Improved Direct Competitive Enzyme-Linked Immunosorbent Assay for Cyclopiazonic Acid in Corn, Peanuts, and Mixed Feed

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An improved direct competitive enzyme-linked immunosorbent assay (dc-ELISA) was developed for the analysis of cyclopiazonic acid (CPA) in corn, peanuts, and mixed feed. Two new approaches were used for the preparation of enzyme markers; one involved coupling CPA−bovine serum albumin (CPA-BSA) conjugate to horseradish peroxidase (HRP) using either the glutaraldehyde (GA) or the periodate (PI) method, and the other involved conjugating CPA carboxymethyl oxime (CPA-CMO) derivative to an ethylenediamine-modified HRP using a water-soluble carbodiimide (WSC) method. The concentrations causing 50% binding inhibition of the labeled HRP to the antibody by CPA in the dc-ELISAs using markers prepared by PI, GA, and WSC methods (IC_{50}) were 0.42, 0.68, and 0.93 ng/mL, respectively. The dc-ELISAs using CPA-BSA-HRP prepared by either PI or GA method were more effective and subsequently used for the analysis of CPA in corn, peanuts, and mixed feed. Four extraction solvent systems with 70−80% of methanol in different buffers at pH 7.4−8.5 showed no adverse effects on the dc-ELISA, and sample extracts after dilutions could be directly used in the assay. Using CPA-BSA-HRP prepared according to the GA method in the dc-ELISA, the detection limits of CPA in corn, mixed feed, and peanuts were estimated to be around 100, 300, and 600 ng/g (ppb), respectively. The mean analytical recoveries (200−5000 ppb range) for CPA added to the corn, mixed feed, and peanuts were found to be 97.6, 92, and 93%, respectively.

Keywords: *CPA; ELISA; immunoassay; corn; peanuts; mixed feed*

INTRODUCTION

Cyclopiazonic acid (CPA), a toxic secondary fungal metabolite produced by a number of *Aspergillus* and *Penicillium* species commonly occurring in a variety of agricultural commodities, was originally isolated from *Pencillium cyclopium* and *Aspergillus flavus* (Cole and Cox, 1981; Holzapfel, 1968). The toxin causes degeneration and necrosis of the liver, lesions of the myocardium, and neurotoxic effects through alteration of calcium homeostasis and cellular transduction processes in many animal species (CAST, 1989; Cullen et al., 1988; Peden, 1990; Porter et al., 1988; Low et al., 1992; Riley et al., 1992). CPA has been found in a number of foods and feeds (Balachandran and Parthasarathy, 1996; Chang-Yen and Bidasee,1990; Lansden, 1986; Norred et al., 1987; Rao and Husain, 1987; Still et al., 1978; Urano et al., 1992a,b) and also can be transmitted into milk and eggs after animals receive the toxic feed (Dorner et al., 1994). Simultaneous production of CPA and aflatoxin by some toxigenic fungi has led to the suggestion that CPA and aflatoxin may have some additive toxic effects to animals (Cole and Cox, 1981; Bradburn et al., 1994; Goto et al., 1996; Gallagher et al., 1978; Huang et al., 1994). To minimize the potential risk to human and animal health, there is a need for a simple and rapid method for routine screening of CPA in foods and feeds. Recent studies using nonaflatoxigenic *A. flavus* as an agent to control formation of aflatoxins in the field reiterated such need because some of these nonaflatoxigenic *Aspergillus* species may produce CPA but not aflatoxins, which may create another potential health hazard to humans and animals (Brown et al., 1991; Dorner et al., 1992).

Analysis of CPA, however, has been a difficult task because it has no fluorescence and its UV absorption maximum (284 nm) is not very specific. Most chemical methods involve derivatization and extensive cleanup treatment of the sample and thus are time-consuming and not very sensitive. The detection limits ranged from 80 ppb for spectrophotometric to 125 ppb for TLC methods (Chang-Yen and Bidases, 1990; Lansden, 1984, 1986; Rao and Husain, 1987). Although high-performance liquid chromatography (HPLC) is sensitive and can detect as low as 25 ppb of CPA in some commodities, it requires a lengthy sample cleanup and the use of expensive instruments (Goto et al., 1987; Lansden, 1984; Matsudo and Sasaki, 1995; Norred et al., 1987; Urano et al., 1992a).

