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Determination of ochratoxin A in grain by monoclonal antibody-based enzyme-linked immunosorbent assay

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Abstract— The simple rapid and sensitive enzyme-linked immunosorbent assay (ELISA) methods, direct and indirect ELISA, for quantitation of ochratoxin A in cereal had been developed by the utilization of monoclonal antibody on immunomicroplate. Direct ELIAS was found to be less time consuming than indirect ELISA. For direct ELISA, recovery of 1－500 ppb OA added to wheat was 78.9－100.0% and rice was 88.9－120.0%. For indirect ELIAS, recovery of 1－500 ppb OA added to wheat was 79.0－110.0% and rice was 82.0－120.0%. The minimal detection level for OA was 1 ppb. Analyses of 31 samples that caused humanintoxicant for OA showed that the ELISA results agreed well with those obtained by thin-layer chromatography.

Keywords: enzyme-linked immunosorbent assay (ELISA); ochratoxin A; monoclonal antibody; cereal.

1 Introduction

It is widely accepted that ochratoxin A (OA) is a tumorigenic mycotoxin (Bendele, 1985; Boorman, 1988) produced by several fungal species, such as *Aspergillus* and *Penicillium* and exhibits a high acute toxicity to a number of animal species (Ueno, 1987). OA is also a potent nephrotoxin and hepatotoxin and is believed to be involved in mould-induced nephropathy (Krogh, 1978). A positive correlation between the residues of OA in human blood and the incidence of Balkan endemic nephropathy and urinary system tumors in Bulgaria has been reported (Petkova-Bocharova, 1988). The toxin has been found in barley, wheat, oat, maize and other agricultural commodities (Steyn, 1984). The presence or absence of OA in such products can be monitored only by analytical mythology which detects the toxin. For this reason the techniques used for determination must be simple, sensitive, specific and less dependent on elaborate extract procedures, therefore, can be used for the mass survey of OA in cereals, food and feed.

The present analytical methods which are commonly used for OA determination are thin-layer chromatography (TLC) (Nesheim, 1973) and liquid chromatography (LC) (Osborne, 1980). These methods often require an extensive clean up, expensive

instruments and are time- consuming. The immunological basis of the ELISA method makes it highly specific, less dependent on elaborate clean up procedures and also offers the advantages of increased sensitivity and speed over existing methods (Kawamura, 1990; Lee, 1984). We have also prepared monoclonal antibodies against OA with high specificity (Yang, 1992). In this study, the competitive indirect ELISA and direct ELISA procedures using these antibodies to detect OA in wheat and rice spiked with the toxin or naturally contaminated were developed. The results are confirmed by TLC procedure.

2 Materials and methods

2.1 Samples

A total of 44 samples of grains (wheat and rice) that caused humanintoxicant and normal grain were analyzed. These samples were obtained from both flooded areas in Anhui, Henan provinces and retail outlets.

2.2 Apparatus

ELISA reader: microplate photometer, model 511, Shanghai No. 3 Analytical Instrument Factory, Shanghai 200031, China.

Microtiter plates: 96 flat bottom well plates, Flow Laboratories Inc. Mclean, Virginia 22102, USA.

TLC: TLC prewashed glass plates, 20×20 cm; rectangular glass developing tank with lid.

2.3 Reagents

2.3.1 Chemicals

Ochratoxin A, bovine serum albumin (BSA), horseradish peroxidase (HRP), 1- ethyl-3, 3-dimethylaminopropyl carbodiimide (EDPC), 3,3′,5,5′- tetramethylbenzidine (TMB), goat anti-mouse IgG-HRP conjugate were purchased from Sigma Chemical Co. St. Louis, Mo. All other chemicals either were reagent grade or chemical pure.

2.3.2 ELISA buffer system

Coating buffer: 50 mmol/L carbonate-bicarbonate buffer, pH 9.6; PBS-T buffer: 50 mmol/L sodium phosphate buffer saline containing 0.05% tween-20 (v/v), pH 7.4; sample and standard toxin dilution buffer: 20% methanol in PBS-T buffer; substrate buffer: 100 mmol/L of Na_2HPO_4 plus 50 mmol/L of citric acid, pH 5.0; enzyme substrate solution: stock solution of TMB (10 mg/ml) in dimethylformamide was stored frozen, 50 μl TMB and 10 μl 30% H_2O_2 were mixed with 10 ml substrate buffer, and used within 1 h; stopping solution: 2 mol/L H_2SO_4.

2.3.3 Standard solution

OA (1 mg/ml) was prepared with methanol and stored at −20 ℃; working

solutions of OA (100 μg/ml) were prepared with dilution buffer and stored at 5 ℃; working standard solution of 1－500 ng/ml of OA was prepared on the day of ELISA assay with dilution buffer.

2.3.4 Monoclonal antibodies and OA-BSA conjugate

The hybridoma cell line 6G11 producing specific monoclonal antibody for OA was prepared in our laboratory. HRP labelled monoclonal antibody (6G11) was conjugated by a simplified periodite method. OA conjugated to BSA was used for coating microtiter plates. This conjugate was prepared by the same method as Chu *et al.* (Chu, 1976).

