Development of Cost-Effective and Simple ELISA-based Technologies for the Estimation of Aflatoxins and Ochratoxin A in Foods and Feeds

By

K Thirumala-Devi

Thesis submitted to the Osmania University
for the degree of Doctor of Philosophy in Microbiology

MICROBIOLOGY DEPARTMENT
OSMANIA UNIVERSITY
HYDERABAD, INDIA

2001

Dedicated to

Dr D V R Reddy

Whose continued support, inspiration, invaluable guidance and life giving words has made this possible

Dr Gopal Reddy
Assoc. Professor
Microbiology Department
Osmania University
Hyderabad - 500 007
Andhra Pradesh, India

Dr DVR Reddy
Principal Scientist
Mycotoxins & Virology, Crop Health Unit, GREP
International Crops Research Institute
for the Semi-Arid Tropics
Patancheru - 502 324, India

CERTIFICATE

This is to certify that the thesis entitled ***"Development of Cost-Effective and Simple ELISA-based Technologies for the Estimation of Aflatoxins and Ochratoxin A in Foods and Feeds"*** submitted by ***Mrs K Thirumala-Devi***, for the award of degree of ***Doctor of Philosophy in Microbiology***, Osmania University, is a record of work done by her during the period 1998-2001, under our supervision and that it has not previously formed the basis for the award of any degree or diploma or associate or fellowship or other similar title.

Dr Gopal Reddy

Dr DVR Reddy

DECLARATION

I hereby declare that the thesis entitled ***"Development of Cost Effective and Simple ELISA-based Technologies for the Estimation of Aflatoxins and Ochratoxin A in Foods and Feeds"*** submitted for the degree of ***Doctor of Philosophy in Microbiology***, Osmania University, is a record of the bonafied research work done by me at Department of Microbiology, Osmania University and International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru under the supervision of Dr. Gopal Reddy and Dr. D V R Reddy. This thesis has not formed in whole or in part, the basis for the award of any degree or diploma, earlier to this date.

K. Thirumala-Devi

Department of Microbiology
Osmania University
Hyderabad - 500 007
AP, INDIA
E-mail: **T.DEVI@CGIAR.ORG**

ACKNOWLEDGEMENTS

It is with great pleasure and respects, I wish to express my gratitude to my research supervisors, Dr Gopal Reddy and Dr DVR Reddy for their inspiring guidance, constant encouragement, moral support, critical analysis and care which contributed to successful completion of this thesis.

I would like to express my profound gratitude and heart felt thanks to Dr Mike Mayo, Senior Principal Virologist, Scottish Crop Research Institute (SCRI), Dundee, Scotland, United Kingdom, for his able guidance, encouragement and care during my stay at SCRI.

I would like to express my gratitude and reverence to Dr Anjali Karande, Indian Institute of sciences, Bangalore for her able guidance to produce monoclonal antibodies.

I am grateful to the Dean, Faculty of Science, Osmania University for his help and encouragement for the submission of the thesis.

I am grateful to Dr L Venkateshwar Rao, Head of the Department and Professor G Seenayya and Dr Padma Sridhar, former Heads of the Department of Microbiology, Osmania University, Hyderabad for their help and encouragement.

I am extremely grateful to my husband K L Ch Sanjeev Rao whose moral support, encouragement and understanding has made this possible. I would like to express my gratitude to my parents who gave me all the moral support I needed and love to my one year old daughter K V S Apurva for bearing so many hours of my absence at home.

I wish to express my gratitude to A Sudarshan Reddy and Philippe Delfosse and special thanks to S Veera Reddy, Mycotoxin diagnostics and virology lab, ICRISAT for their support and help.

I sincerely thank Jane Miller, Angelica Ziegler and Michele Linsey for their help and useful discussions for the selection of aflatoxin mimotopes and their care during my stay at SCRI.

I acknowledge the able technical support from Y Ramachandraiah, S Prabhakar Reddy and Ch Ravinder Rao of Mycotoxin diagnostics and Virology lab ICRISAT.

I gratefully acknowledge the Department for International Development, UK for providing me the fellowship and funding for the research under the holdback grant R7083, awarded to ICRISAT and SCRI.

