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Development of a Sensitive Enzyme-Linked Immunosorbent Assay for the Determination of Ochratoxin A

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Polyclonal antibodies for ochratoxin A (OTA) were generated from rabbits after the animals had been immunized with either OTA-γ-globulin or OTA- keyhole limpet hemocyanin (KLH). A competitive direct enzyme-linked immunosorbent assay (cdELISA) and a competitive indirect ELISA (ciELISA) were used for the characterization of the antibodies and for analysis of OTA in various agricultural commodities. The antibody titers in the serum of rabbits immunized with OTA-γ-globulin were considerably higher than those in rabbits immunized with OTA-KLH. The antibodies from the rabbits immunized with OTA-γ-globulin were further characterized. In the cdELISA, the concentrations causing 50% inhibition (IC_{50}) of binding of OTA-horseradish peroxidase to the antibodies by OTA, ochratoxin B (OTB), and ochratoxin C (OTC) were found to be 0.90, 110, and 0.54 ng/mL, respectively. When 10 to 250 ng/g of standard OTA was spiked to soybean samples and then extracted with 50% aqueous methanol, the recovery rate of OTA was found to be 85.9% in the cdELISA. Analysis of OTA in various agricultural commodities showed that 12 of the 20 examined samples were contaminated with OTA at levels from 16 to 160 ng/g. The efficacy of cdELISA was also confirmed by the high-performance liquid chromatography method.

KEYWORDS: Ochratoxin A; antibodies; ELISA.

INTRODUCTION

Ochratoxin A (OTA) is a naturally occurring toxic metabolite produced primarily by *Aspergillus ochraceus* and *Penicillium verrucosum*. It has been found as a common contaminant of cereals or in other products such as coffee beans, nuts, wine, and animal organs (*1*−*3*). Numerous studies have revealed the role of OTA as a major causative factor in mycotoxic porcine nephropathy in many European countries (*4*, *5*). Toxicological studies indicate that OTA is a teratogenic, mutagenic, and carcinogenic mycotoxin, which is generally absorbed from the gastrointestinal tract in animals and has strong toxic effects on their livers and kidneys. (*1*, *2*, *4*−*7*). Although acute renal failure due to inhalation of OTA in humans has been rarely reported (*8*), OTA, with a long half-life of 840 h in human blood, is frequently found at high levels in serum samples obtained from people living in regions where Balkan Endemic Nephropathy occurs. OTA is also associated with an increased incidence of tumors of the upper urinary tract in human (*9*−*11*). The International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B). The European Union has also enacted a regulatory limit for the levels of OTA in cereals (5 μg/kg), roasted coffee (5 μg/kg), and instant coffee (10 μg/kg) (*12*, *13*).

To help minimize the risk of human and animal exposure to OTA, extensive research has been conducted to develop sensitive and specific methods for detection of OTA in food and feed samples. High-performance liquid chromatography (HPLC) fluorescence detection, with good accuracy and reproducibility, is most widely employed for monitoring OTA (*14*−*18*). However, HPLC methods require highly qualified personnel and extensive sample cleanup as well as expensive equipment. Development of immunochemical approaches has led to more simple and rapid methods to monitor and quantify OTA in contaminated food and feed. Although several groups have developed immunoassays for OTA detection (*19*−*24*), most of the assays rely on the competitive indirect ELISA (ciELISA) in which OTA-protein conjugates were coated onto microplates as solid phase. Compared with the competitive direct ELISA (cdELISA), the ciELISA is more time-consuming. To effectively analyze OTA levels in various agricultural commodities, in the present study a new method for the production of polyclonal antibodies against OTA was developed, and a sensitive cdELISA was also established.

MATERIAL AND METHODS

Materials. Ochratoxin A (OTA) and ochratoxin B (OTB) (**Figure 1**) were purchased from Sigma Chemical Co. (St. Louis, MO). A standard solution of OTA at 20 μg/mL in toluene-acetic acid (99:1) was prepared and assayed according to the AOAC method (*25*). Bovine serum albumin (BSA), γ-globulin, gelatin, ovalbumin (OVA),

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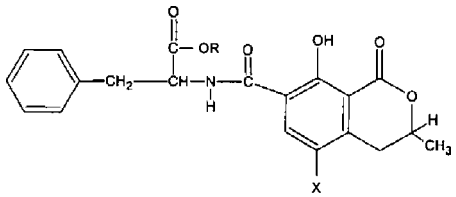

