APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1984, p. 1161–1163
0099-2240/84/051161-03$02.00/0

Vol. 47, No. 5

Detection of T-2 Toxin in *Fusarium sporotrichioides*-Infected Corn by Enzyme-Linked Immunosorbent Assay

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Received 9 December 1983/Accepted 13 February 1984

A competitive enzyme-linked immunosorbent assay was used to screen for T-2 toxin in *Fusarium sporotrichioides*-infected corn. The assay detected T-2 toxin in diluted methanol extracts of corn samples at concentrations of 0.05 ng/ml. In infected corn samples, enzyme-linked immunosorbent assay and gas-liquid chromatography estimations of T-2 toxin concentrations were similar.

T-2 toxin (T-2) and other trichothecene mycotoxins are a group of sesquiterpenoid fungal metabolites produced by various fungal genera, including *Fusarium* spp. T-2 has been implicated in a number of mycotoxicoses and has been found in a number of agricultural commodities (6). Analytical methods developed to detect T-2 include various biological assays, thin-layer chromatography, high-pressure liquid chromatography, gas-liquid chromatography (GLC), and gas chromatography-mass spectroscopy (6). These methods either lack sensitivity or specificity or are laborious and require expensive equipment, and they are therefore inadequate as rapid screening assays. The development of a radioimmunoassay (2) and an enzyme-linked immunosorbent assay (ELISA) (8) for T-2 has shown that immunological assays can be useful alternatives for detection of this toxin. We report herein the comparison of an ELISA screening method for T-2 after a simple extraction of corn infected with T-2-producing strains of *Fusarium sporotrichioides* with a GLC analysis of the same corn after an extensive cleanup.

T-2 (MycoLab Co., Chesterfield, Mo.) was converted to T-2–hemisuccinate (T-2HS) and conjugated to fatty acid-free bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo.) by the method of Chu et al. (2). Antisera made to this conjugate were prepared as described by Chu et al. (2) and purified by precipitation with ammonium sulfate (4) by means of a 35% saturated ammonium sulfate mixture.

T-2HS was conjugated to horseradish peroxidase by means of a modification of the method of Pestka et al. (8). In this variation, 150 μg of T-2HS dissolved in 1.0 ml of ethanol–3.0 ml of water was mixed with 150 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDPC) (Sigma) and 3.0 mg of type VI horseradish peroxidase (Sigma) in 1.0 ml of 25% aqueous ethanol. The mixture was stirred for 30 min at room temperature, and then an additional 150 mg of EDPC was added. This mixture was then stirred for 20 h at 4°C and then dialyzed against three changes of 0.01 M sodium phosphate buffer (pH 7.5) for 3 days. The T-2HS–horseradish peroxidase conjugate (0.3 mg/ml) was stored frozen in 1-ml aliquots. As needed, 1-ml aliquots were thawed and distributed into single-use aliquots (30 μl), and these were frozen. The 1:20 ratio of T-2HS/horseradish peroxidase resulted in less nonspecific binding of the conjugate in the ELISA than did the 3:10 ratio used by Pestka et al. (8).

Antisera titration and T-2 quantitation by ELISA were modified from the method of Pestka et al. (8). Falcon 3070 polystyrene microtissue culture plates (Becton Dickinson and Co., Oxnard, Calif.) were prepared by air drying 50 μl of a fraction V (Sigma) BSA solution (0.2 mg/ml of water) in each well. The wells were then reacted with 50 μl of 0.2% (vol/vol) glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.5) for 30 min, washed extensively with distilled water, air dried, and stored under desiccation. Purified antisera were diluted in PBS, and 50-μl aliquots were air dried in each well under a warm air current (ca. 40°C) and then stored under desiccation. When used, the antisera-coated plates were first washed three times by filling each well with 0.2 ml of 0.05% (vol/vol) Tween 20 in PBS (PBS-Tween 20) and aspirating the contents with a single-well aspirator under a vacuum of 580 mmHg (ca. 77,314 Pa). Nonspecific binding was decreased by incubating each well for 1 h at 37°C with 0.2 ml of 1% (wt/vol) BSA in PBS, followed by two more washes with PBS-Tween 20.

Antisera were titrated by diluting the T-2HS–horseradish peroxidase conjugate 1:300 with 5% (wt/vol) BSA and 0.1% (vol/vol) Tween 20 in PBS (PBS-BSA-Tween 20) and adding 50-μl aliquots to wells previously treated with serial dilutions of antisera or preimmune sera. Incorporation of Tween 20 with the conjugate was found to allow less nonspecific conjugate binding than did the method of Pestka et al. (8). In the competitive ELISA, this step was performed by simultaneously incubating 25 μl of T-2 standard or extracts of infected corn diluted in 10% methanol in PBS with 25 μl of the T-2HS–horseradish peroxidase conjugate diluted 1:150 in PBS-BSA-Tween 20. In both assays, incubation at 37°C for 1 h followed. The plates were then washed six times with PBS-Tween 20 as described above. Bound horseradish peroxidase per well was assayed (7), and absorbance at 410 nm was determined with a Microelisa Mini Reader MR590 (Dynatech Laboratories, Inc., Alexandria, Va.).