K Thirumala Devi

Safe to Eat or Why Chickens Die and Human Beings Suffer from Liver Cancer : Development of Cost-Effective and Simple ELISA-based Technologies for the Estimation of Aflatoxins and Ochratoxin A in Foods and Feeds

Many agricultural commodities are vulnerable to attack by a group of fungi that are able to produce toxic metabolites called mycotoxins. Among various mycotoxins, aflatoxins and ochratoxin A have assumed economic importance. They are harmful to human beings, poultry and livestock. Aflatoxins are potent carcinogens and immunosuppressive agents and ochratoxin A is a nephrotoxin. A recent report from India showed clearly the risk the population in Indian subcontinent is exposed to liver cancer as a result of hepatitis B virus and aflatoxin B1 (Kathyar *et al.* 2000). Unfortunately aflatoxins as well as ochratoxin A enter into milk and subsequently into many milk-based foods. One of the important effects of these toxins on human beings is suppression of immune system, which will lead to many disorders.

In order to protect animal and human health, it is important to be able to estimate mycotoxin content in various foods as well as feeds so that strategies can be devised for their reduction/elimination. Among many methods available for the estimation of mycotoxins, immunochemical methods are simple, cost-effective and adaptable to situation in developing countries. The major requirements for the application of immunochemical methods are high quality antibodies and methodologies to use the antibodies for the estimation of mycotoxins. Therefore this thesis was focused on these aspects.

This thesis is divided into 9 chapters containing subsections. Chapters 1 and 2 contain the "Introduction" and "Review of Literature", chapter 3 and 4 "Production of Antibodies for Aflatoxins and Ochratoxin A", chapter 5 "Development of Enzyme Linked Immunosorbant Assays", chapter 6 "Analysis of Foods and Feeds for Aflatoxins and Ochratoxin A". Chapter 7 contains "Discussion". The "Summary and Conclusions" are presented in chapter 8, and the "References" in chapter 9.

Out line

CONTENTS

List of Tables

List of Figures

Chapter 1

Introduction

INTRODUCTION

1.1. Background

Agricultural products are always at risk of contamination with fungi, and some of these are able to produce toxic metabolites. These compounds are called **mycotoxins** and the diseases caused are called **mycotoxicoses**. Although it has been known from more than 100 years that some kinds of mouldy grains, when eaten, may cause illness, intensive study of mycotoxins and mycotoxicoses only dates from the 1960s, following the occurrence of 'Turkey-X disease' in the United Kingdom. The disease resulted from the presence of aflatoxin B1 (Sargeant *et al.*, 1961) in the feed imported from Brazil. Propelled by the discovery of the aflatoxins, much effort has been directed to the investigation of the association between exposure to naturally occurring mycotoxins and long-term adverse health effects in human beings and livestock. Among various mycotoxins, aflatoxins and ochratoxin A have assumed economic importance (Van Egmond 1991; Bosch and Peers 1991). They are harmful to human beings, poultry and livestock. Aflatoxins are potent carcinogens and ochratoxin A is a nephrotoxin. A recent report from India showed clearly the risk the population of the Indian subcontinent is exposed to liver cancer as a result of hepatitis B virus infection and ingestion of aflatoxin B1 (Kattyar *et al.*, 2000).

Virtually all countries engaged in international commerce have enacted or have proposed permissible levels of mycotoxin contamination in foods and feeds. Indeed, aflatoxin contamination levels are used in international trade to fix the price of various commodities. Thus, it becomes imperative that methods are available that can monitor effectively the mycotoxin content of a range of commodities in both the exporting and importing countries.

Many different methods are available currently for the estimation of mycotoxins. Biological detection methods are based on the toxic effect of mycotoxins, which include death, pathological lesions, changed biochemical events or immunotoxic effects (Chu, 1978). Often these methods are not specific and their sensitivity is generally low compared to that of other methods. As a result they

are used only as a tool for screening general toxicity. Physicochemical assay methods such as thin layer chromatography, high performance liquid chromatography, gas chromatography or mass spectrometry are laborious and require expensive instrumentation and clean-up of the samples. Thus these methods are of limited use in routine safety and quality control screening for mycotoxins in agricultural commodities. To overcome the difficulties with biological and physicochemical methods, new immunochemical methods have been developed. Serological methods that use specific antibodies are gaining wide acceptance for quantitative estimation of mycotoxins because of their sensitivity and specificity (Chu, 1986).