	R	X
Ochratoxin A	H	Cl
Ochratoxin B	H	H
Ochratoxin C	CH_2CH_3	Cl

Figure 1. Structures of ochratoxin A, ochratoxin B, and ochratoxin C.

ammonium biocarbonate, Tween 20, trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), 1,1′-carbonyldiimidazole (CDI), 1-ethyl-3-[3-dimethylaminopropyl]-carbodimide (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Chemical Co. (St. Louis, MO). Goat anti-rabbit−peroxidase conjugate and keyhole limpet hemocyanin (KLH) were obtained from Pierce Chemical Co. (Rockford, IL). Horseradish peroxidase (HRP) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, ID.). HRP substrate solution 3,3′,5,5′-tetramethylbenzidine (TMB) was obtained from Neogen corporation (Lexington, KY). Ammonium sulfate, absolute ethanol and HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Microtiter plates and strips (low and high protein binding) were obtained from Nunc (Roskilde, Demark). ELx 50 ELISA washer was purchased from Bio-Tek instruments (Winooski, VT). Vmax automatic ELISA reader was purchased from Molecular Devices Co. (Menlo Park, CA). Seven-week-old, female New Zealand white rabbits were obtained from Deer-Ho farm (Taichung, Taiwan). Freund's complete adjuvant containing *Mycobacterium tuberculosis* (H37 Ra) and Freund's incomplete adjuvant were obtained from Gibco BRL (Grand Island, NY). The HPLC equipment consisted of a Beckman System Gold 126 solvent module (Fullerton, CA) and an FP-1520 fluorescence detector (Jasco, Japan). The 250 × 4.6 mm i.d., 5 μm, Lichrospher C18 reverse phase column in conjunction with a 40 × 4.0 mm i.d., 5 μm, Lichrospher C18 guard column were obtained from Merck (Darmstadt, Germany). The 0.45 μm syringe filter was obtained from Gelman Science (Ann Arbor, MI). C18 Sep-Pak cartridges were obtained from Waters (Milford, MA). All other chemicals and organic solvents used were of reagent grade or better.

Preparation of Ochratoxin C (OTC). Ochratoxin C (OTC) (**Figure 1**) was prepared by esterification of OTA according to Fuchs et al. (*26*). OTA, 0.5 mg, was dissolved in 20 μL of absolute ethanol and added to a solution of 50 μL of thionyl chloride in 2 mL of absolute ethanol. After 3 h, the reaction mixture was added drop by drop to 0.5 mL ice-cold water and then OTC was extracted with 1 mL of chloroform. After the chloroform was evaporated, the residue containing OTC was redissolved in 0.5 mL of absolute ethanol. The purity of the OTC was checked with HPLC by the disappearance of the OTA peak at the retention time of 8.3 min and the appearance of the OTC peak at 23.5 min. The conversion of OTA to OTC was about 98%.

Preparation of Various OTA Conjugates. *Conjugation of OTA to γ-globulin.* OTA was conjugated to γ-globulin in the presence of water-soluble carbodiimide, EDC, and NHS under the following conditions (*27*). In a typical experiment, EDC solution (1.5 mg of EDC in 0.02 mL of DMSO) and NHS solution (1.2 mg of NHS in 0.02 mL of DMSO) were freshly prepared and added to an OTA solution (1.0 mg of OTA in 0.2 mL of DMSO). The reaction was kept at room temperature for 2 h and then at 4 °C overnight. Then the mixture was added slowly to 3.0 mg of γ-globulin, which was dissolved in 1.5 mL of 0.1 M carbonate buffer (pH 9.6) and kept at room temperature for another 2 h. After reaction, the mixture was dialyzed against 2 L of 0.01 M phosphate buffer containing 0.15 M NaCl (PBS, pH 7.5) for 72 h with two exchanges of buffer and then lyophilized.

Conjugation of OTA to KLH. OTA was conjugated to KLH in the presence of EDC under the following conditions (*27*). EDC solution (1.0 mg of EDC in 0.02 mL of double-distilled H_2O) was freshly prepared and added to an OTA solution (1.0 mg of OTA in 0.4 mL of 25% ethanol). Then the mixture was added slowly to 2.0 mg of KLH, which was dissolved in 0.4 mL of conjugation buffer (0.1M 2-*N*-morpholinoethane-sulfonic acid [MES], 0.9 M NaCl, pH 4.7) and kept at room temperature for another 2 h. After reaction, the mixture was dialyzed against 2 L of 0.01 M phosphate buffer containing 0.15M NaCl (PBS, pH 7.5) for 72 h with two exchanges of buffer and then lyophilized.