Since other trichothecenes might be present in samples of *F. sporotrichioides*-infected corn, various standards were tested by competitive ELISA to determine cross-reactivity in the T-2 ELISA. The trichothecenes tested were HT-2 toxin, verrucarol, diacetoxyscirpenol, and roridin A, all from Sigma Chemical Co., deoxynivalenol from MycoLab, and acetyl T-2, neosolaniol, T-2 triol, and T-2 tetraol, prepared as previously described (1, 11). The structures of these trichothecenes are shown in Table 1. The reactivities of these trichothecenes in the ELISA relative to T-2, as indicated by the concentration required for 50% inhibition of

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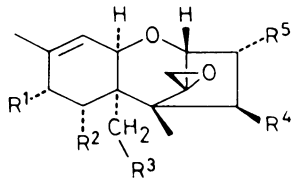
TABLE 1. Structures of trichothecenes tested



Name	R^1	R^2	R^3	R^4	R^5
T-2	$OCOCH_2CH(CH_3)_2$	H	$OCOCH_3$	$OCOCH_3$	OH
T-2HS	$OCOCH_2CH(CH_3)_2$	H	$OCOCH_3$	$OCOCH_3$	$OCOCH_2CH_2COOH$
Acetyl T-2	$OCOCH_2CH(CH_3)_2$	H	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$
HT-2	$OCOCH_2CH(CH_3)_2$	H	$OCOCH_3$	OH	OH
T-2 triol	$OCOCH_2CH(CH_3)_2$	H	OH	OH	OH
Neosolaniol	OH	H	$OCOCH_3$	$OCOCH_3$	OH
T-2 tetraol	OH	H	OH	OH	OH
Verrucarol	H	H	OH	OH	H
Diacetoxyscirpenol	H	H	$OCOCH_3$	$OCOCH_3$	OH
Deoxynivalenol	=0	OH	OH	H	OH
Roridin A	H	H	A 14-carbon diester		H

conjugate binding, is summarized in Table 2. Both T-2HS and T-2 inhibited conjugate binding at 50 pg/ml. Only trichothecenes containing the isovaleroxy moiety at the R^1 position (Table 1) inhibited conjugate binding at a concentration comparable to that of T-2. As an illustration of this point, neosolaniol, similar in structure to T-2 except for this moiety, inhibited conjugate binding at only 0.1% of the level of T-2. However, as indicated by the low affinity of T-2 triol for the T-2 antibody (Table 2), not all analogs with the isovaleroxy moiety are cross specific. The minor reaction of trichothecenes without the C-8 isovaleroxy moiety would be insignificant in assays employing this ELISA method. The macrocyclic trichothecene roridin A was the only trichothecene tested that did not inhibit conjugate binding at 500 μg/ml. Similar cross-reactivities of various trichothecenes for T-2 antibody have been found in T-2 radioimmunoassay (2) (Table 2), suggesting that no significant specificity differences occur when radiolabeled or horseradish peroxidase-labeled T-2 is used as an immunoassay ligand.

The effectiveness of ELISA in detecting T-2 in *F. sporotrichioides*-infected corn was tested. Ears of corn (inbred B79) were inoculated at the milk stage of development by inserting toothpicks infested with a single strain of *F. sporotrichioides* through the husks into the center of the ear (3). *F. sporotrichioides* strains used were NRRL 3299, F27, T-2, F38, and T-340. Strains T-2 and T-340 were obtained from E. B. Smalley and R. W. Caldwell (Department of Plant Pathology, University of Wisconsin); strains NRRL 3299 and F38 were obtained from C. J. Mirocha (Department of Plant Pathology, University of Minnesota); and strain F27 was from the culture collection of L. P. Hart.

For ELISA determinations, 25 g of infected kernels were extracted by blending for 5 min with 250 ml of methanol-water (60:40). The solids were removed by filtration through no. 4 filter paper (Whatman, Inc., Clifton, N.J.), and the extracts were diluted to various degrees (up to 4×10^6 times) with 10% methanol in PBS.

ELISA of 60% methanol-extracted, noninfected corn revealed that the extract could be diluted minimally (sixfold) with PBS without causing significant interference of peroxidase conjugate binding in the assay. However, when the methanol was evaporated out of the extract and then readded to the extract to 10% (vol/vol), interference with peroxidase conjugate binding occurred. Therefore, preparations for ELISA reported herein did not include methanol evaporation. Preliminary experiments with *F. sporotrichioides* F38-infected and T-2-spiked, noninfected corn samples indicated that T-2 could be detected by ELISA in samples prepared by methanol extraction with recoveries of 30 to 60%. Both infected and spiked samples had equivalent concentrations of T-2 (ca. 20 ppm [20 μg/g]).

