ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR MYCOTOXINS USING MONOCLONAL ANTIBODIES

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Abstract

Specific monoclonal antibodies against aflatoxin B_1 (AFB_1), T-2 toxin (T2) and ochratoxin A (OA) were used in competitive enzyme-linked immunosorbent assays (ELISAs) to detect 0.1 ng ml^{-1} AFB_1, 10 ng ml^{-1} T2 or 1 ng ml^{-1} OA extracted together from barley grain. Acetonitrile-0.5% KCl-6% H_2SO_4 (89+10+1) extracts of barley grain were either diluted to 1:10 for direct assay or were treated by a simple cleanup procedure which concentrated the extract 10:1 before assay. Mean recoveries from barley artificially contaminated with pure AFB_1, T2 and OA were 93.8%, 80.6% and 95.8%, respectively. The mean within-assay, inter-assay and subsample coefficients of variation by ELISA were <12% for AFB_1 and OA but up to 17% for T2.

Introduction

Mycotoxins are toxic secondary metabolites of fungi produced in grain before harvest and during storage. Chemical methods for quantifying mycotoxins in cereal grains, e.g., thin-layer chromatography, liquid chromatography or gas chromatography, are time consuming, require extensive cleanup of samples and are expensive. By contrast, immunochemical methods based on antigen-antibody reactions are fast, specific, sensitive and inexpensive (Pestka, 1988).

Using polyclonal antibodies, enzyme-linked immunosorbent assays (ELISA) have previously been described for aflatoxin B_1 (Pestka, 1980; Ram *et al.*, 1986), zearalenone (Liu *et al.*, 1985), trichothecenes (Pestka, 1981, Xu *et al.*, 1988) and for ochratoxin A (Lee and Chu, 1984; Morgan *et al.*, 1983). However, polyclonal antisera can be produced in only limited amounts, and can change in affinity and specificity from bleeding to bleeding. Monoclonal antibodies are of consistent affinity and specificity and have the potential to be produced in unlimited quantities. Monoclonal antibodies are now available for aflatoxins (Candlish *et al.*, 1985; Groopman *et al.*, 1982; Lubet *et al.*, 1983), trichothecenes (Fan *et al.*, 1988; Gendloff *et al.*, 1987; Goodbrand *et al.*, 1987; Hack *et al.*, 1989; Hunter *et al.*, 1985), ochratoxins (Candlish *et al.*, 1988; Chiba *et al.*, 1985) and zearalenone (Dixon

et al., 1987).

Cereal grain can often be contaminated with several mycotoxins and their simultaneous extraction and/or determination saves time and costs. We have used the monoclonal antibodies for aflatoxin B_1 (AFB_1), T-2 toxin (T2) and ochratoxin A (OA) developed by Candlish and his colleagues at the University of Strathclyde, to optimize ELISA for the assay of the three mycotoxins in a single extract from barley grain. Further details may be found in Ramakrishna *et al.* (1990).

Materials and methods

Monoclonal antibodies and conjugates

Hybridoma cell lines 4E1, 15E7 and 10E2 producing specific monoclonal antibodies (McAb) for AFB_1, T2 and OA, respectively, were developed by Candlish *et al.* (1985; 1988) and Goodbrand *et al.* (1987). The McAb of AFB_1 and T2 were conjugated to horse-radish peroxidase (HRP) by a simplified periodate method (Kurstak, 1985). The conjugates are referred to in text as AFB_1-McAb-HRP and T2-McAb-HRP.

Mycotoxin conjugates

Mycotoxin-Bovine Serum Albumin (BSA) conjugates were used for coating microtitre plates. The conjugates AFB_1-BSA and T2-BSA were purchased from Sigma Chemical Co. OA was coupled to BSA by the modified water-soluble carbodiimide method of Chu *et al.* (1976)

ELISA protocols

A direct competitive ELISA was developed for AFB_1 and T2 after conjugation of their McAb to HRP while an indirect competitive ELISA was used for OA.

