Sensitive ELISA Test for Determination of Ochratoxin A

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A direct, competitive enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody has been developed for quantitative determination of ochratoxin A (OA) in different cereals. A dichloromethane/citric acid mixture was used for extraction of cereals. This cleanup procedure proved to be as effective for ochratoxin A extraction as protocols using strong acids. The mean within-assay and interassay coefficients of variation for the standard curve was <10%. The range of this test is 1−10 ng/g, with a detection limit of 0.5 ng/g OA. The toxin recovery from cereals infected with 5−100 ng/mL OA varied between 90 and 130%.

Keywords: *Ochratoxin A; ELISA; cereals*

INTRODUCTION

Ochratoxins are dangerous byproducts of several species of some storage fungi such as *Aspergillus* and *Penicillium*. Among them, ochratoxin A (OA) is the most toxic and known to be hepatotoxic, nephrotoxic, and teratogenic and mutagenic to a wide variety of mammalian species (Clarke et al., 1993). Surveys have demonstrated the presence of OA in human blood (Breitholtz et al., 1991; Hult et al., 1982; Kovács and Ványi, 1994) and in mother's milk (Kovács and Ványi, 1994; Gareis et al., 1988) as well. The detection of OA is essential to avoid the risk of consumption since it is frequently found in cereals such as barley, oats, maize, and wheat (Ramakrishna et al., 1990). Although conventional analytical techniques (TLC, GC, HPLC) are officially accepted, they are expensive and time-consuming and need appropriate instrumentation and/or trained personnel. Proposed legislation in some countries concerning "acceptable" limits of OA such as those in Denmark for pig kidneys (<10 ppb) suggests that the assay must have high sensitivity. Immunoassays such as ELISA fulfill these requirements and have several other advantages including simplicity, low cost, reliability, low requirements for technical skills, and simple equipment. The sensitivity of these techniques enables the detection of OA at a concentration at or below proposed regulatory values. Although many reports have been published about the development of ELISA using polyclonal (Clarke et al., 1993; Lee and Chu, 1984; Märtlbauer and Terplan, 1988; Pestka et al., 1981) or monoclonal (Ramakrishna et al., 1990; Candlish et al., 1988; Kawamura et al., 1989) antibodies against OA, only a few of them have been commercialized (EZ-SCREEN ochratoxin A, EDS, U.S.A.; RIDASCREEN ochratoxin A, R-Biopharm, Germany; ochratoxin A assay kit, BioKits, U.K.).

Five years ago our institute started a research program to develop sensitive ELISA methods and test kits for routine detection of mycotoxins that are frequent contaminants in Europe. As a result, two reagent kits for measurement of *Fusarium* T-2 and F-2 toxins have been completed and have been reported elsewhere (Barna-Vetró et al., 1994).

In this report the development of a sensitive, direct, competitive ELISA based on monoclonal antibody for quantitative measurement of OA is described.

MATERIALS AND METHODS

Chemicals. OA, coumarin, and L-β-phenylalanine were purchased from Sigma Chemical Co. (St. Louis, MO). Ochratoxin B (OB) and ochratoxin α were generous gifts from M. E. Stack (U.S. Food and Drug Administration, Washington, DC); monoclonal anti-OA (used for control of enzyme-labeled OA conjugate) was kindly supplied by A. A. G. Candlish (University of Strathclyde, Scotland).

Toxin Label. The carboxylic group of OA was conjugated directly to horseradish peroxidase (RZ = 3.0) according to a mixed anhydride method as described by Märtlbauer et al. (1988). The working dilution of this conjugate was assessed in direct ELISA.

Antibody. Hybridoma supernatants were screened using the direct competitive ELISA. Detailed information regarding antibody production was published elsewhere (Gyöngyösi et al., 1996). Hybridoma selection was carried out as briefly outlined: From each supernatant 100−100 μL was added to two microplate wells previously coated with rabbit Ig anti-mouse Ig (IgM + IgG + IgA) (self-made) and incubated for 1 h at room temperature (RT). After washing, 50 μL PBS buffer (zero toxin concentration, B_0) was pipetted to one well, and to the other well was added 25 ng of OA (50 μL of 500 ng/mL OA in buffer). Immediately thereafter, 50−50 μL of OA−peroxidase conjugate in working dilution was added to each well and incubated for 1 h at RT. After removal of unbound fraction by washing, the enzyme activity of the bound enzyme conjugate was measured using tetramethylbenzidine (TMB/H_2O_2) chromogen substrate. Those antibodies were selected where OD_{450nm} at 0 toxin concentration (B_0) was >0.5 and the percent of inhibition at 25 ng toxin level was >90%. The specificity of the selected antibody (5/9G 4A4H) toward OA metabolites was tested in a direct ELISA. The cross-reactions with OA and OB were 100 and 9.3%, respectively; no cross-reaction was measured with ochratoxin α, coumarin, or L-β-phenylalanine.