To overcome the problems encountered in the chemical and biological methods, antibodies against CPA have been generated and sensitive direct competitive enzyme-linked immunosorbent assay (dc-ELISA) and indirect competitive ELISA (idc-ELISA) have been developed. The polyclonal antibody (pAb)-based dc-ELISA established by Hahnau and Weiler (1991), which has a detection limit of 200 ng/mL of agar, was considerably more sensitive than the monoclonal antibody (mAb)-based dc-ELISA developed by them (Hahnau and Weiler, 1993). A high-affinity mAb against CPA was generated in our laboratory, and we have established a sensitive idc-ELISA with a detection limit of 0.067 ng/

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mL for the detection of CPA in fungal cultures (Huang and Chu, 1993; Huang et al., 1994). Nevertheless, protocols for the application of different ELISAs of CPA in corn, peanuts, and mixed feed have not been established.

In the present study, several different mAb-based dc-ELISA protocols for CPA were established and the efficacy for using CPA−enzyme conjugates prepared by different methods was compared. The effect of different extraction solvent systems and sample matrix, i.e., corn, peanuts, and mixed feed, on the newly developed ELISA was studied. We also tested the efficacy of this method for the analysis of CPA in corn that previously had been analyzed using HPLC method. Details for the preparation of the conjugates, ELISA protocols for the analysis of the toxin in different samples, and their analytical recoveries are presented in this paper.

MATERIALS AND METHODS

Materials. Cyclopiazonic acid, bovine serum albumin (BSA, RIA grade, No. A-7511), Tween 20, and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma Chemical Co. (St. Louis, MO). Carboxymethoxylamine hemihydrochloride and 1-ethyl-3-(dimethylamino)propylcarbodiimide hydrochloride (EDPC) were from Aldrich Chemical Co. (Milwaukee, WI). Horseradish peroxidase (HRP) was obtained from Boeringer Mannheim GmbH Biochemicals (Mannheim, Germany). Microtiter ELISA plates were obtained from Nunc Co. (no. 4-69914; Roskilde, Denmark). Cyclopiazonic acid-free blank corn, mixed feed, and peanuts were kindly provided by Dr. J. W. Dorner (National Peanut Research Laboratory, USDA, Dawson, GA). All other chemicals and solvents were of reagent grade or better.

Production and Purification of mAb against CPA. Monoclonal antibody for CPA was produced by hybridoma cell line 5C8D1 in Dulbecco modified Eagle's medium with 10% fetal calf serum, 1 mM sodium pyruvate, 0.375% sodium bicarbonate, and penicillin−streptomycin at a final concentration of 100 units /mL each as described previously (Huang and Chu, 1993). After separation of the cell debris by centrifugation at 10000*g* for15 min, the supernatant fluid was precipitated with saturated ammonium sulfate to a final concentration of 50% saturation and then dialyzed against 2 L of 0.01 M MES (pH 5.6). The dialysate was then loaded on a column packed with 10 g of BakerBond ABx (J. T. Baker, Phillipsburg, NJ) that had been previously equilibrated with MES. After a washing with ca. 500 mL of MES buffer to remove the contaminated proteins ($A_{280nm} < 0.1$), the antibody was eluted from the column as a single protein peak, with a gradient starting with 100% of MES (200 mL) and ending with 100% of 0.25 M KH_2PO_4 (pH 6.8, 200 mL), which recovered all of the anti-CPA binding activity and 18% of original proteins. The purified mAb was then dialyzed against 1 L of distilled water for 0.5 h and against 2 L of 0.01 M phosphate buffer (PB) overnight in the cold room, lyophilized, and stored at −20 °C.

