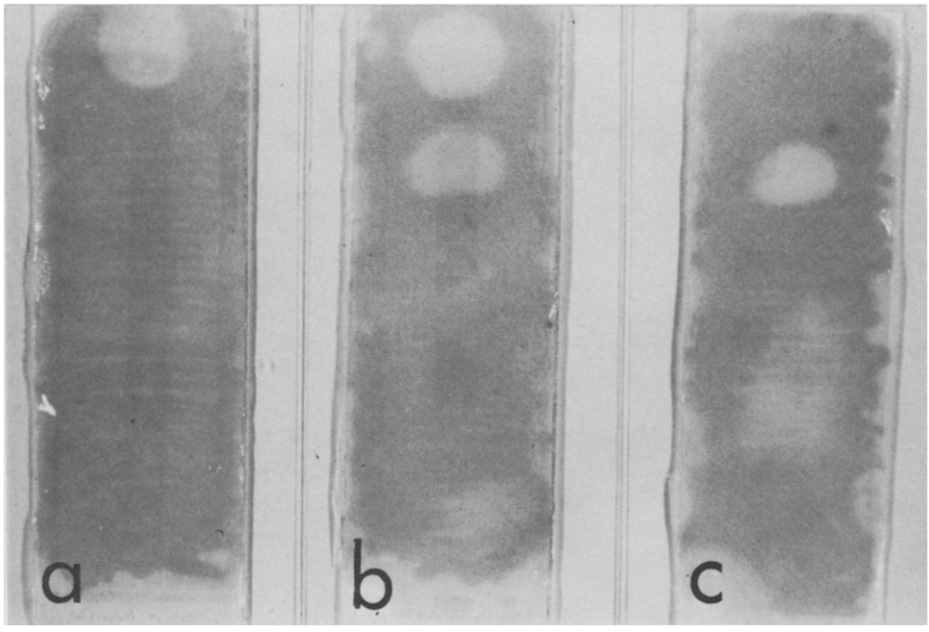

Fig. 3. TLC of trichothecene mycotoxins showing separation of T-2 toxin and HT-2 toxin and bioautographic detection with *K. fragilis* on YPD agar. Spots contained (a) 2.0 nmol T-2 toxin, (b) 2.0 nmol T-2 toxin and 40 nmol HT-2 toxin, (c) 40 nmol HT-2 toxin.

inhibition was a single region of radioactivity from [^{3}H]T-2 toxin (Fig. 1) indicating that growth inhibition is due to T-2 toxin.

A calibration graph (Fig. 2) was constructed for T-2 toxin. The zone of growth inhibition is logarithmically proportional to the toxin concentration in a range from 0.2 to 160 nmol. *K. fragilis* is used as the test organism from 0.2 to 40 nmol, while *S. cerevisiae* is used from 40 to 160 nmol. With both test organisms there was an excellent correlation between log of T-2 toxin concentration and zone of growth inhibition. Values shown in Fig. 2 represent the mean of 3 replicates. The standard error of these values never exceeded 10% of the mean.

To test the calibration curve T-2 toxin and HT-2 toxin were mixed. TLC and bioautography permitted the separation, detection and quantification of T-2 toxin and its major breakdown product, HT-2 toxin (Fig. 3). The larger zone of inhibition is produced by T-2 toxin. The $R_F \times 100$ value of T-2 toxin is 86 while that of HT-2 toxin is 71. In all solvent systems reported T-2 toxin has a larger R_F value than HT-2 toxin[10]. The zone of growth inhibition produced with T-2 toxin is 560 mm^2, corresponding to a T-2 toxin concentration of 1.95 nmol. The calculated concentration is very close to the actual concentration of 2.0 nmol.

At present using visible light T-2 toxin can be detected at levels of 2.0 nmol per spot with cerium(IV) sulphate[20] and 0.2 nmol per spot with CA[13] and NBP[141]. Using ultraviolet light T-2 toxin can be detected at levels of 0.4 nmol per spot with *p*-anisaldehyde[21], 0.1 nmol per spot with CA[13] and 0.05 nmol per spot with nicotinamide-2-pyridine (NAP)[15]. Our bioautographic detection permits visualization at levels comparable to most of these reagents but offers several practical advantages not present in other methods. First, the method of detection is very simple. Unlike the other methods no chemical developing reagents, high temperatures or extensive handling of the plates are required. Second, for reference purposes the TLC plates can be dried and the zone of inhibition remains visible for over 1.5 years. When T-2 toxin is detected with NBP or CA the color remains visible for 3 h[14] and a few weeks[13], respectively. The fluorescence observed when T-2 toxin is detected with NAP is stable for less than 4 h[15]. Third, using bioautography the detection range for T-2 toxin is 0.2–160 nmol per spot, which is an increase over the range of 0.2 to 20 nmol per spot reported for NBP[14] and over the range of 0.2 to 25 nmol per spot reported for bioautographic detection with *P. wickerhamii*[18]. Fourth, the cleanups steps required to avoid reactions with extraneous compounds[16] are eliminated as bioautographic detection identifies the compounds based on toxicity. A final point in considering bioautographic detection of T-2 toxin is that some of the compounds used in other procedures are human health hazards.

Using TLC and bioautography T-2 toxin can be separated from its major breakdown product HT-2 toxin. This coupled with the stability of the plates and the increased detection range makes bioautographic detection especially suited for use in situations demanding the ability to quantify T-2 toxin and detect HT-2 toxin.

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