2.3.5 TLC developing solvent

Longitudinal developing: toluene-ethyl acetate-formic acid-water (6:3:1.2:0.07).

Horizonatal developing: diethyl ether.

2.4 OA determination

2.4.1 Enzyme-linked immunosorbent assay

2.4.1.1 Extraction of samples

Portions of finely ground grain (20g) were weighted in 250 ml flask, and 30 ml petroleum ether, 100 ml methanol-water (55:45) were added. The mixture was extracted for 30 min on a wrist-action shaker and filltered. The aliquots of methanol-water (20 ml) was extracted with 25 ml chloroform again, and shaken for 2 min in a separatory funnel and allowed to separate. This fractionated OA from chloroform was further treated with 50 ml 4% NaCl solution. The chloroform fraction was then evaporated to remove the organic solvent and diluted with dilution buffer.

2.4.1.2 ELISA methods

One hundred μl of 10 μg/ml OA-BSA dissolved in coating solution was added to each well of 96 microtiter plate. The plate was put at 4 ℃ overnight, and then used for the following experiments.

Direct and indirect ELISA using those plates were performed as follows:

Procedure of indirect ELISA:

Added 50 μl of serially working standards solution of toxin; added 50 μl of the 6G11 monoclonal antibody (diluted to 2×10^4 fold with PBS-T); incubated for 1 h at 37 ℃; washed the plate three times with PBS-T; added 100 μl of goat anti-mouse IgG-HRP conjugate solution; incubated for 1.5 h at 37 ℃; washed the plate three times with PBS-T; added 100 μl of enzyme substrate solution; reacted for 10 min at 37 ℃, then added 50 μl of stopping solution; measured absorbance at 450 nm using microplate photometer.

Procedure of direct ELISA

Added 50 μl of serially working standard solutions of toxin; added 50 μl of

HRP labelled 6G11 monoclonal conjugate diluted to 400 fold with PBS-T; incubated for 1.5 h at 37 ℃; washed the plated three times with PBS-T; added 100 μl of enzyme substrate solution; reacteted for 10 min at 37 ℃, then added 50 μl of stopping solution; measured absorbance at 450 nm using microplate photometer.

2.4.2 TLC analysis

2.4.2.1 Extraction of samples

A portion of the chloroform extract used for ELISA analysis was evaporated. The residue was dissolved in 200 μl benzene-acetoni trile (98:2), and used for TLC.

2.4.2.2 TLC methods

Samples were analyzed according to Chinese National Standard "Method for Determination of Ochratoxin A in Cereal and Beenen", GB 13111-91.

3 Results and discussion

3.1 Standard curves

The standard curves of OA at different dilutions of HRP labelled 6G11 monoclonal antibody conjugate (direct ELISA) or antibody (indirect ELISA) are shown in Fig. 1.

The standard inhibition curves by ELISA (direct and indirect) are sensitive in the range of 1 − 500 ng/ml of OA. These sensitivities are obtained at HRP labelled 6G11 monoclonal antibody conjugate dilution of 1: 400 or when monoclonal antibody is used at a dilution of 1:20000. The intra-assay and inter-assay coefficients of variation of the standard cruves of OA are given in Table 1.

3.2 Recovery tests

The mean recoveries from the wheat, rice samples spiked with the OA standards in the range of 1 − 500 ppb are shown in Table 2.

These samples were directly assayed with ELISA (direct and indirect) after extraction, no clean up procedure was needed. Thus, particularly useful for routine screening of large numbers of grain

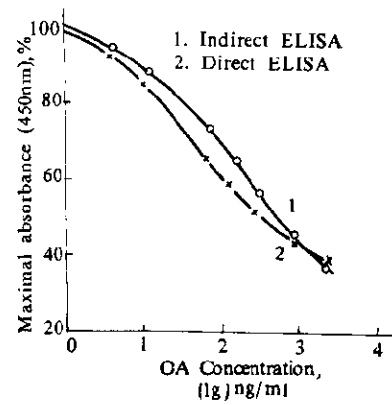

Fig.1 Standard curves for indirect ELISA and direct ELISA of ochratoxin A. results are average of quadruplicate analyses

Table 1 Estimates of intra-assay and inter- assay variablility of OA standard curves by ELISA

Methods	Concn. of competing mycotoxin, ng/ml	Mean ± *SD*, ng/ml (%CV) intra-assay	inter-assay
Indirect ELISA	1.00	0.95 ± 0.20(21.3)	0.92 ± 0.16(17.5)
	10.00	10.50 ± 2.00(19.2)	8.90 ± 1.10(12.3)
	100.00	103.50 ± 18.40(17.8)	95.30 ± 11.20(11.8)
	500.00	5.43.50 ± 65.80(12.1)	488.00 ± 92.20(18.9)
Direct ELISA	1.00	0.89 ± 0.19(20.8)]	0.85 ± 0.17(19.5)
	10.00	9.30 ± 1.83(19.7)	9.10 ± 1.30(14.3)
	100.00	115.40 ± 13.73(11.9)	102.30 ± 7.80(7.6)
	500.00	497.50 ± 42.30(8.5)	455.40 ± 24.60(5.4)