In most developing countries, limited or no facilities exist for monitoring aflatoxins or ochratoxin in foods and feeds. Commercial kits utilising immunological methods are expensive, in addition to the problems related to importing them. The maximum allowed limits of aflatoxins and ochratoxin A range from zero to 10 µg/kg. Therefore, for consumption as well as for export, it is essential to monitor the foods and feeds for mycotoxin content. The main aim of this study was to develop highly sensitive and cost-effective ELISA-based methods for quantitative and qualitative estimation of aflatoxins and ochratoxin A in foods and feeds.

1.2. Objectives

The presence of mycotoxins in agricultural commodities is particularly unavoidable because of the uncontrollable environmental conditions that favour mycotoxin contamination. In order to protect animal and human health, it is important to be able to detect and quantify mycotoxins in foods and feeds. Thereafter, the contaminated materials can be handled in a way to eliminate the toxins. Immunochemical methods that use specific antibodies and suitable assay systems that are sensitive and cost-effective are therefore vital to reduce the risk of aflatoxins and ochratoxin A contamination. The objectives of the present study were therefore:

- Production of polyclonal and monoclonal antibodies for aflatoxins
- Production of polyclonal antibodies for ochratoxin A

- Development of ELISA based test procedures for the estimation aflatoxins or ochratoxin A
- Analysis of foods and feeds for contamination with aflatoxins ochratoxin A

Chapter 2

Review of Literature

REVIEW OF LITERATURE

2.1. Mycotoxins

Mycotoxins are a group of chemically diverse secondary metabolites of fungi. They exhibit a wide array of biological effects and individual mycotoxins can be mutagenic, carcinogenic, embryo-toxic, teratogenic, oestrogenic or immunosuppressive (Hohler *et al.*, 1998). Several human mycotoxicoses have been described, e.g., 'ergotism' which has been wide spread in Europe and the Far East from the Middle ages to the early 20th century; 'stachybotryotoxicosis' in the 1930's (Rodricks and Eppley, 1974); and 'alimentary toxic aleukia' in the mid-1940's (Joffe, 1971), both in the Soviet Union. The aflatoxin problem came to light in the 1960's when there was a severe outbreak of Turkey 'X' disease in England killing over 100,000 turkeys and other farm animals (Blount, 1961). The cause of this disease was traced to a feed component in peanut meal that was infested heavily with *Aspergillus flavus*. This very singular event triggered intensive research on mycotoxins. These studies include the occurrence, chemical structure, biosynthesis, factors affecting the biosynthesis and health hazards of aflatoxins. Table 1 shows commonly occurring mycotoxins and the fungi that produce them. The UN Food and Agriculture Organization has estimated that up to 25% of the world's foods are significantly contaminated with mycotoxins (Mycotoxins in human nutrition and health, 1994).

Table 1. Natural occurrence of selected mycotoxins

Mycotoxins[a]	Major producing fungi	Typical substrate in nature	Biological effect
Alternaria (AAl) mycotoxins	*Alternaria alternata*	Cereal grains, tomato, animal feeds	M, Hm
Aflatoxin (AF) B_1 and other aflatoxins	*Aspergillus flavus*, *A. parasiticus*	Peanuts, corn, cottonseed, cereals, figs, most tree nuts, milk, sorghum, walnuts	H,C,M,T
Citrinin (CT)	*Penicillium citrinum*	Barley, corn, rice, walnuts	Nh,C,(?),M
Cyclopiazonic acid (CPA)	*A. flavus*, *P. Cyclopium*	Peanuts, corn, cheese	Nr,Cv
Deoxynivalenon (DON)	*Fusarium graminearum*	Wheat, corn	Nr
Cyclochlorotine (CC)	*P. islandicum*	Rice,H,C	
Fumonisins (FM)	*F. moniliforme*	Corn, sorghum	H,Nr,C,(?),R
Luteoskyrin (LT)	*P. islandicum*, *P. rugulosum*	Rice, sorghum	H,C,M
Monilitormin (MN)	*F. moniliforme*	Corn	Nr,Cv
Ochratoxin A (OTA)	*A. ochraceus*, *P. verrucosum*	Barley, beans, cereals, coffee, feeds, maize, oats, rice, rye, wheat	Nh,T
Patulin (PT)	*P. patulum*, *P. urticae*, *A. clavatus*	Apple, apple juice, beans, wheat	Nr,C,(?),M
Penicillic acid (PA)	*P. puberulum*, *A. ochraceus*	Barley, corn	Nr,C,(?),M
Penitrem A (PNT)	*P. palitans*	Feedstuffs, corn	Nr
Roquefortine (RQF)	*p. roqueforti*	Cheese	Nr
Rubratoxin B (RB)	*P. rubrum*, *P. purpurogenum*	Corn, soybeans	H,T
Sterigmatocystin (ST)	*A. versicolor*, *A. nidulans*	Corn, grains, cheese	H,C,M
T-2 Toxin	*F. sporotrichioides*	Corn, feeds, hay	D,ATA,T
12-13, Epoxytrichothecenes (TCTC) other than T-2 and DON	*F. nivale*	Corn, feeds, hay, peanuts, rice	D,Nr
Zearalenone (ZE)	*F. graminearum*	Cereals, corn, feeds, rice	G,M