The detection of T-2 in corn by ELISA was compared with detection by GLC for nine different samples of corn inoculated in the field with various isolates of *F. sporotrichioides* (Table 3). Visibly molded kernels were removed from infected ears and dried at 65°C for 2 days, followed by dry chopping for 45 s in a blender set at low speed. For sample 1 (Table 3), the entire cob, which was extensively molded, was dry chopped for 90 s. Each sample was divided into two subsamples, and ELISA determination of T-2 concentration was made on one subsample after dilution of the methanol-

TABLE 2. Specificity and sensitivity of T-2 antibody in ELISA for T-2 and other trichothecenes

Trichothecene	% Cross-reactivity relative to T-2[a]	Minimum sensitivity (ng/ml)[b]
T-2	100 (100)[c]	0.05
T-2HS	100 (ND)	0.05
Acetyl T-2	100 (ND)	0.05
HT-2	3.4 (17.5)	0.1
Neosolaniol	0.1 (0.2)	100
T-2 triol	0.1 (2.1)	50
T-2 tetraol	<0.1 (0.07)	500
Deoxynivalenol	<0.001 (<0.01)	10,000
Verrucarol	0.003 (ND)	5,000
Diacetoxyscirpenol	0.001 (<0.01)	10,000
Roridin A	<0.001 (ND)	>100,000

[a] Nanograms of T-2 required for 50% inhibition/nanograms of trichothecene required for 50% inhibition × 100.

[b] Nanograms of trichothecene per milliliter required for first significant inhibition ($P = 0.05$ by Student's t test on four replications per ELISA) of peroxidase conjugate binding.

[c] Numbers in parentheses indicate percentage of cross-reactivity relative to T-2 determined for T-2 radioimmunoassay by Chu et al. (2). ND, Not determined.

TABLE 3. Comparison of ELISA and GLC methods for determination of total trichothecenes in *F. sporotrichioides*-infected corn samples[a]

Sample	*F. sporotrichioides* strain	ELISA (mean [3 determinations])[b]	GLC[c]			
			T-2	HT-2	T-2 triol	Neosolaniol
1	NRRL 3299	20.0 (15, 20, 25)	9.5	10.0	tr[d]	
2	F27	23.3 (15, 25, 30)	11.4	1.6	tr	4.2
3	F27	<0.05 (<0.05, <0.05, <0.05)	10.9	0.3		tr
4	T-2	0.08 (0.1, 0.1, 0.05)	1.4	1.3		
5	T-2	0.5 (0.3, 0.6, 0.6)	2.3	0.4		
6	T-2	25.0 (20, 25, 30)	19.9	21.2		
7	T-340	33.3 (15, 25, 60)	47.7	42.4	3.5	2.8
8	T-340	1.2 (1.0, 1.0, 1.5)	2.0	4.0		
9	T-340	0.3 (0.4, 0.4, 0.15)	1.0	0.9		

[a] All values are in parts per million. ELISA results are of total trichothecenes in the sample with affinity for the T-2 antibody.
[b] Each determination is the average of four replications of infected corn extract per ELISA plate compared with replications on the same plate of noninfected corn extracts diluted to the same degree and spiked with various amounts of T-2.
[c] Levels uncorrected for spike recoveries (85% for T-2, 76% for HT-2). GLC analysis was made with heptafluorobutyryl derivatives and a ^{63}Ni electron capture detector (9).
[d] tr, Trace (<0.5 ppm).

water extract as described above. The other subsample was subjected to GLC determination of toxin concentration after extraction and cleanup procedures as described by Scott et al. (9). GLC was performed with heptafluorobutyryl derivatives and a ^{63}Ni electron capture detector (9). Thin-layer chromatography by the method of Takitani et al. (10) was used to confirm the presence of the various trichothecenes tested. The efficiency of recovery by the GLC method in samples spiked with 2.5 ppm was 85% for T-2 and 76% for HT-2. The results obtained by the two methods are shown in Table 3. A linear regression analysis of ELISA versus GLC for T-2 concentration was significant at $P = 0.01$ (df = 1, 7; $F = 13.95$). The regression equation was $Y = 2.716 + 0.747X$, where X = T-2 concentration as determined by GLC. The correlation coefficient (r) was 0.816.

For sample 3 (Table 3), T-2 was not detected by ELISA, but 10.9 ppm was detected by GLC. This disparity might be related to sampling problems. Further samples from the same lot were not available for reanalysis. It is also possible that this isolate might produce substances that interfere with the interaction of T-2 with antibody during ELISA or cause a peak at the same retention time as T-2 heptafluorobutyrate during GLC analysis.

This is the first report of T-2 detection in infected grains by immunological methods. The results presented indicate that ELISA could be used as a semiquantitative tool for the detection of T-2 in *F. sporotrichioides*-infected corn. Acetyl T-2, which also may occur in grains (5), could also be detected by this ELISA. The high sensitivity exhibited is much greater than is required to detect T-2 at toxic concentrations (6), and the extraction procedures are simpler than those used in other assays (6, 8). This assay could therefore be very useful for routine screening of large numbers of corn samples for T-2 in the time required to analyze a much smaller number of samples by conventional chemical means (6).

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