Direct Competitive ELISA. Microplate wells (Immunoplate F-8, Maxisorp, Nunc, Denmark) were coated with 150 μL of rabbit Ig anti-mouse Ig (10 μg/mL) for 18 h at RT and then washed three times with 0.05% Tween 20 in distilled water. Afterward, 120 μL of diluted anti-OA (1:500) ascites

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fluid was pipetted to each well and incubated for 18 h at RT. After washing with distilled water, the plates were dried and stored in a foil bag for up to several months at 4 °C.

Fifty microliters of toxin standards or extracted samples was incubated simultaneously in wells with 50 μL of OA−peroxidase conjugate (1:4000) diluted in PBS for 1 h at RT. After four washing steps, the wells were incubated with 150 μL (TMB)/H_2O_2) substrate per well for 15 min. The color reaction was terminated by adding 50 μL of 6 N sulfuric acid, and OD_{450nm} was measured with an automated microplate reader (Labsystems Multiscan PLUS, Finland).

The standard curve of OA was obtained by plotting $\log_{10}$ concentration (x-axis) against B/B_0 (y-axis)

$$B/B_0 = (\text{OD of standard or sample})/(\text{OD of blank [no toxin added]}) \quad (1)$$

where optical density (OD) is the mean A_{450nm}.

The concentration of OA in sample extracts was calculated using the calibration curve and was expressed in nanograms per gram (ng/g). The nanogram per milliliter value was multiplied either by 5 (where sample extract was used directly) or by 50 (at 1:10 sample dilution). The slope of the standard curve was the color change per of unit concentration.

Sample Preparation and Measurement. Cereal samples (maize, barley, soy, mixed concentrates, etc.) were triturated in a grinder, and 2 g was transferred into a 50-mL Erlenmeyer flask. Thereafter, 10 mL of dichloromethane and 5 mL of 1 M citric acid were added to the flask, and the sample was vortex mixed for 5 min, sealed with parafilm, and agitated for 2 h at RT (about 22 °C) on a horizontal shaker. The whole suspension was then transferred to a centrifuge tube and centrifuged for 30 min at 4500*g*. Three phases, aqueous (upper), sample cake (middle), and dichloromethane (lower), were obtained after centrifugation. The upper aqueous phase was discarded, the sample cake was cut through, and 2 mL of the dichloromethane phase was transferred to another conical tube. Two milliliters of 1% sodium bicarbonate buffer was added to this, sealed with parafilm, and shaken for 30 min, during which time OA was transferred into the aqueous solution. The mixture was centrifuged (20 min at 4500*g*) to obtain a clear buffer solution. Four hundred and ninety microliters of the upper buffer solution was pipetted into a test tube, and 10 μL of 1 N HCl added. The sample was mixed thoroughly, and 50 μL of this solution was used directly in the ELISA. If the expected OA concentration was >10 ng/g, the final sample solution was diluted 1:10 with 1% sodium bicarbonate buffer (pH 7.5−8.0).

Recovery of OA Toxin from Artificially Infected Cereals. Pure OA (0.5−100 ng/g) was added to 2 g of finely ground cereals, and the mixture was homogenized 1 day prior to extraction. Samples were extracted and assayed as above.

HPLC Analysis of OA Toxin from Artificially Infected Cereals. HPLC analyses were carried out using a Hewlett-Packard HP 1090 Series II liquid chromatograph equipped with a binary solvent delivery system, an autoinjector, an autosampler, a temperature-controlled column compartment, and a fluorescence detector (HP 1046 A). The samples were analyzed on a BST (Bio Separation Technic Co., Budapest, Hungary), rutin C_{18} BD column (250 × 4 mm, 10 μm) (basic desactivated) according to the procedure of Nesheim et al. (1992).