Table 2 Recoveries of OA from artificially contaminated grain samples as determined by ELISA

Grain sample	Added ng/g	Direct ELISA Detected, ng/g	Recovery, %	Indirect ELISA Detected, ng/g	Recovery, %
Wheat	1	1.0 ± 0.1	100.0	1.1 ± 0.1	110.0
	10	7.89 ± 0.2	78.9	7.9 ± 0.2	79.0
	50	46.6 ± 2.3	93.2	39.6 ± 1.7	79.2
	100	84.7 ± 8.3	84.7	79.1 ± 7.9	79.1
	500	396.2 ± 7.0	79.2	534.5 ± 38.7	106.9
Rice	1	1.2 ± 0.1	120.0	1.2 ± 0.1	120.0
	10	11.2 ± 0.6	112.0	8.2 ± 0.5	82.0
	50	49.9 ± 2.6	99.8	56.0 ± 4.4	112.0
	100	99.5 ± 7.2	99.5	99.5 ± 10.1	99.5
	500	444.6 ± 14.5	88.9	557.7 ± 29.4	111.5

samples.

The results for the comparison of the two methods of ELISA are also given in Table 2. No significant difference is existed between them ($P > 0.05$). Because direct ELISA is easy to handle and advantageous from the view point of both time and economy, it is suggested that the direct ELISA developed here could be used as a more usefully practical method.

3.3 Samples assay

Six rice and twenty-six wheat samples that caused human-intoxicant were detected

with direct ELISA. The samples were also confirmed by TLC methods. The results are shown in Table 3.

Table 3 ELISA and TLC analysis of OA in grain samples

Samples	No.	Concn. of OA, ng/g		Samples	No.	Concn. of OA, ng/g	
		ELISA	TLC			ELISA	TLC
Rice	1	4.0	0.0	Wheat	17	11.2	8.0
	2	0.0	0.0		18	0.0	0.0
	3	0.0	0.0		19	0.0	0.0
	4	0.0	0.0		20	0.0	0.0
	5	0.0	0.0		21	4.0	0.0
	6	0.0	0.0		22	0.0	0.0
Wheat	7	19.9	18.0		23	12.5	8.0
	8	4.0	0.0		24	70.8	64.0
	9	31.6	24.0		25	15.8	0.0
	10	25.1	18.0		26	0.0	0.0
	11	5.0	0.0		27	0.0	0.0
	12	25.0	24.0		28	5.6	0.0
	13	0.0	0.0		29	0.0	0.0
	14	15.9	12.0		30	0.0	0.0
	15	199.5	160.0		31	0.0	0.0
	16	44.7	40.0		32	0.0	0.0

A statistical comparison between TLC and ELISA results is given in Table 4.

Table 4 Statistical comparison of the results obtained by ELISA and TLC

No. of samples	Correlation coefficient	Probability P (T-test for paired data)
31	0.995	0.153

The correlation coefficient value of 0.995 for samples demonstrate a high degree of coincidence between the two systems. In addition, the results of T-test (for paired data) also showed that there is no significant difference between the results of the two systems.

The minimum detection level of ochratoxin A by TLC is 10 ng/g. So, it is comprehensible that the samples shown by ELISA to ochratoxin A at concentrations of 4−6 ppb failed to give a positive response with the TLC.

In conclusion, TLC procedure for ochratoxin A is time-consuming and may not be suitable for routine assays. The ELISA method established here is highly specific, sensitive and speed over TLC method, thus suitable for large numbers of sample determination.

The results suggest that it is necessary to monitor the contamination of grains by ochratoxin A in China, and to work out the maximum residue limit in food stuffs in order to ensure the consumer's health.

References

Bendele AM, Carlton WW, Krogh P, Litliehoj EB. Journal of the National Cancer Insitute, 1985; 75:733.

Boorman G. Toxicology and carcinogenesis studies of ochratoxin A in F344/N rats (gavages studies). Washington, DC.: NIH publication No. 88−2813 NIH, 1988

Chu FS, Chang FCC, Hinsdill RD. Appl Environ Microbiol, 1976; 31:831

Kawamura O, Sato S, Nagura M, Kishimoto S, Ueno I, Sato S, Uda T, Ito Y, Ueno Y. Food and Agricultural Immunology, 1990; 2:135.

Krogh P. Acta Pathol Microbiol Scand Sect A, 1978; 269 (Suppl.): 6

Lee SC, Chu FS. J Assoc Off Anal Chem, 1984; 67(1):45

Nesheim S, Haradin NF, Francis UJ, Langham WS. J Assoc Off Anal Chem, 1973; 56:817

Osborne BG. Food Cosmet Toxicol, 1983; 18:615

Steyn PS. Mycotoxins-production, Isolation, separation and purification (Ed. by Bentina V). France: Elsevier Publication, Bartsch, 1984:183

Ueno Y. Mycotoxins, in Toxicological aspects of food (Ed. by Miller K). Elsevier, Amsterdam, 1984:139

Yang CH, Luo XY, Liu C, Li YP. Biomedical Environmental and Sciences (in press)

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