Conjugation of OTA to OVA for Indirect ELISA. OTA was conjugated to OVA by the water-soluble carbodiimide method and used as a solid-phase antigen for the indirect ELISA (*27*). In a typical reaction, 0.5 mg of OTA in 0.2 mL of conjugation buffer was mixed with 2.5 mg of OVA first, and then 1 mg of EDC in was added to the mixture with constant stirring. The coupling reaction was carried out at 25 °C for 2 h. The mixture was dialyzed as described above for 72 h against 0.01M PBS and then lyophilized.

Preparation of OTA−Peroxidase. Conjugation of OTA to HRP was achieved by the CDI method (*28*). Briefly, 0.2 mg of OTA in 0.05 mL of acetone was mixed with 0.4 mg of CDI, and then an HRP solution (0.8 mg of HRP in 0.3 mL of 0.1 M, pH 9.6, carbonate buffer) was added. After being stirred at room temperature for 2 h, the mixture was dialyzed against 0.01 M PBS (pH 7.5) for 72 h and then lyophilized.

Production of Polyclonal Antibody. The schedule and methods of immunization were the same as those described previously (*29*). Two immunogens, OTA- γ-globulin and OTA-KLH, were tested in 4 rabbits, with 2 rabbits for each immunogen. Each rabbit was injected intradermally at multiple sites on the shaved back (30 sites) with 500 μg of the immunogen in 1 mL of 0.01M PBS mixed with 1 mL of Freund's complete adjuvant. For booster injections, the same amount of immunogen in PBS solution was mixed with an equal volume of Freund's incomplete adjuvant and injected subcutaneously at 4 sites on the thigh of each rabbit at the 5th and 12th weeks. Antisera were collected from the ears of the rabbits from the fifth week after the initial injection. The antisera were subjected to sequential steps of ammonia sulfate precipitation. The final precipitate in 35% ammonia sulfate was redissolved in distilled water equal to half of the original serum volume and then dialyzed against 2 L of PBS for 72 h at 4 °C with two changes of buffer.

Monitoring of Antibody Titers by Indirect ELISA (iELISA). The protocol for the iELISA was similar to that described previously (*29*). In general, 0.1 mL of OTA-OVA conjugate (1 μg/mL in 0.01 M PBS, pH 7.5) was added to each well of microtiter plate and kept at 4 °C together overnight. After the plate had been washed 4 times with Tween-PBS (0.35 mL per well; 0.05% Tween 20 in 0.01 M PBS) using an automated ELISA washer, 0.17 mL of gelatin−PBS (0.17 mL per well; 0.1% gelatin in 0.01 M PBS) was added and allowed to incubate at 37 °C for 30 min. Then the plate was washed 4 times as described above, and 0.1 mL of diluted anti-OTA antiserum was added. After incubation at 37 °C for 1 h and washing with Tween-PBS, 0.1 mL of goat anti-rabbit IgG-HRP conjugate (1:20 000 dilution) was added and incubated at 37 °C for 45 min. The plate was washed 4 times with Tween-PBS again, then 0.1 mL of TMB substrate solution (1 mM 3,3′,5,5′-tetramethylbenzidine and 3 mM H_2O_2 per liter of potassium citrate buffer, pH 3.9) was added. After 10 min of incubation at room temperature, 0.1 mL of 1 N hydrochloric acid was added to stop the reaction. Absorbance at 450 nm was determined in a Vmax automatic ELISA reader.

cdELISA. The protocol for cdELISA was essentially the same as previously described (*29*). The antibody collected from the 15th week was diluted in 0.01 M PBS, pH 7.5, (1:1000 dilution) and 0.1 mL of the diluted form was coated onto each well. After the plate had been incubated at 4 °C overnight, it was washed with Tween-PBS followed by blocking with gelatin−PBS (0.17 mL per well; 0.1% gelatin in 0.01M PBS) at 37 °C for 30 min. The plate was washed again with Tween-PBS 4 times, then OTA standard (0.05 mL per well in 0.01M PBS) concentrations from 0.01 to 100 ng/mL or samples were added to each well, then OTA−HRP conjugate (1:10 000 dilution, 5 ng/mL, in 0.01M PBS, 0.05 mL per well) was added to all wells and incubated at 37 °C for 50 min. The plate was washed 4 times with Tween-PBS, and 0.1 mL of TMB substrate solution was added. After incubation at room temperature in the dark for 10 min, the reaction was terminated

by adding 0.1 mL of 1 N HCl. The absorbance at 450 nm was determined in the Vmax automatic ELISA reader.