Preparation of CPA Enzyme Markers. *1. Preparation of CPA-BSA-HRP Conjugate. 1.1. Preparation of CPA-BSA Conjugate.* CPA was conjugated to BSA via the Mannich reaction according to the method of Huang and Chu (1993). Briefly, 10 mg of BSA in 2 mL of sodium acetate buffer (0.1 M, pH 5.0) was first activated with 50 μL of 37% formaldehyde at room temperature with gentle stirring for 5 min, and then 1.0 mg of CPA in 200 μL of dimethyl sulfoxide (DMSO) was added over a period of 5 min. The reaction mixture was kept at room temperature overnight, followed by dialysis against 2 L of 0.01 M phosphate-buffered saline (PBS, pH 7.4) with four changes of buffer. Precipitates in the dialysate were removed by filtration through a 0.45 μm membrane (Gelman Sciences, Ann Arbor, MI). The CPA conjugated to BSA was estimated to be no less than 4 mol/mol from the increase in absorptivity at 284 nm using the molar extinction coefficient of 2.04×10^4 at 284 nm (Cole and Cox, 1981). The final solution was divided into small aliquots, lyophilized, and stored at −20 °C.

1.2. Coupling CPA-BSA to HRP by the Glutaraldehyde Method (GA-CPA-BSA-HRP). Conjugation of CPA-BSA to HRP was accomplished by reacting 1.0 mg of HRP with 1.0 mg of CPA-BSA in 0.5 mL of 0.1 M PB (pH 6.9) in the presence of 30 μL of 5% glutaraldehyde at room temperature for 2 h. The reaction was stopped by dialyzing the product against 2 L of PBS with four changes of buffer. After dialysis, an equal volume of glycerol was added and the solution was then kept at −20 °C for further use.

1.3. Coupling CPA-BSA to HRP by Periodate Method (PI-CPA-BSA-HRP). This method was essentially the same as that described by Tsang et al. (1995) for the preparation of IgG-HRP except that dialysis was used instead of column chromatography. Briefly, 4.0 mg of HRP in 0.2 mL of citric acid/sodium citrate buffer (0.1 M, pH 5.0) was warmed to 37 °C, to which 26 μL of sodium periodate (30 mg/mL) was added. Five minutes after the reaction, 13 μL of 1% ethylene glycol was added, followed immediately by dialysis of the oxidized enzyme against 1 L of citric acid/sodium citrate buffer (0.001 M, pH 5.0) for 1 h. The oxidized HRP was then reacted with CPA-BSA solution (1 mg of CPA-BSA solution in 1 mL of 0.1 M sodium carbonate buffer, pH 10.0) at 4 °C for 48 h, followed by addition of 10 μL of sodium cyanoborohydride solution (10 mg/mL), and reacted for another 2 h at 4 °C. The reaction mixture was then dialyzed against PBS and stored in 50% glycerol/PBS as described above.

2. Preparation of CPA−Carboxymethyloxime−Ethylenediamine-Modified HRP (CPA-CMO-EDA-HRP) Conjugate. 2.1. Preparation of CPA-CMO Derivative. CPA-CMO was prepared as described by Hahnau and Weiler (1991). In a typical experiment, 1.3 mg of CPA was reacted with 2.1 mg of carboxymethoxylamine hemihydrochloride in 0.5 mL of dry ethanol/pyridine (1:1, v/v) at room temperature. Cyclopiazonic acid converted instantly to its CMO derivative as monitored by TLC. The R_f of the CPA and the reaction product in a developing solvent system of ethyl acetate/propan-2-ol/25% ammonia solution (10:3:2, v/v/v) were 0.47 and 0.07, respectively. After the solvents were removed from the reaction mixture under reduced pressure, CPA-CMO was extracted with ethyl acetate; the excess unreacted reagent was removed by washing the ethyl acetate solution four times with distilled water. After the solvent was evaporated, the residue was dissolved in 200 μL of dry *N*,*N*-dimethylformamide.

