Production of Polyclonal Antibodies against Ochratoxin A and Its Detection in Chilies by ELISA

K. Thirumala-Devi,[†] M. A. Mayo,[‡] Gopal Reddy,[§] S. V. Reddy,[†] P. Delfosse,[†] and D. V. R. Reddy*[,†]

International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra pradesh 502 324, India, Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK, and Osmania University, Hyderabad, Andhra Pradesh 500 007, India

Polyclonal antibodies were produced for Ochratoxin A (OA) by injecting OA−bovine serum albumin (BSA) conjugate subcutaneously at multiple sites into a New Zealand White inbred rabbit. Antiserum could be used at a dilution exceeding 1:100 000 in an indirect competitive enzyme-linked immunosorbent assay (ELISA), and detected OA concentrations up to 0.1 ng/mL. The 50% inhibition binding (I_{50}) of OA was 5 ng/mL. Antibodies did not react with ochratoxin B, coumarin, 4-hydroxycoumarin, L-phenylalanine, and aflatoxin B1. OA contamination in chilies (*Capsicum annum* L.) collected from commercial markets and cold storage units was determined. The mean recoveries from OA-free chilies spiked with 1 to100 μg of OA per kg of chili sample were 90−110% with a standard deviation of <10%. Of 100 chili samples tested, 26 were found to contain over 10 μg/kg of OA. In 12 samples the OA concentration varied from 10 to 30 μg/kg, in 10 samples from 30 to 50 μg/kg, in 3 samples from 50 to100 μg/kg, and in one sample it was 120 μg/kg. This is the first record in India of OA in chilies, a major component of cooked foods in this country, and it is noteworthy that OA contamination exceeded the permissible limit for human consumption of less than 20 μg/kg in over 26% of the market samples tested.

Keywords: *Ochratoxin A; chilies; ELISA; polyclonal antibodies; mycotoxin*

INTRODUCTION

Ochratoxin A (OA) is a mycotoxin produced by certain species of *Aspergillus* and *Penicillium* (Munro et al., 1974). It has been shown to be nephrotoxic, hepatotoxic, teratogenic, carcinogenic, mutagenic, and an immunosuppressive agent (Kuiper-Goodman and Scott, 1989). Of greatest concern for human health is its implicated role in an irreversible and fatal kidney disease referred to as "Balkan Endemic Nephropathy". OA has been found to occur in foods of plant origin, in edible animal tissues, and in human blood sera, tissues, and milk. Therefore, OA contamination of foods is a potential hazard for humans. The Provisional Tolerable Daily Intake (PTDI) for humans, proposed by the World Health Organization, is 16 ng OA/kg body weight/day (Hohler, 1998).

A variety of foods are susceptible to mycotoxin contamination, and these include spices. The molds isolated from spices are predominantly *Aspergillus* and *Penicillium* species (Flannigan and Hui, 1976). Chili is a popular spice in the Indian subcontinent and is consumed by many people whose incomes are below the poverty line. Additionally, chilies are exported from India to some neighboring countries. The only mycotoxins currently known to occur in chilies in India are aflatoxins (Madhyastha, 1985). The occurrence of OA has not yet been reported. Concentration of OA can be determined by analytical methods such as thin-layer chromatography, liquid chromatography, gas chromatography, and mass spectrometry (Balzer et al., 1978; Josefsson and Moller, 1979; Osborne, 1979). These techniques require extensive sample preparation and are expensive. Enzyme-linked immunosorbent assay (ELISA) is gaining wide acceptance for estimating the concentrations of mycotoxins because of its sensitivity and specificity, and it is less expensive to perform than other analytical methods. Using polyclonal antibodies, indirect competitive ELISA has been successfully used for OA estimation in barley and wheat (Morgan et al., 1983; Lee and Chu, 1984). This paper reports the production of high-titered polyclonal antibodies against OA and the adoption of an indirect competitive ELISA procedure for estimation of OA in chilies. To assess the extent of ochratoxin A contamination in chilies in commercially available samples, a survey was conducted in the two major chili-producing regions of southern India.