ATA, Alimentary toxin aleukia; C, carcinogenic; C(?), carcinogenic effect questionable; Cv, cardiovascular lesions; D, dermatoxin; G, genitotoxin and estrogenic effects; H, hepatotoxic; Hr, hemorrhagic; M, mutagenic; Nh, nephrotoxin; Nr, neurotoxins; R, respiratory; T, teratogenic

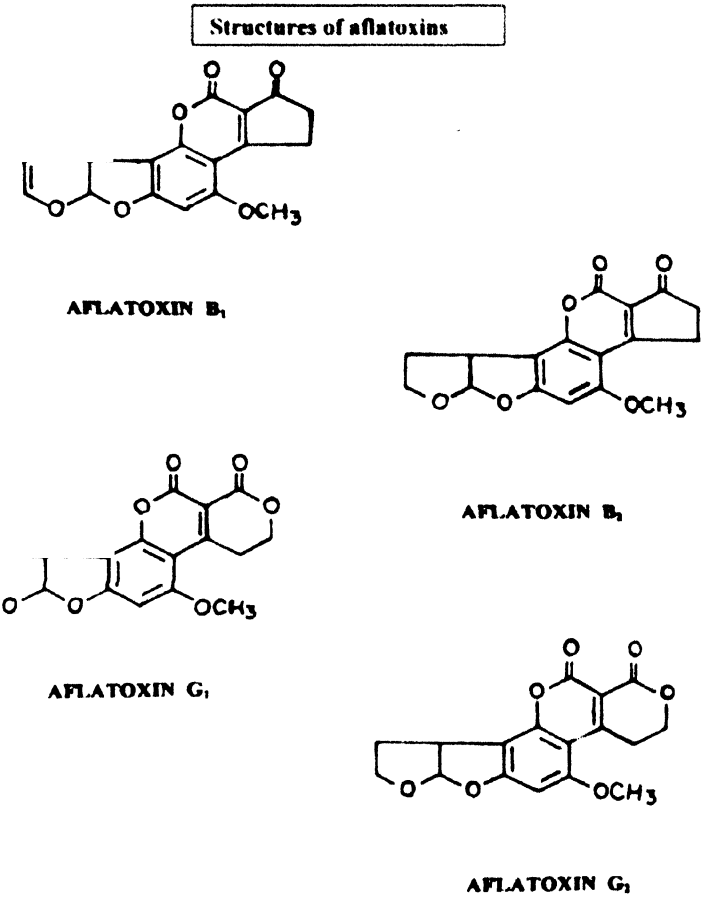
[a]The optimal temperatures for the production of mycotoxins are generally between 24 and 28°C, except for T-2 toxin, which is produced at 15°C.

