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An Indirect Enzyme Immunoassay for the Mycotoxin Citrinin†

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An indirect competitive enzyme immunoassay using rabbit antisera could detect citrinin in buffer solutions at 1 to 13 ng/ml (0.05 to 0.65 ng per assay). Cross-reactivity with austdiol, alternariol, ochratoxin A, and deoxynivalenol was <0.1% relative to citrinin. Recovery of citrinin added to wheat flour at 200 to 2,000 ng/g was 89 to 104%, with a coefficient of variation of 6.9 to 13%.

The nephrotoxin citrinin is produced by *Aspergillus* and *Penicillium* species (1) and is often found together with another mycotoxin, ochratoxin A. Citrinin occurs in wheat flour (13), rye, oats (17), barley (6), corn (12), and feed grains (7) and has been assayed by thin-layer chromatography techniques (2, 4, 5, 18). Liquid chromatography has been used to estimate citrinin levels in cereals (8, 11), biological fluids (14), and fermentation media (20). Immunochemical methods provide a convenient and sensitive alternative for detecting many mycotoxins (3), but no practical immunoassay for citrinin has yet been reported.

Immunization. Keyhole limpet hemocyanin (KLH [6.0 mg]) from *Megathura crenulata* was dissolved in 0.8 ml of sodium acetate (0.1 mM [pH 4.2]). To this was added 1.0 mg of citrinin in 0.2 ml of methanol and 320 μl of 37% formaldehyde solution (10, 21). After 24 h at 37°C, the mixture was dialyzed at 4°C for 3 days against three changes of phosphate-buffered saline (PBS [0.01 mM phosphate, 0.1 mM NaCl at pH 7.3]). The resulting citrinin-KLH conjugate, which contained precipitates, was made up to 1.5 ml with saline and emulsified with 4.5 ml of Freund's complete adjuvant. KLH is sparingly soluble in acidic solution in native form and tends to precipitate during most conjugation reactions. Nevertheless, the precipitates were used for immunization, because previous work had shown examples of precipitated KLH coupled to haptens in a potent immunogenic form (16). Three rabbits (female Chinchilla; Savo Ivanovna, Kisslegg, Germany) each received 2 ml of the emulsion described above (containing 2 mg of citrinin-KLH) intradermally at 20 to 30 sites on their backs, which had been shaved.

Labeled antigen. For citrinin labeling, glucose oxidase from *Aspergillus niger* (GOX) was selected for its high solubility at pH 4 to 5 and its high molecular mass (1.86×10^5 Da). GOX (3.7 mg) was conjugated to citrinin as described above, except the mixture was incubated for 72 h at 22°C. After dialysis, the protein content (9) was 0.9 mg/ml. The difference in A_{330} between the conjugate and an equivalent amount of GOX was used to estimate the level of protein-bound citrinin, assuming the A_{330} for citrinin to be 8,000. Approximately three molecules of citrinin appeared to be bound per molecule of GOX.

EIA. The citrinin-GOX conjugate was diluted 1:1,000 with sodium carbonate buffer (0.05 mM [pH 9.6]), and 100 μl was added per well to microtiter plates, which were kept overnight at >90% relative humidity. Free protein-binding sites were blocked with casein sodium salt (2% in PBS) for 30 min before being washed with 0.85% NaCl solution containing Tween 20 (250 μl/liter) and decantation. Sera from the rabbits 13 weeks after the initial injection were diluted with PBS, and 100 μl was added per well. After 1 h, each plate was washed with NaCl-Tween solution and decanted. Antibody against rabbit immunoglobulin G developed in goats and conjugated to horseradish peroxidase (Sigma-Aldrich) was diluted 1:5,000 in PBS containing 1% casein sodium salt, and 100 μl was added per well. After 1 h, each plate was washed with NaCl-Tween, and 100 μl of an enzyme substrate solution containing 3 mM H_2O_2 and 1 mM 3,3′, 5,5′-tetramethylbenzidine in potassium citrate

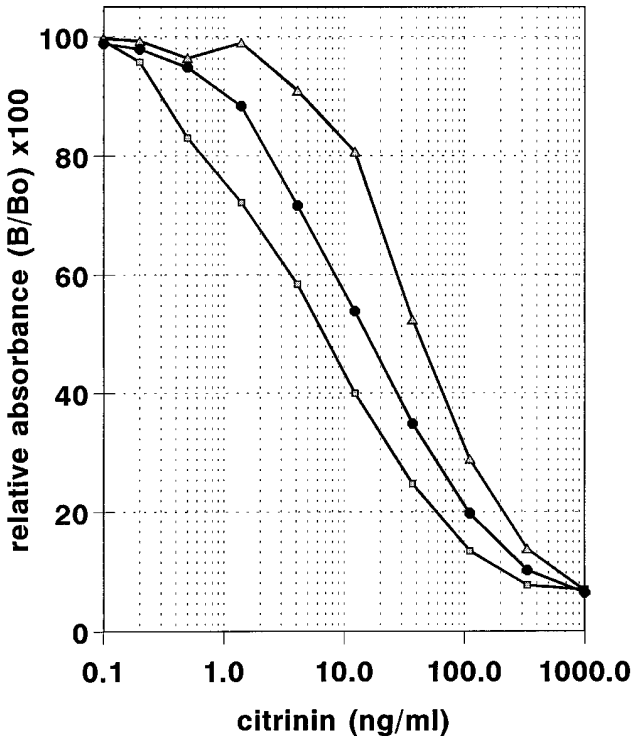

FIG. 1. Standard curves for competitive EIA detection of citrinin with antiserum samples from three rabbits. ●, rabbit 1; ▲, rabbit 2; ■, rabbit 3. B/B_o, absorbance of test relative to that of the negative control.