MATERIALS AND METHODS

Materials. Ochratoxin A, ochratoxin A-BSA conjugate, Freund's complete adjuvant, Freund's incomplete adjuvant, goat anti-rabbit IgG-ALP conjugate, *p*-nitrophenyl phosphate, and bovine serum albumin (BSA), were all purchased from the Sigma Chemical Co., St. Louis, MO. Microtiter plates (Maxisorp F96) were obtained from Nalge Nunc International, Denmark. All other chemicals were reagent grade or chemically pure.

Production of Polyclonal Antibodies. Commercially available OA-BSA conjugate (Sigma cat. no. 0−3007) was used. OA-BSA (250 μg) in 250 μL of sterile 0.01 M phosphate-

* Address for correspondence: Dr. D. V. R. Reddy, Principal Scientist, GREP, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, India; Tel: +91-40-329-6161, ext. 2669; Fax: +91-40-329-6182; E-mail: D.REDDY@CGIAR.ORG.

[†] International Crops Research Institute for the Semi-Arid Tropics.

[‡] Scottish Crop Research Institute.

[§] Osmania University.

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buffered saline (PBS) was emulsified with an equal volume of complete Freund's adjuvant and injected into a New Zealand White inbred rabbit subcutaneously on the dorsal side at multiple sites. Subsequent immunizations were given with incomplete Freund's adjuvant. After four immunizations at weekly intervals followed by a booster after three weeks, the rabbit was bled at weekly intervals and the titer checked by indirect competitive ELISA. Booster injections were given when a drop in the titer was noticed. Serum was lyophilized and stored at −20 °C until utilized.

Monitoring Antibody Titers. An indirect ELISA procedure similar to that reported for aflatoxins (Devi et al., 1999) was used. Microtiter plate wells were coated with 1 μg/mL of OA-BSA in 0.2 M sodium carbonate buffer, pH 9.6 (150 μL/well; Hobbs et al., 1987) and incubated overnight in a refrigerator. Subsequent steps were performed at 37 °C for 1 h. Antiserum was diluted in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and 0.2% BSA (PBS-T BSA) and held for 45 min at 37 °C. Antiserum dilutions in 50-μL volumes were added to 100 μL of OA at concentrations ranging from 100 ng/mL to 100 pg/mL. Goat antirabbit immunoglobulins (GAR IgG) conjugated to alkaline phosphatase were used at a 1:1000 dilution to detect rabbit antibodies attached to OA. *p*-Nitrophenyl phosphate was used as a substrate at 1 mg/mL and allowed to develop for 1 h at room temperature. Absorbance was recorded at 405 nm (A_{405}) with an ELISA plate reader (Titertek Multiskan, Labsystems, Finland).

Specificity of Antibody. To evaluate the cross-reactivity of the antibody with ochratoxin B, coumarin, 4-hydroxycoumarin, L-phenylalanine, and aflatoxin B1, it was essential to determine the optimum conditions for neutralization. These included coating antigen (OA-BSA) concentration and the optimum dilution of the antibody required for neutralization. The optimum dilution required to obtain the maximum sensitivity was determined by 50% displacement values of *B*/*B0*, where *B* is the extinction of the well containing OA, and *B0* is the extinction of the well without toxin, derived from the slope of the calibration curves.

Collection of Chili Samples. Dried chili pods were collected from market yards and cold storage units of the two major chili-growing areas in the state of Andhra Pradesh in India, namely the Guntur and Khammam districts. From 50-kg bags, approximately 1-kg samples were drawn from several points. A total of 80 samples (70 from market yards and 10 from cold storage units) were collected and graded into three types: grade 1, grade 2, and grade 3, based on the quality of the pods. Samples categorized as grade 1 were of good quality, having fully matured pods (mostly from the first plucking), that were unbroken, bright red colored, and absolutely free from pod diseases, insect damage, and molds. Grade 2 had samples of medium quality and size and only a few pods were discolored. Grade-3 samples contained moldy, damaged, and discolored pods left over when sound pods were mechanically separated from the produce. In addition, 20 different brands of chili powders were obtained from retail shops.