2.2. Coupling CPA-CMO to EDA-Modified HRP. Ethylenediamine-modified HRP was prepared according to the method for the preparation of EDA-BSA previously described by us (Chu et al., 1982). Briefly, 2.1 mg of HRP in 1.0 mL of sodium acetate buffer (NaOAc, 0.1 M, pH 5.5) was reacted with 10 μL of 10% EDA in the presence of 15 mg of EDPC at room temperature overnight. The reaction was stopped by dialysis against 2 L of NaOAc buffer in a cold room overnight. Conjugation of CPA-CMO to EDA-HRP was achieved by reacting 50 μL of the CMO-CPA solution with EDA-HRP in NaOAc buffer in the presence of 10 mg of EDPC at room temperature for 4 h. The final product was dialyzed against 2 L of 0.01 M PBS as above and stored at −20 °C until use. The ratio of the absorbance at 280 nm (due to HRP and CPA) and 402 nm (due to HRP) of the final product was found to be 0.79. In contrast, a ratio of 0.3 was found for the original EDA-HRP. Such change was due to the increase in absorptivity at 280 nm as a result of coupling of CPA to the HRP.

Direct Competitive ELISA. Protocols for the dc-ELISAs were essentially the same as we previously described for aflatoxin B_1 (AFB_1) (Chu et al., 1987). The optimal concentrations of mAb and enzyme marker used in the assay were determined using a checkerboard titration test. In a typical dc-ELISA, the microtiter plate wells were each coated with 100 μL of purified anti-CPA mAb (0.5 μg/mL IgG diluted in PBS) at 4 °C. After overnight incubation, the wells were washed four times by filling each with 300 μL of PBS containing 0.05% (v/v) Tween 20 (PBST), followed by addition of 200 μL each of the blocking solution [0.1% (m/v) BSA in PBS (BSA-PBS)] and incubation at 37 °C for 0.5 h. The wells

were washed four times with PBST as above. Fifty microliters of CPA standards or samples dissolved in 7.0% MeOH/PBS together with 50 μL of CPA-BSA-HRP conjugate (diluted in BSA-PBS) was added to each well. After incubation at 37 °C for another 1 h, the wells were washed again, to which 100 μL of K-blue substrate solution (ELISA Technologies, Lexington, KY) was added. The reaction was terminated by the addition of 100 μL of 1 M HCl to each well after 15 min of incubation at room temperature. The absorbance at 450 nm was determined using 650 nm as a reference wavelength in an automatic ELISA reader (Molecular Devices Co., Menlo Park, CA). Samples and standards were run in triplicate. Standard curves were calculated from the raw data using a four-parameter (sigmoidal) equation.

Extraction and Sample Preparation. For analysis of CPA in different commodities, 10 g of the ground sample (80 mesh) was mixed with 100 mL of 70% methanol in PBS and kept overnight at 4 °C. The sample was then shaken on an orbital shaker at a speed of 120 rpm for 30 min. The mixture was filtered through Whatman no. 1 filter paper, and the filtrates were diluted 1:10 times with PBS. Depending on the level of CPA per gram of sample and sample matrix, the solution was further diluted with 7.0% MeOH/PBS, and 50 μL portions of the diluted solution were used for ELISA. In the recovery experiment, various amounts of CPA in methanol or PBS were spiked into 10 g of blank samples and treated the same way as described above.

Analysis of CPA and Aflatoxin in Naturally Contaminated Corn Samples. A total of 13 corn samples that have previously been shown to contaminate CPA and aflatoxin were subjected to ELISAs. These samples were collected in August 1990 from Tifton, GA, and had been subjected to a collaborative study for the correlation of co-contamination of both CPA and aflatoxin in corn with an HPLC method (Urano et al., 1992b). The samples were shipped to our laboratory with coded numbers. Analytical data were not provided to us until we finished all of our analyses. For the analysis of CPA, the ELISA protocols described for the analysis of CPA in corn were used. For aflatoxin, a pAb-based ELISA using the protocol described by Chu et al. (1987) was used.