Source F.S. Chu, 1995

2.2. Aflatoxins

Aflatoxins are potent carcinogenic, mutagenic, teratogenic and immunosuppressive agents. Chemically they are a group difuranocoumarins (Figure 1) produced as secondary metabolites by the fungus species *Aspergillus flavus* and *Aspergillus paraciticus* (Diener and Davis 1969; Busby and Wogan, 1979) on a variety of agricultural commodities (Butler, 1974). A positive correlation has been shown between exposure to aflatoxin and the incidence of liver cancer in humans (Anon, 1962; Smith and Moss, 1985; Mackenzie, 1988; Bosch and Peers, 1991; IARC, 1993; Kativar *et al.*, 2000). Presently 18 different types of aflatoxins have been identified among which aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 are the most common. Of these, the four naturally occurring major aflatoxins are aflatoxin B_1, B_2, G_1 and G_2 (Heathcote and Hibbert, 1978). The rank order of toxicity, carcinogenicity and mutagenicity is AFB1 > AFG1 > AFB2 > AFG2 indicating that the unsaturated terminal furan of AFB1 is critical for determining the degree of biological activity of this group of mycotoxins (Heathcote and Hibbert, 1978).

Figure 1. Structures of aflatoxins and ochratoxin A



2.2.1. Occurrence

Aflatoxins are produced on a variety of substrates at an optimum temperature of 30-35° C and at a relative humidity of 80-85%. Among the substrates, rice, groundnut and maize have been shown to yield substantial amounts of aflatoxins under laboratory conditions (Hesseltine *et al.*, 1966). In investigations on trout fish in the United States, Sinnhuber *et al.*, (1968) demonstrated that rations containing cottonseed meal and peanut meal were responsible for the outbreaks of hepatomas and demonstrated the presence of aflatoxin. A field survey of local foods in the Philippines (Campbell *et al.*, 1970) showed that aflatoxins were present in a variety of peanut products, cassava, cocoa, corn, rice and other foods. Similar distributions of aflatoxins in food have been found in other parts of the world. Although rice is a cereal of very widespread usage, the levels of aflatoxin appear to be less than in other foods with the possible exception of parboiled rice. High levels of contamination (1.7 mg/kg) have been reported in cassava from Uganda, which was associated with a case of acute liver disease (Serick-Hassen *et al.*, 1970). In field samples there is always a wide variation in the levels of aflatoxin (Shank *et al.*, 1972a).

2.2.2. Effects of aflatoxins in animals

The history of aflatoxin investigations began with the discovery of their harmful effects in turkey poults. Aflatoxins have been shown to be lethal to many domestic and experimental animals (Goldblatt, 1969; Wogan, 1977). Animal susceptibility to carcinogenesis by aflatoxins varies with the sex, age, species, strain within the species, hormonal and nutritional status of the animal. The duckling is the most susceptible species and the mouse the most resistant one to the lethal toxicity of aflatoxin (Patterson and Allcroft, 1970). Protein deficiency was observed to sensitise the animal to acute toxic effects of aflatoxin but was protective towards its carcinogenic effects (Madhavan and Gopalan, 1968). Consumption of mycotoxin-contaminated feed has resulted in a number of disease outbreaks in farm and domestic animals. Outbreaks mostly attributable to aflatoxins have been reported in turkeys (Austwick, 1978), horses (Vesonder, 1991), dairy cattle, poultry, rabbits, dogs and camels (Bhat and Nageswara Rao, 1989).

2.2.3. Effects of aflatoxins in humans

Several attempts have been made in various parts of the world to correlate the consumption of various foods contaminated with aflatoxins with acute or chronic diseases in humans such as primary liver cancer, Indian childhood cirrhosis, chronic gastritis, kwashiorkor (a wide spread and serious disorder of children in the tropics), ryes syndrome (Encephalopathy with fatty degeneration of the viscera) and some respiratory diseases. Cases in which acute poisoning occurred due to the consumption of foods heavily contaminated with aflatoxins were reported mainly from tropical countries such as Taiwan (Tung and Ling, 1968), Uganda (Serck-Hanssen, 1970) and Thailand (Shank *et al.*, 1972b). In India, an acute aflatoxicosis outbreak occurred, that affected man and dogs due to consumption of aflatoxin contaminated maize (Krishnamachari *et al.*, 1975). Data on aflatoxins and human cancer have shown a positive correlation between aflatoxin ingestion and liver cancer in population studies in which aflatoxin intake and the incidence of primary liver cancer were estimated concurrently (Shank *et al.*, 1972c; Peers and Linsell, 1973; Van Rensburg *et al.*, 1974; Campbell and Stoloff, 1974). A recent report from India showed clearly the risk the population in the Indian subcontinent is exposed to liver cancer as a result of infection by hepatitis B virus and ingestion of aflatoxin B1 (Katiyar *et al.*, 2000). The possible relationships between aflatoxin exposure and kwashiokar and Indian childhood cirrhosis were discussed by Raisuddin, (1993) and Adhikari *et al.*, (1994). The possibility that some cases of Reye's syndrome might be due to aflatoxin ingestion has been reported by Dvorackova *et al.*, (1977) in Czechoslovakia, where they detected aflatoxins in the livers of patients with Reye's syndrome. Aflatoxin B_1 has also been found to be a potent mutagen. Mutagen studies in bacteria suggest that the possible mechanism of mutagenesis may be initiated by the ability of the aflatoxins to bind to DNA. Aflatoxins are implicated in genetic mutations in people suffering from primary liver cancer particularly in areas of sub-Saharan Africa and Southeast Asia (Harris, 1991; Hsu *et al.*, 1991; Bressac *et al.*, 1991).