Preparation of Chili Samples for ELISA. *Dried Chili Pods.* Samples were dried at 40 °C for a week. Pods were thoroughly mixed, three aliquots of 50 g were drawn and ground to a fine powder, of which 15 g was processed for ELISA.

Chili Powders. Commercially available samples were purchased in 200-g quantities. After a thorough mixing, three 15-g sub-samples were drawn from each 200-g sample. Each 15-g quantity (in finely ground form) was extracted in 75 mL of a mixture of methanol−water and KCl (70:30:0.5%) by blending in a Waring blender, followed by shaking for 30 min. The extract was filtered through Whatman No. 41 filter paper and diluted to 1:4 with PBS-T-BSA for processing by ELISA.

Indirect Competitive ELISA Procedure for Processing Chili Samples. The protocol was similar to that for determining antibody specificity (as described above) with the exception that OA standards in 100-μL volume, ranging from 100 ng/mL to 100 pg/mL, were prepared in a diluted extract from chilies. Only chili samples which did not contain ochratoxins were used. They were extracted in methanol as described above, filtered, and used at a 1:10 dilution prepared in PBS-T BSA. Test chili samples were diluted to 1:4 in PBS-T BSA prepared in OA-free chili extract. A 100-μL aliquot of each sample was added to a well containing 50 μL of antiserum. Standard curves were obtained by plotting $\log_{10}$ values of OA standards against optical density at A_{405}. Concentration of OA in the sample extract was determined from the standard curves and expressed in μg/kg using a formula: OA concentration (ng/mL) in sample extract × dilution with buffer × extraction-solvent volume used (mL)/sample weight (g). To test the recovery of OA from spiked chilies, OA standards were added to finely ground 15-g samples, at concentrations ranging from 0.1 to 100 μg/kg, then extracted and assayed as above.

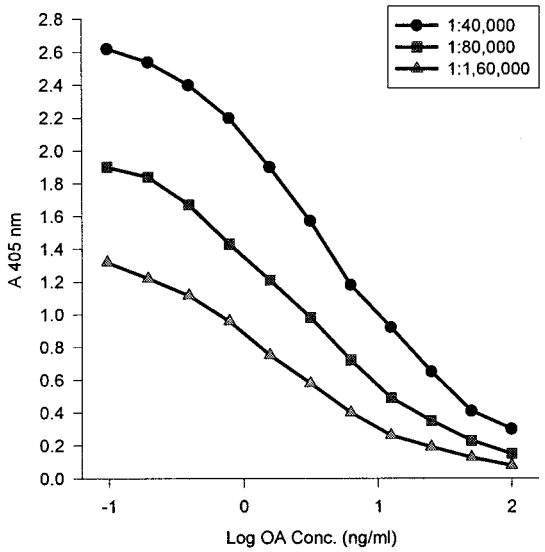

Figure 1. Standard curves for OA by indirect competitive ELISA at three different dilutions of antibody: 1:40 000 (●); 1:80 000 (■); 1:160 000 (▲).

RESULTS AND DISCUSSION

Production of Antibody. The protocol used for immunization gave an antibody titer of 1:132 000, 34 weeks after initiation of immunization.

Optimum OA-BSA Concentration for Coating ELISA plates and Antibody Dilution for ELISA. OA-BSA conjugate was tested at concentrations ranging from 10 μg/mL to 1 ng/mL in 10-fold intervals. In five independent tests, an OA-BSA conjugate concentration of 1 μg/mL was found to be optimum for coating the plates. Antiserum at a dilution of 1:100 000 gave optimum results. The curves for OA standards at different dilutions of the antibody are shown in Figure 1. Linear inhibition curves were obtained for OA concentrations ranging from 1 to 100 ng/mL. Using conjugate at 1μg/mL and antiserum at 1:100 000 dilution, 50% inhibition of binding of OA was estimated to occur at 5 ng/mL.