RESULTS AND DISCUSSION

All immunoassays are based on the reaction of analyte with its specific antibody. The quality of this antibody (affinity, avidity, cross-reactivity) is one of the most important factors for the development of sensitive assays. High-quality antibody can be obtained by using the appropriate immunogen, immunization protocol, and screening procedures. In general, indirect or indirect competitive ELISA is used for screening monoclonal antibodies against mycotoxins (Kawamura et al., 1989).

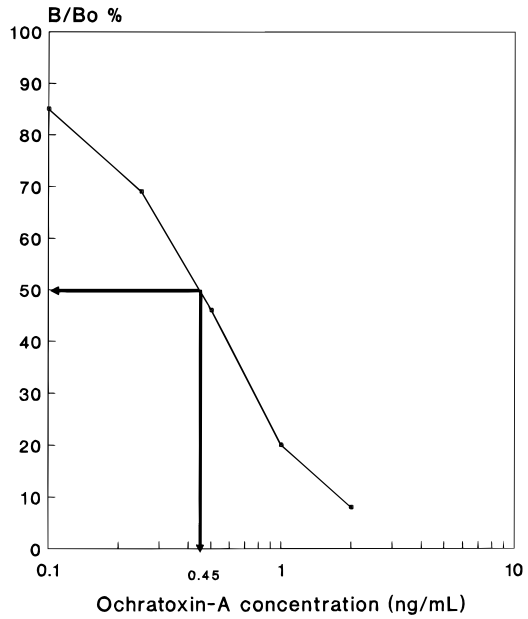

Figure 1. Dose−response curve of ochratoxin A. Fifty percent inhibition value of OA was 0.45 ng/mL; coefficients of variation for standard concentration of OA (0.1−2 ng/mL) varied between 7.3 and 8.5%.

Although highly sensitive monoclonal antibodies against other mycotoxins have been obtained by our group using this selection method and applied in the direct competitive ELISA (Barna-Vetró et al., 1994), we could not raise the appropriate quality monoclonal antibodies against OA. In this study the direct ELISA was therefore used for antibody selection (Gyöngyösi et al., 1996) since the objective of the study was to develop a direct competitive ELISA for OA. This procedure avoided the selection of nonspecific antibodies against the carrier proteins (BSA, HSA, KLH) as only the OA-specific monoclonal antibodies react. In addition to a specific antibody, a very good quality OA−peroxidase conjugate was necessary as well. The selected monoclonal antibodies were ranked according to the following criteria: assay range, the slope of calibration curves, and 50% displacement values of B/B_0 (I_{50}). Using this selection strategy, several monoclonal antibodies were obtained; the I_{50} values varied between 10 and 0.4 ng/mL OA. Monoclonal antibody (hybridoma cell line 5/9G4A4H) with an I_{50} value of 0.4 ng/mL OA was used for further studies. The first step in developing a direct, competitive ELISA is to determine the optimal dilution of antibody and mycotoxin−peroxidase. In our experiment the monoclonal antibody was coated indirectly onto the microplate using rabbit Ig anti-mouse Ig as the capture antibody; thereby, the slope of standard curve was near the optimal and the B_0 value was higher compared to test systems in which the specific antibody was coated directly onto the microplate. Figure 1 shows the dose−response curve of OA with the detection limit in buffer solution (0 ± 2 SD) being 0.042 ng/mL. The most accurate measurement is in the middle range of the curve, where the I_{50} value was 0.45 ng/mL OA. Sensitivity, defined as the slope of the standard curve at the inflection point (i.e. middle of the range), was estimated by the Statistical Graphics program (Statgraphics 5.0) and proved to be 0.95. The within-assay and interassay coefficients of variation for standard concentration of OA (0.1−2 ng/mL) were <10%. The correlation coefficient (r) of the linear part of the calibration curve was 0.991. A very important step of validation is the