RESULTS AND DISCUSSION

Efficacy of Different HRP-Labeled CPA Markers on the dc-ELISA of CPA. Two types of enzyme markers were tested in our initial studies. One type was prepared by direct coupling of CPA to HRP via the Mannich reaction and the other by direct coupling of CPA-CMO to HRP via a water-soluble carbodiimide method. However, neither marker was recognized by the mAb elicited from cell lines of 5C8B2, 5C8D1, 5C8D3, or 5C8F3 in the dc-ELISA. We rationalized that the failure in binding of the marker enzyme to the antibodies could be due to the short distance of the bond between the hapten and the carrier enzyme. Subsequently, two new approaches for the preparation of the markers were made, and all of the approaches aimed to extend the bond distance. One involved coupling CPA-BSA to the HRP via the GA or PI method, and the other involved conjugating the CPA-CMO derivative to an ethylenediamine (EDA)-modified HRP in which the carboxyl groups in HRP were transformed to amino groups with the addition of a two-carbon spacer (Chu et al., 1982).

Results as shown in Figure 1 indicate that the newly synthesized enzyme markers were effective. The concentrations causing 50% inhibition of binding of the marker to the solid-phase antibody by CPA (IC_{50}) in the PI-CPA-BSA-HRP, GA-CPA-BSA-HRP, and CPA-CMO-EDA-HRP ELISA systems were found to be 0.42, 0.68, and 0.93 ng/mL, or 0.021, 0.034, and 0.046 ng per assay, respectively. Comparison of the IC_{50} values reveals that

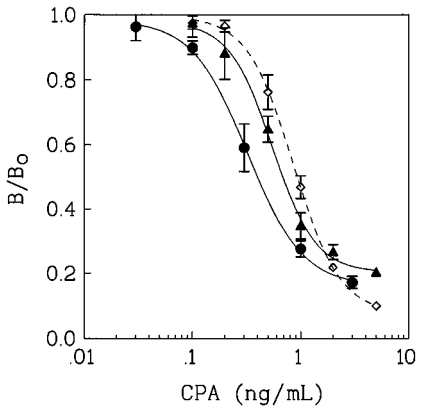

Figure 1. Standard curves for the dc-ELISA of CPA using different markers. The experiments were carried out under the following conditions: One hundred microliters of 5C8D1 mAb and 50 μL of enzyme marker were used in each assay. In the experiment using GA-CPA-BSA-HRP (▲), the concentrations of mAb and marker were 1.25 μg and 0.2 μg/mL, respectively. For PI-CPA-BSA-HRP (●), the concentrations of mAb and marker were 0.5 μg and 0.18 μg/mL, respectively. For CMO-CPA-EDA-HRP (◇), the concentrations of mAb and marker were 1.25 μg and 2.5 μg/mL, respectively. B/B_0 represents the ratio of absorbency of standard solution at different concentrations (B) to absorbencies in the absence of CPA (B_0), which were 1.0, 1.2, and 0.8 for GA-CPA-BSA-HRP, PI-CPA-BSA-HRP, and CMO-CPA-EDA-HRP, respectively. The IC_{50} values of CPA for the displacement of GA-CPA-BSA-HRP, PI-CPA-BSA-HRP, and CMO-CPA-EDA-HRP were estimated to be 0.68 ng/mL (0.034 ng per assay), 0.42 ng/mL (0.021 ng per assay), and 0.93 ng/mL (0.046 ng per assay), respectively.

the PI-based dc-ELISA is about 10 and 2400 times more sensitive, respectively, than pAb-based and mAb-based dc-ELISAs established by Hahnau and Weiler (1991, 1993). Using a reduction of 3 standard deviations of the mean absorbance of the blank samples as a basis (Fleeker, 1987), the detection limits for CPA in the PI-CPA-BSA-HRP, GA-CPA-BSA-HRP, and CPA-CMO-EDA-HRP dc-ELISA systems were found to be around 0.08, 0.2, and 0.3 ng/mL, respectively.

Among the three markers tested, CPA-BSA-HRP conjugates prepared by either PI or GA method were more effective; fewer antibodies and enzyme conjugates were needed in the dc-ELISA in these two systems. Diluting of the enzyme markers with BSA-PBS greatly enhanced the binding of CPA-BSA-HRP to the antibodies, hence improving assay performance. Although PI-CPA-BSA-HRP marker provided a more sensitive ELISA system for CPA, GA-CPA-BSA-HRP is much easier to prepare. Thus, GA-CPA-BSA-HRP was used in all of the dc-ELISAs described below.