Specificity of Antiserum. To determine the cross-reaction of the antiserum with molecules resembling OA, it was decided to test it against ochratoxin B, L-phenylalanine, coumarin, 4-hydroxy coumarin, and Aflatoxin B1. Various dilutions of these were used in ELISA but the antibodies did not react with any of them (Figure 2).

Effect of Chili Extract on ELISA. The ELISA procedure reported for AFB1 estimation (Devi et al., 1999) did not at first give accurate results due to the interference of substances present in the chili extract. To confirm that interference in ELISA was due to chili extract, we compared OA standards prepared in PBS-T

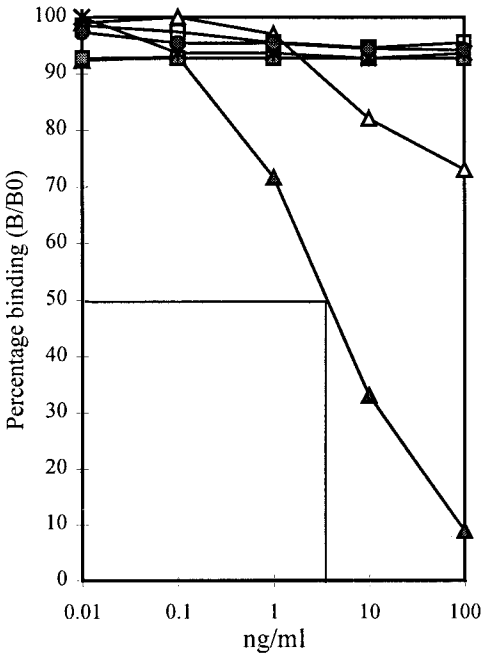

Figure 2. Cross reactivity of OA antiserum (▲) to OB (△), coumarin (■), 4-hydroxy coumarin (□), L-phenylalanine (∗), and AFB1 (●).

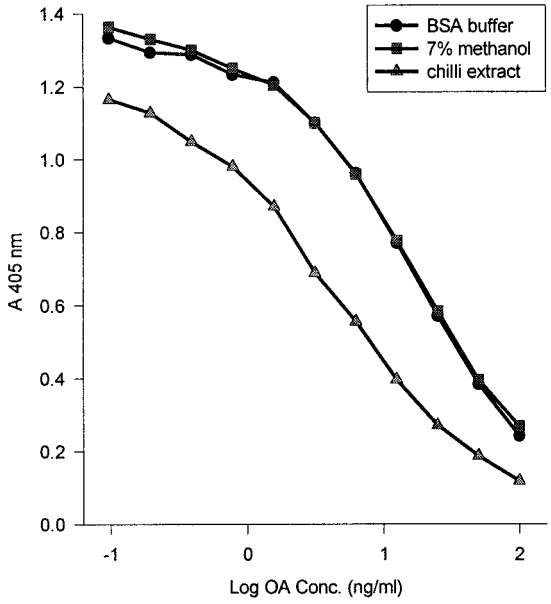

Figure 3. Effect of chili extract on the standard curve of OA by indirect competitive ELISA. Standard curves were prepared for OA standards, either by diluting in BSA PBS-T (●), or in BSA PBS-T containing 7%methanol (■), or in BSA PBS-T containing chili extract (▲).

BSA with and without chili extract. Preparation of chili extract for this purpose is described in materials and methods. Curves were found to be influenced by substances present in the chili extract. In the absence of chili extracts nonspecific absorption was noticed (Figure 3). As a result it was essential to prepare the standard solutions of OA in chili extract devoid of OA.

Chili extracts have been shown to contain substances which can bind to aflatoxins (Shantha, 1999). It was apparent that test samples require preparation in extracts from OA-free chilies to prevent interference from substances present in chilies. We met with a similar situation when indirect ELISA was used for

Table 1. Recovery of OA from Artificially Contaminated Chili Samples as Determined by ELISA

sample no.	concn. of OA used for spiking (μg/kg)	concn. of OA estimated (μg/kg)[a]	percent recoveries of OA in spiked samples[b]
1	1	1.26 ± 0.16	110 ± 4.7
2	5	4.6 ± 0.37	92 ± 7.4
3	10	9.36 ± 0.94	94 ± 8.9
4	50	52.1 ± 0.87	103 ± 1.6
5	100	125 ± 3.9	103 ± 3.3

[a] Each sample was spiked with a known concentration of OA, extracted in 70% methanol, and assayed. Data represent means of three replications ± SD. [b] Determined by formula: detected OA (μg/kg) / the concentration of OA used for spiking × 100. Values are means ± SD.