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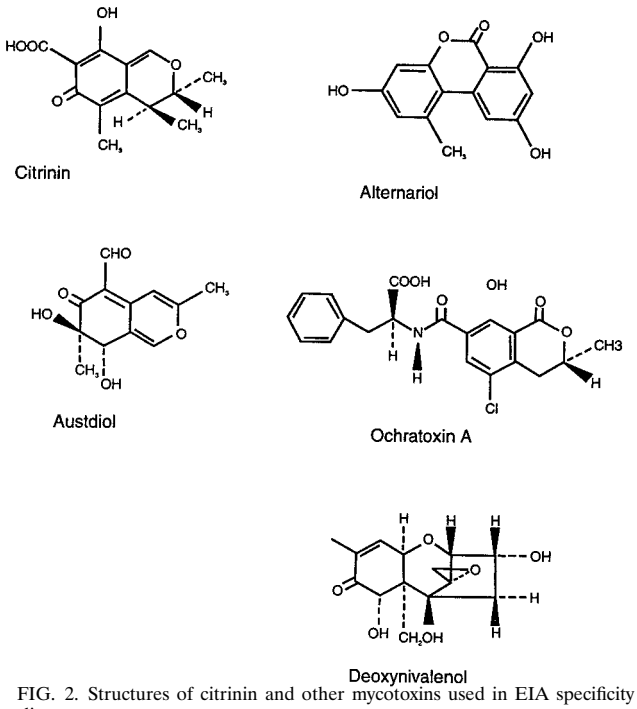

FIG. 2. Structures of citrinin and other mycotoxins used in EIA specificity studies.

buffer (0.2 mM [pH 3.9]) was added per well. After 15 min, the reaction was stopped with 1 M H_2SO_4 (100 μl per well), and the A_{450} was measured.

Optimum dilutions for coating microtiter plates with citrinin-GOX and for the antisera were determined with checkerboard titrations with and without citrinin added. Maximum assay sensitivities and B_o (negative control) values of 1.0 to 1.2 resulted from a citrinin-GOX dilution of 1:1,000 and antiserum dilutions of 1:10,000 (rabbits 1 and 2) and 1:1,000 (rabbit 3). For the standard curves (Fig. 1), 50 μl of these diluted sera per well was used. Solutions of citrinin in 10% methanol in PBS were then added (50 μl per well). The enzyme immunoassay (EIA) was conducted as described above. With Student's *t* test, concentrations of citrinin at the detection limit were 1 (0.05 ng per assay [rabbit 3]), 8 (0.4 ng per assay [rabbit 1]), and 13 (0.65 ng per assay [rabbit 2]) ng/ml; respective concentrations causing 50% inhibition of binding were 6, 15, and 40 ng/ml at a confidence level of 95%. The linear parts of the standard curve were in the ranges of 1 to 40 ng/ml (rabbits 1 and 3) and 20 to 200 ng/ml (rabbit 2), and intra-assay coefficients of variation for standard concentrations were generally below 7.5% (n = 4).

By using an indirect assay format, a simple and reliable procedure with a low level of intraplate color intensity variations was established. The citrinin-GOX conjugate was pure enough to have sufficient antibody binding capacity and had an acceptable low level of background color reaction. Plates coated as described above could be stored for 3 weeks at 4°C without loss of activity. Because ~25 ml of serum could be obtained from a single bleeding, the antiserum dilutions (1:10,000 and 1:1,000) used in this indirect EIA ensured ample availability of the immunoreagent.

Cross-reactivity. EIA specificity was studied with different mycotoxins as competitive antigens. Austdiol and alternariol (Sigma-Aldrich), produced by *Aspergillus ustus* and *Alternaria alternata*, respectively, were selected because of structural similarities to citrinin (Fig. 2) and possible co-occurrence with citrinin in agricultural commodities. Ochratoxin A also shares structural features with citrinin and commonly co-occurs with citrinin in cereals (1). Deoxynivalenol was included because it is the most common mycotoxin in cereals on a global basis (19). None of these toxins inhibited antibody binding to the solid-phase antigen in concentrations up to 10,000 ng/ml.

TABLE 1. Recovery of citrinin from artificially contaminated wheat flour

Amt of citrinin added (ng/g)	n^a	Amt of citrinin found		
		Mean recovery (%)	SD (ng/g)	Coefficient of variation (%)
200	6	104	16.4	7.9
500	6	93	56.0	12.0
1,000	6	89	114.0	13.0
2,000	8	94	130.0	6.9

[a] n, number of determinations.

Application to wheat flour. Wheat (17) and wheat products (13, 15) appear to be good substrates for citrinin production, and so wheat flour was selected as a matrix for testing EIA performance. Samples (2 g) of commercial wheat flour were artificially contaminated with methanol solutions of citrinin to give final concentrations of 0, 200, 500, 1,000, and 2,000 ng of citrinin per g of flour. Each sample was stirred with 10 ml of 10% methanol in PBS for 30 min. After filtration through Whatman 1 paper, the extract was diluted 1:10 (or as appropriate) for the EIA with serum from rabbit 3 diluted 1:1,000. Duplicate samples were assayed for 3 to 4 consecutive days; the results are indicated in Table 1. With a previous thin-layer method (17), 63 and 86% recoveries from wheat flour were found for citrinin added at 500 and 1,000 ng/g, respectively. With a previous liquid chromatography procedure for ground corn (11), recoveries were 92.0% (coefficient of variation, 10.6%), 69.5% (coefficient of variation, 10.9%) and 75.8% (coefficient of variation, 22.8%) for citrinin added at 200, 1,000 and 2,000 ng/g, respectively (n = 4). Our EIA produced higher levels of recovery of citrinin added to wheat flour at similar concentrations, although the methods are not strictly comparable because of different matrices.

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