Effect of pH and Methanol on the ELISA. To evaluate the effect of pH and methanol concentration on the ELISA, CPA standards in 0.01 M PBS at various pH values and methanol concentrations were subjected to the analyses. We found that the dc-ELISA performed satisfactorily under all of the pH values tested (6.0−8.5). Only a slight shifting of standard curves was observed. The IC_{50} values for CPA at pH 6, 7.5, 8.0, and 8.5 were found to be 1.0, 0.62, 0.71, and 0.56, respectively. In contrast, Hahnau and Weiler (1993) found a sharp decrease in sensitivity at pH >8 in the dc-ELISA developed by them. Because the dc-ELISA developed in the present study can run at pH 8.5, the solvent system (2% $NaHCO_3$/MeOH, pH 8.2) developed by Urano et al. (1992a,b) could easily be applied for extraction of the toxin from the sample.

Table 1. Analytical Recovery of CPA from Mixed Feed Extracting with Four Different Solvent Systems

solvent system	method A[a]		method B[a]	
	mean (%)	SD[b]	mean (%)	SD
A[c]	86.4	7.7	80.9	3.7
B	96.4	1.8	89.7	5.0
C	83.0	6.7	89.2	4.8
D	84.0	12.6	89.9	4.7

[a] ELISA CPA was performed in 7% CH_3OH/PBS (method A) and 7% CH_3OH/TBS (method B). [b] SD, standard deviations, $n = 4$. [c] Solvent systems: A, CH_3OH/2% $NaHCO_3$ (pH 8.2, 7:3, v/v); B, CH_3OH/0.05 M TBS (pH 8.0, 7:3, v/v); C, CH_3OH/0.01 M PBS (pH 7.4, 7:3, v/v); D, CH_3OH/0.01PBS (pH 7.4. 8:2, v/v).

Increased methanol concentration in the incubation medium resulted in a gradual decrease in assay sensitivity; for example, the standard curves became flat. However, the IC_{50} of the assays remained constant. Results obtained in a medium containing <10% methanol for samples and standard solutions were similar to those obtained in the buffer. An effective ELISA standard curve was obtained even in the presence of 30% methanol (data omitted). Our data are consistent with early reports that some mAb-based ELISAs can be made in solutions containing a relatively high concentration of methanol (Chu, 1996). It should be reiterated, however, that the pH and methanol levels should be maintained at a constant and identical level for both the standard and the assay samples.

Selection of Extraction Solvent. CPA can be extracted from the sample matrix with either a hydrophobic solvent such as chloroform or a hydrophilic, water miscible solvent such as methanol (MeOH) or acetonitrile. Because extracts obtained from the latter solvent system could be used directly in the ELISA, the efficacy of four solvent systems, which contained 70 or 80% methanol in different buffers at pH values of 7.4−8.2, for the extraction of CPA was evaluated in the ELISAs. We found that all four solvent systems have similar extraction efficiencies (Table 1). The mean analytical recoveries of CPA from the mixed feed (1 ppm added) were found to be 87.5 and 87.4%, respectively, when 7% MeOH/PBS and 7% MeOH/TBS were used in the ELISA**.** Our data confirmed early reports that solvent system A (CH_3OH/2% $NaHCO_3$, pH 8.2, 7:3, v/v) is a good solvent for the extraction of CPA from corn and peanuts (Urano et al., 1992a,b).

Effect of Sample Matrix on the dc-ELISA. Results for the effect of sample matrix on the dc-ELISA are shown in Figure 2. Three different CPA-free matrices, e.g. corn, mixed feed, and peanuts, were tested. The samples were extracted with solvent C (70% MeOH/PBS) and then diluted with PBS (1:10). Subsequent dilutions were made with 7% MeOH/PBS before the dc-ELISA was performed. Thus, the final concentrations of methanol in the standards and samples were maintained at 7%. No significant change in binding of the marker enzyme to the solid-phase mAb was observed when each milliliter of assay solution contained less than 5, 1, and 0.5 mg of corn, mixed feed, and peanut extracts, respectively. The overall data showed that corn extract contained the fewest interference materials affecting the binding of marker enzyme to the coated antibodies, whereas peanut extracts contained the most interference materials. Because the lower detection limit of CPA in buffer solution was estimated to be around 0.2 ng/mL in the GA-ELISA system, the detection limits of CPA in corn, feed, and peanuts were estimated as 40 (i.e. 0.2 ng/5 mg), 200 (0.2 ng/1 mg), and 400 ppb (0.2/0.5 mg), respectively.