Preparation of microtitre plates. AFB_1-BSA, T2-BSA or OA-BSA at concentrations of 1-2 $\mu g\ ml^{-1}$ in Tris HCl buffer, pH 9.0 (coating buffer) was adsorbed onto microtitre plates (Nunc Immunoplate MaxiSorp, 150 μl/well) at 4 °C overnight or at 32 °C for 3 h. The plates were then washed four times with buffer, containing 0.02 M Tris, 0.05% Tween-20 and 0.15 M NaCl, pH 7.4 (Tris HCl buffer). The washed plates were treated with 2 mg ml^{-1} BSA in coating buffer (150 μl/well) for 30 min at 32 °C, and washed four times with Tris HCl buffer.

Direct competitive ELISA of AFB_1 or T2. Microtitre plates, sensitized with AFB_1-BSA or T2-BSA, were incubated with samples or standards (100 μl/well) together with AFB_1-McAb-HRP or T2-McAb-HRP conjugate (50 μl/well) for 1 h at 32 °C. Neutralized antibody was removed by washing four times with Tris HCl buffer and bound enzyme activity was measured using tetramethyl benzidine + H_2O_2 in NaAc buffer (150 μl/well). The reaction was stopped after 30 min with 10% H_2SO_4 (50 μl/well) and absorbance measured at 450 nm using a Titertek Multiscan ELISA reader.

Indirect competitive ELISA of OA. Samples or standards (100 μl/well) were first incubated with OA-McAb (50 μl/well) in the microtitre plates sensitized with OA-BSA for 1 h at 32 °C. The plates were washed four times with Tris HCl buffer and incubated with 150 μl/well goat anti-mouse IgG-HRP conjugate (at a dilution of 1:1000) for 1 h at 32 °C. Plates were then washed four times and the bound enzyme activity was measured as above.

On each microtitre plate, two replicate wells were prepared with each standard and three with each sample.

Calculations

Standard curves of AFB_1, T2 and OA were obtained by plotting $\log_{10}$ concentration against absorbance value. Concentrations of AFB_1, T2 and OA in the sample extract were determined from the standard curves and these were used to calculate the amount in the original sample: Toxin conc.(ng g^{-1}) = Toxin conc.(ng ml^{-1}) in sample extract x Sample extract vol. (ml) / Sample wt (g).

Preparation of barley samples for ELISA

Barley grain (10 g) was extracted with 50 ml of Acetonitrile-0.5% KCl-6% H_2SO_4 (89+10+1) mixture by blending for 2 min in a Waring blendor. The extract was filtered through Whatman No. 41 filter paper and diluted 1:10 with Tris HCl buffer for direct assay in ELISA. The acetonitrile extract of grain subjected to water and chloroform cleanup and concentrated 5:1 or 10:1 could also be used in ELISA. For cleanup, 10 ml of acetonitrile extract was transferred to a separation funnel and mixed with 10 ml of distilled water and 10 ml chloroform with shaking for 2 min. The mycotoxins were partitioned into the chloroform, leaving polar contaminants in the upper aqueous phase. The chloroform layer was collected and evaporated to near dryness in a rotary evaporator and redissolved in 1 or 2 ml Tris HCl buffer containing 9% acetonitrile for ELISA.

Recovery of AFB_1, T2 and OA from artificially contaminated barley

Pure AFB_1, T2 and OA were added to 10 g ground barley samples one day before extraction. Samples were extracted, filtered and diluted 1:10 with Tris HCl buffer for direct assay in ELISA. Samples spiked with less than 10 ng g^{-1} AFB_1, 500 ng g^{-1} T2 and 50 ng g^{-1} OA were cleaned up by water and chloroform prior to ELISA.

Estimation of AFB_1, T2 and OA in barley colonized with toxigenic fungi

Barley grain colonized with toxigenic *Aspergillus flavus, Fusarium poae* or *Penicillium verrucosum* were mixed with healthy grain in different proportions to give contaminated samples containing a range of concentrations of AFB_1, T2 and OA. Samples of 100 g each were finely ground and 10 g sub-samples were extracted for AFB_1, T2 and OA. Within-assay, inter-assay and subsample coefficients of variation (CV) were calculated, where CV(%) = (Standard deviation / Sample mean) x 100.