Table 2. Incidence and Range of OA in Chilies as Determined by Indirect Competitive ELISA

	OA contamination		no. of samples with OA contents (μg/kg) in the ranges of			
sample type[a]	incidence	percent	10−29	30−49	50−100	120
chilies grade 1 (market yards)	2/32	6	0	1	1	0
chilies grade 2 (market yards)	2/14	14	1	0	1	0
chilies grade 3 (market yards)	8/23	35	3	5	0	0
chilies (cold storage)	0/10	0	0	0	0	0
chili powders (from super markets)	14/21	66	8	4	1	1

[a] See text for details regarding sample types and sources.

estimating aflatoxins in chilies (Kiranmayi, D.; Reddy, S. V.; Reddy, U.; Thirumala Devi, K.; and Reddy, D. V. R., unpublished). Therefore we presume that chili extracts contain substances which can bind non-specifically to IGg's.

Recovery of OA from Spiked and Naturally Contaminated Chilies. Of four ochratoxin A extraction procedures tried (Lee and Chu, 1984; Candlish et al., 1988; Ramakrishna et al., 1990; Barna-vetro and Solti, 1996) for processing chili samples for ELISA, extraction in 70% methanol followed by 4 times dilution in PBS-T gave 95% recoveries in spiked samples. The mean recoveries from chili samples devoid of OA, spiked with 1 to 100 μg/kg OA, were 93 to 110% (Table 1). Analysis of three replicates of 100 chili samples showed that 26 samples contained OA at levels ranging from 10 to 120 μg/kg (Table 2).

The results of OA analysis of chili samples collected from different locations (market yards, cold storage, and retail shops) are shown in Table 2. It was observed that the incidence of OA contamination in the market yards correlated with the sample grades (6% in grade 1, 14% in grade 2, and 35% in grade 3). In grade 1, two samples were found to be contaminated with OA: 47 μg/kg in one and 93 μg/kg in the other. The occurrence of such high concentrations can be attributed to the presence of inadequately dried pods in the affected lot. None of the samples from cold storage was contaminated by OA. It was observed that the contamination of OA was greater (66%) in the samples obtained from retail shops than in those from market yards. This may be due to differences in storage time, which in the market yards could have been relatively short as the commodity is normally traded-off immediately.

Growth of the mold and the production of ochratoxin are dependent upon a number of factors such as tem-

perature, humidity, handling during harvesting, and conditions during storage. Chilies are produced in countries with tropical climates that have extreme ranges of rainfall, temperature, and humidity. Sun drying of chilies may result in toxin contamination (Atanda et al., 1990). Typically, chilies are spread on the ground for sun-drying in the open air where temperature and humidity favor growth of the mycoflora. To increase their weight, chilies are often wetted by sprinkling with water, a practice likely to promote fungal growth. Guidance on post-harvest technology should be given to farmers, distributors, and retailers concerning proper drying and storage of the chili pods to minimize mold growth.

The importance of red chili in most Indian dishes is evident, as is the need to maintain high quality and freedom from toxic substances such as ochratoxins. It is likely that the lack of sufficient surveillance data on ochratoxins in chilies in India can be attributed to the unavailability of reliable analytical techniques. The present study clearly shows that chilies can contain OA at levels beyond those permissible by public health authorities and emphasizes the need for surveillance of ochratoxin A in chilies. It is currently not known how many other spices that are commonly used in cooking may be contaminated with OA, and research is needed to elucidate this problem and to make producers and consumers aware of the potential health hazards from consuming OA.

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