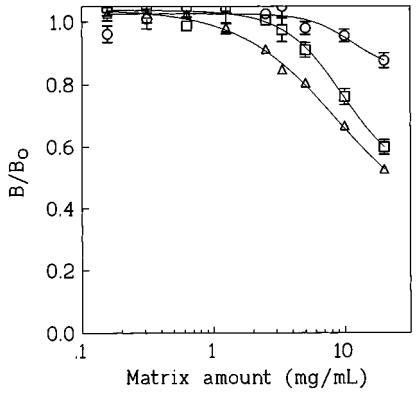

Figure 2. Effect of sample matrix on dc-ELISA of CPA. Each well of the ELISA plate was coated with 100 μL of 5C8D1 mAb (1.25 μg/mL). The enzyme marker GA-CPA-BSA-HRP, diluted with BSA-PBS to a final concentration of 0.18 μg/mL, was used in each assay. Symbols represent corn (○), mixed feed (□), and peanuts (△).

Table 2. Analytical Recoveries (Percent) of CPA in Spiked Corn, Mixed Feed, and Peanuts[a]

CPA added (ppb)	corn			mixed feed			peanuts		
	detd	rec	CV	detd	rec	CV	detd	rec	CV
50	69	138	7.1	134	268	2.0			
100							377	377	14.6
200	193	96.5	12.6	238	119	9.1			
500							672	134	5.7
1000	1000	100	8.1	802	80.2	8.6			
2000							2010	100	7.8
5000	4818	96.4	6.7	3841	76.8	1.2	4298	86.0	8.1
mean rec (%)		97.6[b]			92[b]			93[c]	

[a] Abbreviations used: detd, determined; CPA detected (ppb) by dc-ELISA using GA-CPA-BSA-HRP as marker; rec, recovery of CPA (%); CV, coefficient of variation. [b] Excluding 50 ppb level for the calculation of the mean recovery. [c] Excluding 100 and 500 ppb levels for the calculation of the mean recovery.

Analytical Recovery of CPA Spiked in Corn, Feed, and Peanuts. To test the efficacy of the ELISA developed in the present study, CPA at levels between 50 and 5000 ppb was added to corn, mixed feed, and peanut and then extracted with 70% MeOH/PBS. The sample extracts were diluted to contain 7% MeOH/PBS and then directly subjected to dc-ELISA using CPA-BSA-HRP(GA) as the enzyme marker. Results in Table 2 show that 138−96.4 and 268−76.8%, respectively, of added CPA to corn and mixed feed in the range of 50−5000 ppb were recovered. In peanut samples, 134−86.0% of CPA in the range of 500−5000 ppb was recovered. The mean analytical recoveries for CPA in these samples after the false positives were discarded (>100% recovery) were found to be 97.6, 78.5, and 93.0% for corn, mixed feed, and peanuts, respectively.

Because false-positive data were obtained for corn, mixed feed, and peanuts at levels of 50, 200, and 500 ppb, respectively, the detection limits for CPA are estimated to be around 100 ppb in corn, 300 ppb in feed, and 600 ppb in peanuts. These values are slightly higher than those obtained from the matrix studies (Figure 2) in which we estimate that the detection limits for CPA in corn, feed, and peanuts are around 40, 200, and 400 ppb, respectively.