Recovery of OTA Added to Soybean Samples by cdELISA. A recovery study was carried out to test the efficacy of cdELISA for the analysis of OTA in soybean samples. Five grams each of the ground soybean samples shown to be OTA-free by ELISA were spiked with OTA at concentrations ranging from 10 to 250 ng/g. A control sample with no toxin added was used as the blank. Each of the spiked samples was stored for 1 day and then shaked with 50 mL of extract solvent (methanol/water, 50/50, v/v) for 2 h. Twenty mL of extraction solution was taken and centrifuged at 10 000 rpm for 10 min (*19*, *22*). The supernatant solution, 1 mL of the clear extract, was diluted with 19 mL of 0.01 M PBS and subjected directly to cdELISA. Further dilutions with PBS were made for spiked samples with high levels of OTA before the cdELISA. At least two separate extracts were taken for each sample, and analysis on each extract was determined in triplicate.

cdELISA of Agricultural Commodities Contaminated with OTA. Twenty agricultural commodity samples (maize, barley, bean, and coffee) obtained from local food stores in Taiwan were used to determine their OTA levels. Briefly, each sample (15 g) was shaken with 150 mL of extract solvent (methanol/ water, 50/50, v/v) for 2 h. After centrifugation at 10 000 rpm for 10 min, 1 mL of the supernatant solution was aspirated and diluted with 19 mL of 0.01 M PBS and directly subjected to cdELISA. Further dilutions with PBS were made for samples with high levels of OTA for the cdELISA.

Sample Preparation for HPLC. Ten grams each of selected ELISA positive samples (10 g) were shaken with 100 mL of extract solvent (ethyl acetate/5% acetic acid, 90/10, v/v) for 1 h. Twenty mL of extraction solution was taken and centrifuged at 10 000 rpm for 10 min. The supernatant solution, 4 mL of the clear extract, were then loaded onto a C18 Sep-Pak cartridge that had been washed with 5 mL of acetonitrile and 5 mL of distilled water in sequence (*30*). The cartridge was then washed with 4 mL of distilled water, and OTA was eluted from the cartridge with 4 mL of acetonitrile/water (1/1, v/v) (*30*). This sample solution was kept at −20 °C before HPLC analysis.

HPLC Analysis of OTA. The HPLC equipment consisted of a Beckman System Gold 126 solvent module and an FP-1520 fluorescence detector. The OTA standard and the sample extracts were passed through a low protein binding 0.45 μm filter before injecting into the HPLC. A Lichrospher C18 reverse phase column in conjunction with a Lichrospher C18 guard column was equilibrated with a mobile phase consisting of 50% acetonitrile in 0.05% TFA/water at a flow rate of 1 mL/min (*18*, *31*). The injection volume was 20 μL for both standard solutions and sample extracts. OTA was eluted from the column with 50% acetonitrile in 0.05% TFA for 30 min, and the fluorescence detector was operated at an excitation wavelength of 330 nm and an emission wavelength of 450 nm. A calibration curve was generated using standard OTA of 0.5, 2.5, 5, 25, 50, 250, 500 ng/mL ($R^2 = 0.99$). The lowest detectable limit for the OTA standard was 0.5 ng/mL. OTA concentrations in sample extracts were determined from the calibration curve, using peak area for quantitation.

Chemical Confirmation of HPLC Analysis of OTA. To confirm the presence of OTA, OTA in samples was converted into the methyl ester form according to Pittet et al. (*32*). The OTA standard and the extracts of HPLC-analyzed positive samples (0.5 mL) were evaporated to dryness in the hood under a gentle stream of nitrogen, and the residue was dissolved in 300 μL of boron trifluoride/methanol complex (14% solution in methanol). This solution was heated at 80 °C for 10 min in a heating block and allowed to cool to room temperature (*32*, *33*). The solution was passed through a 0.45 μm filter and analyzed by HPLC.

RESULTS

Production of Polyclonal Antibodies. Sera collected from rabbits immunized with OTA-γ-globulin or with OTA-KLH were subjected to the indirect ELISA (iELISA). Typical titration curves of antibody titers obtained from an OTA-γ-globulin immunized rabbit over a period of 15 weeks are shown in

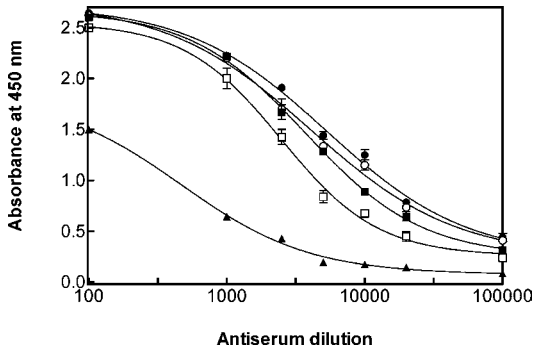

Figure 2. Determination of antibody titers for a representative rabbit after immunization with OTA-γ-globulin by an OTA–OVA based iELISA. The antiserum was obtained 0 (▲), 6 (□), 9 (■), 12 (○), and 15 (●) weeks after immunization.