Results and Discussion

The monoclonal antibodies of AFB_1, T2 and OA have been characterized previously (Table I). The ELISA techniques used were based on competition between mycotoxin-BSA conjugate bound to the ELISA solid phase and free mycotoxin in the sample extract for the specific binding site on the McAb. The neutralized antibody is removed after incubation by washing and the bound antibody is estimated using an enzyme-substrate reaction (Figure 1).

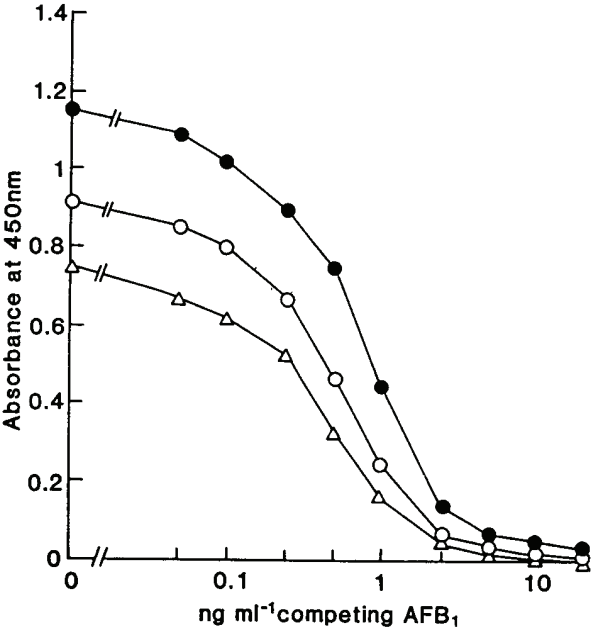

Figure 2. Standard curves for AFB_1 by direct competitive ELISA at different dilutions of the McAb-HRP conjugate: 1:2000 (●), 1:3000 (○) and 1:4000 (△).

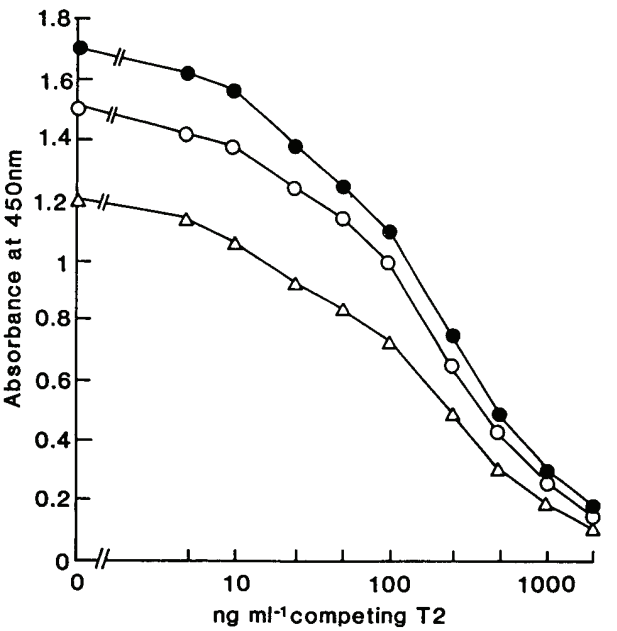

Figure 3. Standard curves for T2 by direct competitive ELISA at different dilutions of the McAb-HRP conjugate: 1:5000 (●), 1:6000 (○) and 1:8000 (△).

Table I. Characteristics of AFB_1, T2 and OA monoclonal antibodies (Candlish *et al.* 1985, 1988; Goodbrand *et al.*, 1987).