Analysis of Naturally Contaminated Corn Samples by dc-ELISA. To test the efficacy of the ELISA

Table 3. ELISA and HPLC Analyses of CPA and AFB_1 in Naturally Contaminated Corn Samples

	CPA			AFB_1		
	ELISA		HPLC[a]	ELISA		HPLC[a]
sample	ppb	SD	ppb	ppb	SD	ppb
11	235	63	120	493	136	760
12	1341	284	770	462	70	515
15	186	21	89	49	7	56
17	126	7	T[b]	4	1	14
19	1077	157	1598	2354	354	2230
21	2867	572	633	219	31	346
22	ND[c]		ND	ND		15
24	ND		ND	7	1	5
25	288	12	225	154	7	138
28	100	2	ND	6	3	34
29	2998	194	2771	1028	98	1600
30	449	65	231	269	23	242
34	117	11	47	19	1	21

[a] Data from Urano et al. (1992b) with a detection limit around 25 ppb. [b] Trace amounts of CPA were detected. [c] ND, none detected; values were lower than the detection limits.

developed in the present study, 13 corn samples naturally contaminated with both CPA and AFB_1 were subjected to the analyses. To confirm previous analytical data, AFB_1 in these samples was also determined using a dc-ELISA developed in our laboratory and the results are given in Table 3. Linear regression analysis revealed that there is an excellent correlation between the AFB_1 level in these samples by the two analytical methods in two laboratories over more than five years. The correlation coefficient between ELISA and HPLC methods for AFB_1 data was 0.968 ($p < 0.0001$) with a regression slope of 1.022. These data indicate that both ELISA and HPLC methods for aflatoxin analysis are sound and that the aflatoxin was very stable during storage.

The correlation between ELISA and HPLC data for CPA was less good with an r value of 0.79 ($p = 0.0012$) and a regression slope of 0.62. The ELISA data for CPA were considerably higher than those of HPLC results. The difference between ELISA and HPLC data for CPA might be due to the following: (1) there may be differences between CPA standards used in the two different laboratories; (2) the recovery of CPA in corn by HPLC was 72−84% (Urano et al., 1992a,b), whereas the recovery by the present dc-ELISA was 97.6% (the regression slope for the ELISA and HPLC for CPA would be in the range of 0.79−0.72 if only 72−84% of analytical recovery in HPLC is considered in the calculation); (3) some matrix interference in the ELISA may occur; (4) antibodies against CPA may cross-react with some CPA-related analogues, which may be present in the samples; and (5) long periods of storage of CPA may lead to some products that are more reactive with the antibodies.

Conclusion. Two new approaches for the preparation of enzyme markers for the dc-ELISA were made in the present study. Both approaches involved an extension of the length of the arm between the hapten and the marker enzyme HRP. The marker enzymes prepared according to these approaches worked well in the dc-ELISA. In contrast, conjugates prepared without the linker arms, including CPA-HRP prepared according to the Mannich method and CPA-CMO−HRP prepared by using the WSC method, were not recognized by mAb. These data further reiterate that the length of the spacer played a role leading to the inability of binding of the marker enzyme to the antibodies. Similar to the present results, Xiao et al. (1995) demonstrated that the sensitivity of the idc-ELISA improved considerably when the coating antigens prepared according to different methods and different carrier proteins were used. Whereas the present ELISA method can detect as low as 0.08 ng of CPA/mL of buffer solution, the assay sensitivity was greatly affected by sample matrixes. Thus, it cannot detect CPA accurately when the levels are less than 100, 300, and 600 ppb in corn, mixed feed, and peanuts, respectively. In a survey of CPA in 45 corn and 50 damaged peanut samples, Urano et al. (1992b) found that 51% of corn and 90% of peanut samples contained CPA with average levels of 467 ppb (ranging from <25 to 2777 ppb) and 460 ppb (ranging from <50 to 2929 ppb), respectively. Of these positive samples, 65% of corn and 22% of peanut samples contained more than 100 and 500 ppb, respectively. High levels of CPA have also been found in moldy corn (1.9−4 ppm) and moldy feed (1.0−2.05 ppm) by Chang-Yen and Bidasee (1990). Thus, the detection limits of the present ELISA should be able to disclose CPA in the samples that are most likely to be contaminated with CPA. Because no cleanup of the sample extract is necessary, the present dc-ELISA could serve as a quick screening method for CPA. The sensitivity of the present ELISA could also be improved by incorporation of a cleanup step in the assay protocol.

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