Figure 2. Antibodies against OTA were detected in the sera of rabbits as early as 6 weeks after initial immunization. The antibody titer increased progressively with time, and the highest titer was found in the sera of rabbits at the 15th week after two subsequent immunizations. The antibody titers of the rabbits immunized with OTA−KLH were found to be considerably lower than those of the rabbits immunized with OTA-γ-globulin.

Characterization of Antibodies. Both the cdELISA and ciELISA were used to determine the specificity of antibodies. Because in the cdELISA the amount of antibody required to coat microtiter plates was much less for the antiserum obtained from OTA-γ-globulin-immunized rabbits (1:1000 dilution) than those from OTA−KLH-immunized ones (1:500 dilution), the 15th week antiserum from rabbits immunized with OTA-γ-globulin was used in the subsequent studies. In the cdELISA, the concentrations causing 50% inhibition (IC_{50}) of binding of OTA−HRP with the antibodies by OTA, OTB, and OTC were found to be 0.90, 110, and 0.54 ng/mL, respectively **(Figure 3A**). The relative cross-reactivities of the antibodies to OTB and OTC were calculated to be 0.8 and 167, respectively. Similar results were also obtained in the ciELISA, in which OTA−OVA was coated on the wells of ELISA plates to serve as solid-phase antigen. The concentrations causing 50% inhibition of binding of antibodies to the solid-phase OTA−OVA by free OTA, OTB, and OTC were found to be 10, 168, and 5 ng/mL, respectively (**Figure 3B**). The relative cross-reactivities of the antibodies to OTB and OTC in the ciELISA were calculated to be 6.0 and 200, respectively. Citrinin and L-phenylalanine, two molecules with chemical structures similar to a part of OTA molecule, at a concentration of 100 μg/mL, did not inhibit the binding of marker antigens with the antibodies in either ELISA system (data not shown).

Recovery of OTA Added to Soybean by cdELISA. To investigate the recovery rate of OTA in cdELISA, OTA were first spiked into soybean samples at concentrations from 10 to 250 ng/g, and then the samples were extracted with 50% aqueous methanol for recovery analysis. The recovery rate for 10−250 ng/g of spiked OTA ranged from 79.2 to 95%, and the overall average was calculated to be 85.9% (CV, 6.5%).

Analysis of OTA in Various Agricultural Commodities with cdELISA. Twenty samples were collected from local food stores and subjected to cdELISA for OTA determination; the results are presented in **Table 1**. Twelve of the 20 examined samples were found to be OTA or OTA analogues positive with levels ranging from 16 to 160 ng/g. Among eight examined corn samples, sample 1 had the highest level of OTA at 63 ng/g, but OTA in samples 3, 4, and 8 were below the detection limit. Buckwheat sample 10 was found to have the highest level

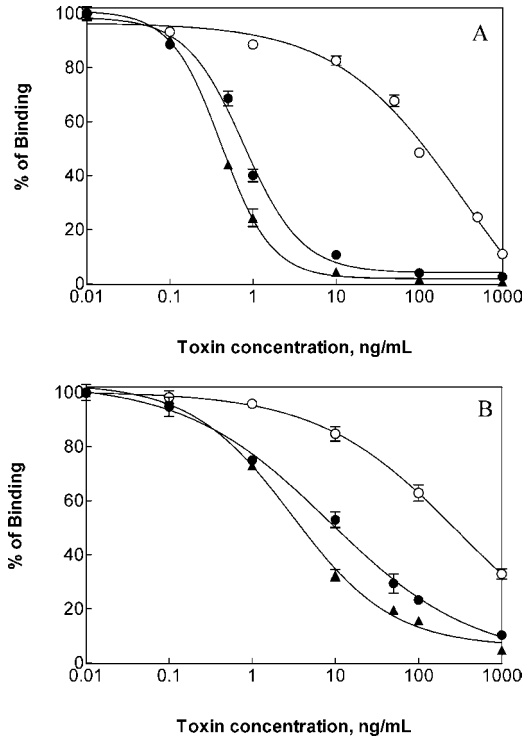

Figure 3. **A**: Cross-reactivity of anti-OTA antibodies with OTA (●), OTB (○), and OTC (▲) in a cdELISA. All data were obtained from the average of 3 sets of experiments. The absorbance of the control, A_0, with no toxin present, was 2.0. **B**: Cross-reactivity of anti-OTA antibodies with OTA (●), OTB (○), and OTC (▲) as determined by a ciELISA. All data were obtained from the average of 3 sets of experiments. The absorbance of the control, A_0, with no toxin present, was 1.8.