McAb to	Cell line	Protein conc (mg ml^{-1})	Class	Subclass	Cross reactivity to related metabolites[1] (%)		
AFB_1	4E1	31.5	IgG	$_{\gamma}G2a(\kappa)$	15 (AFG_1)	13(AFB_2)	1(AFG_2)
T2	15E7	19.4	IgG	$_{\gamma}G1$ (κ)	25 (HT2)		
OA	10E2	15-20	IgG	$_{\gamma}G1$ (κ)	8 (OC)	0.6 (Oα)	

AFG_1 = Aflatoxin G_1, AFB_2 = Aflatoxin B_2, AFG_2 = Aflatoxin G_2, HT2 = HT-2 toxin, OC = Ochratoxin C and Oα = Ochratoxin α.

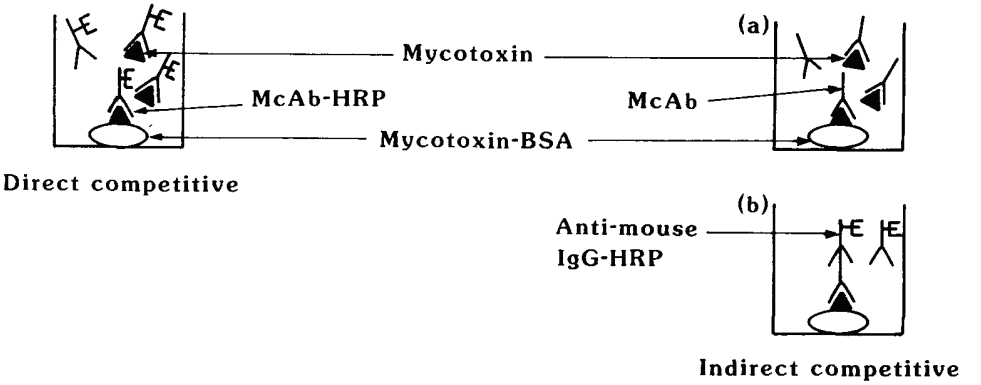

Figure 1. Schematic illustration of direct and indirect competitive ELISA for mycotoxins. In the direct competitive ELISA (AFB_1 or T2) samples or standards are directly incubated with McAb-HRP conjugate in the sensitized microtitre plates while in the indirect competitive ELISA (OA) samples or standards are (a) first incubated with McAb and then with (b) goat anti-mouse IgG-HRP conjugate.

Standard curves of AFB_1, T2 and OA

Figures 2-4 show, respectively, standard curves for AFB_1, T2 and OA by ELISA with different dilutions of the McAb-HRP conjugate or the McAb. The standard inhibition curves by ELISA were sensitive to concentrations of 0.1 to 5 ng ml^{-1} AFB_1 (Figure 2), 10 to 2000 ng ml^{-1} T2 (Figure 3), and 1 to 250 ng ml^{-1} OA (Figure 4). These sensitivities were obtained at McAb-HRP conjugate dilutions of 1:2000 for AFB_1 and 1:5000 for T2 or when OA-McAb was used at a dilution of 1:5000. The sensitivity of AFB_1 and OA ELISA are comparable to those previously reported using either polyclonal (Ram *et al.*, 1986; Morgan *et al.*, 1983; Lee and Chu, 1984; Chu *et al.*, 1987) or McAb (Chiba *et al.*, 1985). The sensitivity of T2 ELISA is similar to that using two other McAb (Hunter *et al.*, 1985; Gendloff *et al.*, 1987) but slightly less sensitive than that using a third McAb (Fan *et al.*, 1988).