Table 1. Recovery of Ochratoxin A from Artificially Contaminated Cereals[a]

added OA (ng/mL)	barley (ng/mL)	detected %[b]	goose feed (ng/mL)	detected %[b]	piglet feed (ng/mL)	detected %[b]	coleseed (ng/mL)	detected %[b]
5	4.81 ± 0.76	96 ± 15	4.5 ± 0.72	90 ± 14.0	6.5 ± 1.2	129 ± 24	6.3 ± 0.8	125 ± 16
10	9.8 ± 0.66	98 ± 6.6	10.3 ± 2.2	103 ± 22	10.1 ± 1.08	101 ± 10.8	11.7 ± 2.3	117 ± 23
20	21 ± 4.0	105 ± 20.0	21 ± 2.8	105 ± 14.0	26 ± 4.5	130 ± 22.5	22.8 ± 4.16	114 ± 21.0
50	64 ± 8.5	128 ± 17.0	57 ± 9.7	114 ± 19.4	69 ± 8.0	138 ± 16.0	64 ± 6.5	128 ± 13.0
100	127 ± 14.0	127 ± 14.0	124 ± 19.0	124 ± 19.0	108 ± 12.0	108 ± 12.0	115 ± 17.0	115 ± 17.0

[a] Each sample was spiked in three parallel experiments and then extracted and assayed in three replicates. Values are means ± SD. [b] Detected OA (ng/g)/added OA (ng/g) × 100.

Table 2. Recovery of Ochratoxin A from Artificially Contaminated Cereals As Determined by ELISA and HPLC

added OA (ng/g)	by ELISA[a]		by HPLC[a]	
	OA (ng/g)	detected (%)	OA (ng/g)	detected (%)
barley 20	18.9 ± 4.0	94.5 ± 20	15.7 ± 1.4	78.7 ± 7
50	50.3 ± 8.0	100.6 ± 16	41.8 ± 2.0	83.5 ± 4
corn 20	22.3 ± 4.1	111.5 ± 20.5	16.8 ± 1.8	84.0 ± 9
50	51.0 ± 9.2	102 ± 18.4	43.0 ± 2.3	85.9 ± 4.6

[a] Values are means ± SD. Each sample analyzed by ELISA and HPLC was done in triplicate.

detection of matrix effects, which should be minimized by the sample preparation procedure. Many reports have been published for extraction of OA from cereals using chloroform-, methanol-, or dichloromethane-based organic solvents supplemented with different acids (HCl, acetic acid, ascorbic acid, phosphoric acid) (Ramakrishna et al., 1990; Lee and Chu, 1984; Märtlbauer et al., 1988; Kawamura et al., 1989; Seidel et al., 1993). Among the several solvents tried in our experiments, the dichloromethane-based extraction method described by Märtlbauer (1988) with modification was applied. Instead of HCl, a milder acid (citric acid) was used and proved to be as effective as HCl. This type of acid has not been previously used for the extraction of OA. In this procedure the centrifugation step is one of the most crucial steps as caution must be taken to avoid the nonspecific reaction caused by incomplete separation of the aqueous buffer solution from the organic phase.

Recovery values of OA from artificially infected cereals averaged 97% as summarized in Table 1. The range of the test was 1−10 ng/g with a detection limit of 0.5 ng/g. The extraction of maize was troublesome in some cases as the sample cake remained diffuse after centrifugation. As a result, the organic extract (lower phase) could not be aspirated properly, resulting in a false positive value. The OA values of some samples measured by ELISA and HPLC were compared (Table 2). Although acceptable recoveries of OA at ELISA proved the effectiveness of our extraction procedure, the standard deviation was somewhat higher than that of HPLC. This method was used for screening of OA-producing *Aspergillus* strains as well (Téren et al., 1996) and human blood samples (Solti et al., 1997).

From the economic and stability point of view, the OA-specific antibody was precoated on the surface of polystyrene strips, the enzyme-labeled OA was freeze-dried and all other reagents (washing buffer, substrate/chromogen and stopping solutions) were kept in concentrated form. The stability of these reagents was 6 months at 4 °C. On one microplate OA concentration of 40 cereal samples can be determined in duplicate.

CONCLUSIONS

The aim of the present work was the development of a sensitive ELISA test that suits the international requirements for OA determination in foods and feeds. Instead of the indirect ELISA used generally for selection of monoclonal antibodies, we applied the direct competitive ELISA, which resulted in highly specific and sensitive monoclonal antibodies against OA. Our extraction method using the milder citric acid was as effective as other methods using stong acids for purification of OA from cereals. This inexpensive and simplified method can be applied for a large number of samples as well for screening of OA in cereals.

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