of OTA at 144 ng/g among four selected cereal samples. In addition, bean samples 13 and 16 had OTA levels at 74 and 43 ng/g, respectively. One of three examined coffee samples contained 160 ng/g of OTA, but the other two were free of OTA.

HPLC Analysis of OTA. To test the efficacy of the cdELISA for OTA detection in various agricultural samples, the OTA standard and selected ELISA-positive samples were further analyzed with an HPLC method. HPLC chromatograms for OTA standard (5 ng), buckwheat sample 10, and roasted coffee sample 20 are shown in **Figure 4**. OTA standard was well-identified with a retention time of 8.3 min under the isocratic elution (**Figure 4A**). The extracts of sample 10 and sample 20 also showed an OTA peak with a retention time of 8.3 min (**Figure 4B**), and the peak areas on the basis of calibration curve were calculated to be 60 and 220 ng/g, respectively. The levels of other examined samples were below the HPLC detection limit, which was around 0.01 ng of OTA per injection (**Table 1**).

Chemical Confirmation of the Presence of OTA in Samples by HPLC. To further ensure the presence of OTA in samples by HPLC, chemical derivatization of OTA into its methyl ester form was conducted with the boron trifluoride method (*32*, *33*). After derivatization and HPLC analysis, the peak of standard OTA decreased and the peak representing the methyl ester form of OTA appeared with a retention time of 18.5 min (**Figure 5A**). The same patterns were observed in the derivatized coffee sample 20 (**Figure 5B**) and buckwheat sample 10 (data not shown). These results identified the existence of OTA in coffee sample 20 and buckwheat sample 10.

Table 1. ELISA and HPLC Analysis of OTA in Different Agricultural Commodities[a]

		ELISA	HPLC
samples	source	OTA and analogues (ng/g ± SD)	OTA (ng/g)
	Corn		
1	Taiwan	63 ± 5	ND[b]
2	Taiwan	24 ± 3	*c*
3	Taiwan	ND[b]	*c*
4	imported	16 ± 3	*c*
5	imported	27 ± 4	c
6	Taiwan	ND[b]	*c*
7	Taiwan	30 ± 3	*c*
8	Taiwan	ND[b]	*c*
	Selected Cereals		
9	imported wheat	84 ± 4	ND[b]
10	imported buckwheat	144 ± 8	60
11	imported barley	35 ± 3	*c*
12	imported barley	43 ± 4	*c*
	Bean		
13	Taiwan (black bean)	74 ± 6	ND[b]
14	Taiwan soybean	ND[b]	*c*
15	Taiwan soybean	ND[b]	*c*
16	Taiwan (red bean)	43 ± 5	ND[b]
17	Taiwan (green bean)	ND[b]	*c*
	Coffee		
18	imported (green coffee)	ND[b]	ND[b]
19	imported (green coffee)	ND[b]	ND[b]
20	imported (roasted coffee)	160 ± 9	220

[a] Each sample was extracted twice and each extract was analyzed in triplicate. [b] ND, not detected or below the limit of detection. [c] Not determined.