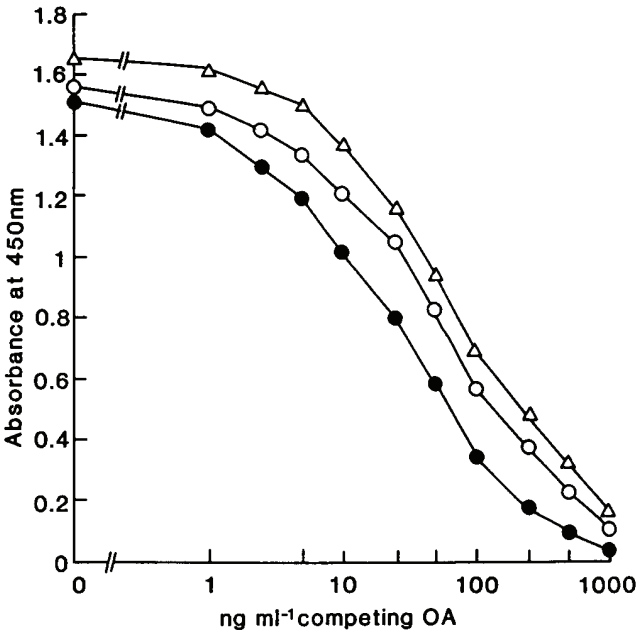

Figure 4. Standard curves for OA by indirect competitive ELISA at different dilutions of the McAb: 1:1000 (△), 1:2000 (○) and 1:5000 (●).

Effect of barley extract on ELISA

AFB_1, T2 and OA have diverse physical and chemical properties. OA is acidic and requires a solvent of <3 pH for efficient extraction from barley grain with organic solvents (Chu, 1974; Takeda *et al.* 1979). We used 1 ml of 6% H_2SO_4 in 100 ml of extraction solvent to give a solvent of 0.90 pH (Table II). Initially we tested extracts of healthy (toxin-free) barley grain for their interference in ELISA. Undiluted acetonitrile extract of barley caused interference in ELISA of all three mycotoxins. Diluting acetonitrile extracts of barley to 1:10 with Tris HCl buffer completely eliminated this interference and samples could then be used for direct assay. Alternatively, the acetonitrile extract, after a water and chloroform cleanup and concentration of 5:1 or 10:1, could be used without any interference in ELISA (Table III).

Table II. pH of grain extract at different stages of sample preparation.

Acid conc in 100 ml extraction solvent	Initial pH	pH of acetonitrile extract		
		undiluted	1: 10 diluted with buffer	on addition of water for cleanup
0%	5.65	-	7.40	-
1 ml 5% H_2SO_4	0.95	2.85	7.35	2.95
1 ml 6% H_2SO_4	0.90	2.35	7.30	2.75
1 ml 10% H_2SO_4	0.90	1.85	7.15	2.65

Table III. Effect of barley extract on the binding of McAb-HRP conjugates of AFB_1 or T2, or OA-McAb to ELISA solid-phase.

Acetonitrile extract diluted[2] or concentrated[3]		% Inhibition[1] of McAb binding in ELISA of: AFB_1	T2	0A
Undiluted		96	95	74
Diluted	1 : 2	99	58	30
	1 : 4	98	10	22
	1 : 8	16	0	1
	1 :10	0	0	0
Concentrated	5 : 1	0	0	0
	10 : 1	3	0	0

[1]Inhibition (%) = 1 - (A sample extract / A no sample extract) X 100;
A = Absorbance at 450 nm.
[2]Extracts were diluted in Tris HCl buffer before assay.
[3]Sample re-extracted in chloroform, evaporated to dryness and reconstituted in Tris HCl buffer containing 9% acetonitrile.

Standard curves of AFB_1 and OA prepared in buffer and buffer containing barley extract, diluted 1:10 or cleaned up and concentrated 5:1, were similar but those with T2 differed slightly from one another. T2 ELISA was also highly sensitive to slight changes in acetonitrile concentration either in the sample or in standard solutions. Therefore standard solutions of T2 were prepared for ELISA using healthy barley extract for ELISA so that both samples and standards had similar concentrations of acetonitrile and barley extract.

Recovery of AFB_1, T2 and OA from artificially contaminated barley

The mean recoveries of AFB_1, T2 and OA were 98%, 78% and 98%, respectively, by direct assay of acetonitrile extracts after 1:10 dilution. The water and chloroform cleanup procedure is particularly useful for improving the lower detection limit by direct assay of T2 from 500 ppb to 50 ppb or less (Table IV).

Table IV. Recovery of AFB_1, T2 and OA from artificially contaminated barley samples as determined by ELISA

Mycotoxin	Without cleanup			With cleanup		
	Conc spiked (ng g^{-1})	Recovery ± SD (%)	CV (%)	Conc spiked (ng g^{-1})	Recovery ± SD (%)	CV (%)
AFB_1	10 - 60	98 ± 10	10.2	4 - 8	88 ± 8.5	9.7
T2	500 - 5000	78 ± 13	16.7	50 - 100	84 ± 12	14.3
OA	50 - 500	98 ± 9	9.2	5 - 10	92 ± 7	7.6

Estimation of AFB_1, T2 and OA in barley colonized with toxigenic fungi

Mycotoxin concentrations in colonized barley ranged from 3 ppb to 21 ppm. The mean within-assay, inter-assay and subsample CVs were smaller for AFB_1 and OA ELISA than for T2 ELISA (Table V). As the T2 McAb is only moderately sensitive (Figure 3), its response to small changes in the concentration of T2 (< 10 ng ml^{-1}) during McAb neutralization may not be linear. Hence, error associated with T2 estimation could be greater than with the other assays. Also the CV in T2 ELISA was greater with barley containing < 1 μg g^{-1} (19%) than with 3 μg g^{-1} T2 (12%). The within-assay and inter-assay CV in T2 ELISA were smaller than those reported using polyclonal antibodies, where the within-assay CV reached 22% (Pestka *et al.*, 1981).

Table V. Variability in ELISA of fungal colonized barley grain.

Mycotoxin	Conc range ($ng\ g^{-1}$)	Coefficient of variation (%): Mean within assay[1]	Mean inter-assay[1]	Subsample[2]
AFB_1	3 - 585	8.7	11.9	8.1
T2	461 - 20750	16.3	17.5	14.7
OA	41 - 5950	10.0	8.9	9.4

[1]Based on concs determined in four wells on three different microtitre plates.
[2]Subsamples extracted separately and conc determined by ELISA in triplicate wells on a microtitre plate.

Multiple immunoassays can thus be applied satisfactorily to a single extract of grain. This decreases the time required for sample preparation to 5 min when assaying several mycotoxins by ELISA for direct assay or 11 min when a cleanup is required. Up to 36 samples can be analyzed in duplicate on a single microtitre plate in 1½ h for AFB_1 or T2 and in 2½ h for OA.

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LES DOSAGES IMMUNOENZYMATIQUES (ELISA) DES MYCOTOXINES, PAR UTILISATION D'ANTICORPS MONOCLONAUX

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RESUME

Les mycotoxines sont des métabolites secondaires et toxiques de champignons produites dans les grains avant la moisson et pendant le stockage. Les immunodosages basés sur les réactions antigène-anticorps sont spécifiques, sensibles et bon marché pour le dosage des mycotoxines. Des anticorps monoclonaux spécifiques ont été élaborés pour l'aflatoxine B1 (AFB1), la toxine T-2 (T2) et l'ochratoxine A (OA) à l'Université de Strathclyde, au Royaume-Uni. Les dosages immunoenzymatiques (ELISA), en utilisant ces anticorps monoclonaux, ont détecté 0,1 ng/ml d'AFB1, 10 ng/ml de T2 ou 1 ng/ml d'OA. AFB1, T2 et OA ont été dosés dans un seul extrait d'orge en utilisant ces tests ELISA avec des anticorps monoclonaux. Des extraits de grains d'orge au mélange : Acétonytrile - 0,5 KCL - 6 % H_2SO_4 (89 + 10 + 1) ont été, soit dilués au 1 / 10 pour un dosage direct, soit soumis à une simple procédure de nettoyage liquide-liquide qui a concentré l'extrait 10 : 1 avant dosage. Les taux moyens de récupération obtenus avec l'orge contaminé artificiellement avec les toxines AFB1, T2 et OA pures étaient de 93,8 %, 80,6 % et 95,8 %, respectivement. Les coefficients moyens de variation entre échantillons et inter-échantillons étaient < 12 % pour AFB1 et OA mais atteignaient 17 % pour T2 avec ce type de test ELISA.