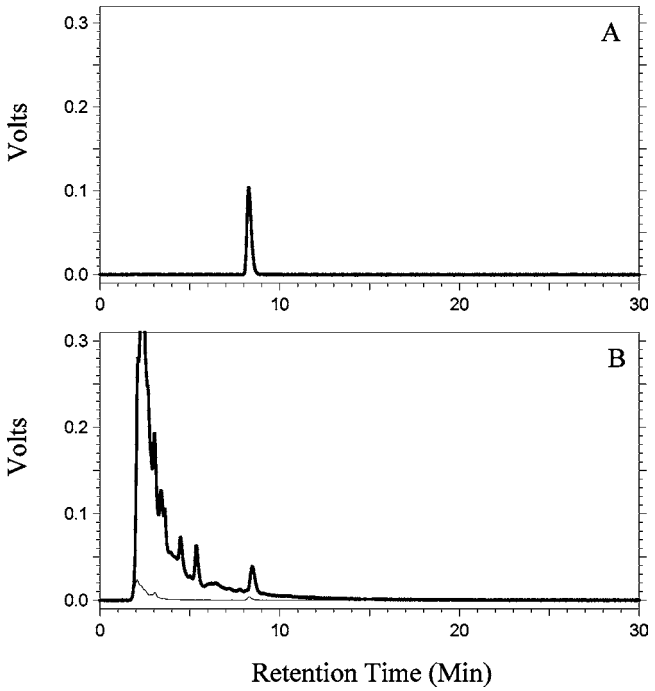

Figure 4. HPLC chromatograms of (**A**) 5 ng of standard OTA and (**B**) natural contamination of OTA in coffee sample 20 (thick line) and buckwheat sample 10 (thin line).

DISCUSSION

Like most other mycotoxins, OTA is a low molecular weight nonimmunogenic toxin, which needs to be conjugated to a protein carrier to render it immunogenic. For the production of antibodies against OTA, most immunogens were prepared via

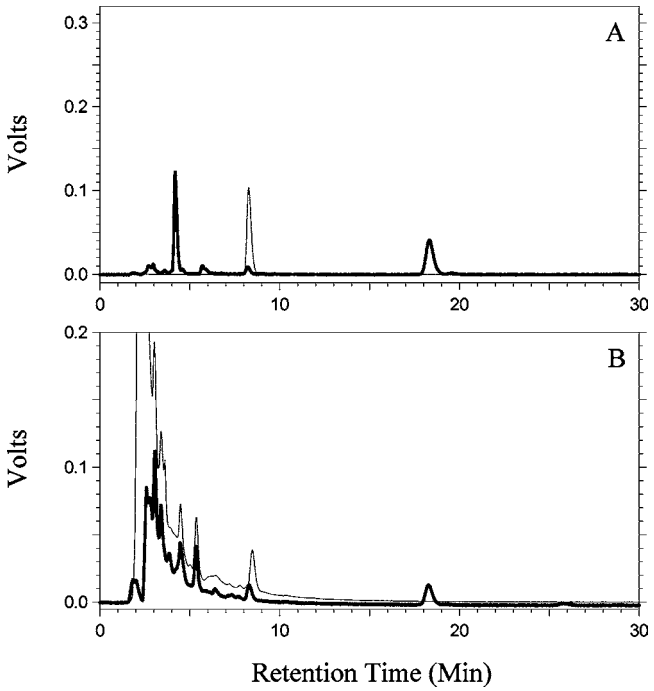

Figure 5. HPLC chromatograms of chemical derivatization of standard OTA and a coffee sample containing OTA. **A**: Standard OTA (thin line) and methyl ester form of standard OTA (thick line). **B**: Coffee sample containing OTA (thin line) and methyl ester form of OTA (thick line).

carbodiimide method by linking OTA to BSA. Chu et al. (*20*) first reported the generation of polyclonal antibodies against OTA in rabbits; OTA was conjugated to BSA via the carbodiimide method by linking the carboxyl groups of OTA to the amino groups of BSA. This method has been adopted by several groups to generate OTA-specific antibodies (*19*, *21*, *22*, *24*). To improve the sensitivity of ELISA for OTA determination, we demonstrated herein that OTA was conjugated to γ-globulin by a combination of carbodiimide and NHS as immunogen for generating specific polyclonal antibodies for OTA. It is known that carbodiimide catalyzes the formation of amide bonds between carboxylic group and amines, and NHS often assists carbodiimide coupling (*34*). Therefore, in this study the combination of carbodiimide and NHS greatly enhanced the coupling efficiency between OTA and γ-globulin, which could then help generate high-affinity antibodies for OTA. Because the basic structure of OTA contains one reactive carboxylic group, the carbodiimide coupling process includes the formation of a stable intermediate active ester by condensation of the carboxylic group at the OTA with the assistance of NHS, and this intermediate then reacts with a primary amine on γ-globulin to form a stable amide bond (*27*, *29*, *34*).

Polyclonal antibodies generated from OTA-γ-globulin-immunized rabbits were applied in cdELISA and ciELISA, because they have a higher affinity to OTA than those obtained from OTA−KLH. To establish a highly sensitive cdELISA, BSA−PBS blocking buffer was replaced by gelatin−PBS in the blocking step to avoid the strong nonspecific binding of OTA to BSA. Good quality of OTA−HRP conjugates also played a key role in cdELISA as well. Several different conjugation methods and various molecular ratios of OTA vs HRP for conjugating OTA to HRP have been tested in the process of developing cdELISA. We found that conjugation of OTA to HRP with the molecular ratio of 25:1 by the CDI method gave the highest sensitivity in cdELISA.

In the cdELISA, the concentrations of OTA and OTC causing 50% inhibition (IC_{50}) of binding of the marker enzymes were 0.9 and 0.54 ng/mL, respectively, suggesting that antibodies showed similar affinities to both toxins (**Figure 3A**). Because OTC was a possible coexistent with OTA in food and feeds (*35*), the established cdELISA could also be applied to screen a large number of samples for the presence of OTC. On the other hand, the antibodies showed a weak cross-reactivity with OTB in both cdELISA (IC_{50} 110 ng/mL) and ciELISA (IC_{50} 168 ng/mL) (**Figure 3**, parts **A** and **B**). These results were consistent with those of Clarke et al. (*21*) and Kwak and Shon (*22*). The only difference between the structures of OTA and OTB is that OTB contains a hydrogen atom instead of chloride on its side chain (**Figure 1**), implying that the chloride atom in OTA or OTC could be the recognition site of our antibodies.

To effectively screen large amounts of agricultural samples, our studies are focused on the development of a cdELISA, which is more rapid, sensitive, and less time-consuming than ciELISA. In the cdELISA, sample extracts prepared in 50% aqueous methanol were generally diluted 10-fold with 0.01M PBS to avoid both matrix and solvent interference. Because cdELISA can tolerate up to 5% methanol in analyzed samples, this system is especially beneficial for those agricultural products contaminated with low levels of OTA; only 10-fold dilution of sample extracts could be analyzed directly by cdELISA. Because good recoveries with an average of 85.9% was obtained at OTA levels above 10 ng/g, the detection limit of cdELISA is calculated to be 0.1 ng/mL based on a 10-fold dilution of the sample extract.

The European Union has established a regulatory limit of 5 μg/kg OTA in cereals and in roasted coffee (*12*, *13*). Several reports also indicated that green and roasted coffee were contaminated with OTA in various amounts (*3*, *35*−*37*). The results obtained from our ELISA showed that 5 out of 20 agricultural samples collected in local food stores contained more than 50 ng/g of OTA, which is at least 10-fold higher than the EU regulatory limit. Besides, all of the examined cereal samples were contaminated with various levels of OTA, implying that cereal samples are subject to the growth of OTA-producing fungi. To verify the results from cdELISA, selected ELISA-positive samples were subjected to HPLC− fluorescence detection. Although the roasted coffee and buckwheat sample were positive in both cdELISA and HPLC, some other samples showing positive in ELISA were found to be negative in the HPLC method (**Table 1**).

Such discrepancies could be due to the presence of OTA analogues such as OTC and OTB in sample extracts. All these OTA analogues reacted with our antibody in cdELISA, but in the HPLC chromatogram only OTA was identified. Another possible reason is that only the sample extracts for HPLC analysis were cleaned with Sep-Pak cartridges; this cleanup step may lead to the partial loss of OTA and its analogues.

Several reports described the use of immunoaffinity columns (IAC) to cleanup the OTA-containing extracts before being subjected to HPLC fluorescence detection (*38*−*40*). Although IAC columns for OTA are commercially available, the column capacity, column expenses, and time-consuming steps strongly restrict their use in sample screening. Here, the developed cdELISA with high sensitivity and specificity could be used for OTA sample analysis to avoid extensive sample cleanup steps. Furthermore, the amount of the antibody used in the ELISA is much less than the amount used in an immunoaffinity cleanup column. Because only 20 samples were analyzed in the present study, further studies with a large number of samples from different sources are needed to evaluate the existence of

OTA in different agricultural commodities. In conclusion, a sensitive and effective cdELISA for OTA was developed for determination of the levels of OTA in agricultural commodity samples; as low as 10 ng/g of OTA or OTC could be easily detected. This ELISA method could also be applied in screening a large number of agricultural commodity samples without sample cleanup.

ABBREVIATIONS USED

BSA, bovine serum albumin; CDI, 1,1′-carbonyldiimidazole; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3 [3-dimethylaminopropyl] carbodimide; ELISA, enzyme-linked immunosorbent assay; cdELISA, competitive direct ELISA; ciELISA, competitive indirect ELISA; iELISA, indirect ELISA; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; NHS, *N*-hydroxysuccinimide; OVA, ovalbumin; TMB, 3,3′,5,5′-tetramethylbenzidine.

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