2.3. Ochratoxin A (OA)

Ochratoxins were discovered during laboratory screening of a large number of fungus cultures for toxicity in South Africa. The toxicity was attributed to

contamination by a strain of *Aspergillus ochraceous* Wilhelm, reported by Scott in 1965, producing OA, the main toxic component in culture extracts (Scott, 1965; Van der Merwe *et al.*, 1965). Work described by Lai *et al.*, (1970) and by Hesseltine *et al.*, (1972), provided evidence that other members of the *A. ochraceous* group produced ochratoxins, whereas Van Walbeek *et al.*, (1968) reported ochratoxin production by a strain *Penicillium viridicatum* Westling. Thus ochratoxins are a group of structurally related metabolites that are produced by *P. viridicatum* and by *A. ochraceous*. The major mycotoxin in this group is OA (Figure 1) and appears to be only one of major toxicological significance. It contains an isocoumarin moiety linked by a peptide bound to phenylalanine. The work of Purchase and Theron (1968) and others (Doster *et al.*, 1972; Munro *et al.*, 1973) characterised OA as a potent nephrotoxin and hepatotoxin.

2.3.1. Occurrence

OA occurs in many food commodities throughout the world, but is found primarily in grains (barley, oats, rye, corn and wheat) grown in northern temperate areas, and results in contamination of breads and cereal products (Kuiper-Goodman and Scott, 1989). In addition to cereals, animal products such as pigs' kidneys and blood sausage can be significant human dietary sources of OA. A survey in 1971 in the U.S.A. showed that barley contained OA at low levels in 18 of 127 samples examined (Nesheim *et al.*, 1971). There was no correlation between the amount of the toxins in samples and their apparent quality. Canadian workers reported OA in mouldy wheat. This had been used as a component of feed for cattle that suffered mortality and was therefore analysed for mycotoxins (Scott *et al.*, 1970). The presence of OA was also confirmed in dried white beans destined for human consumption and in a sample of mouldy peanuts (Scott *et al.*, 1972).

Ochratoxin A residues were detected in the kidneys of 18 of 19 swine from farms where OA-containing feed had been used. The highest residue was 67 ppb. Most of these kidneys showed fibrosis. Liver and adipose tissue showed no pathological changes but the toxin was detected in 7 of 8 samples of adipose tissue (Hald *et al.*, 1972). These findings indicate that human exposure to

ochratoxin A may not occur only through consumption of mouldy plant material but also through consumption of animal products.

2.3.2. Effects of OA on animals

Several investigators studied the toxicological and pathological effects produced by the ochratoxins. All kinds of laboratory animals tested have been shown to be sensitive to injury by ingested OA. In the field however, injury from OA poisoning has been chiefly in poultry and swine. OA has been tested for carcinogenicity by oral administration in several protocols in three strains of mice and one strain of rats. The kidney, and in particular the tubular epithelial cells, was the major target organ for OA induced lesions. OA has been reported to be teratogenic to mice and rats (Hayes *et al.*, 1974) and to the chicken embryo (Verret, 1974). A dose of 200 µg ochratoxin A per kg body weight has been reported to produce nephrosis in pigs (Krogh *et al.*, 1973; Krogh *et al.*, 1974).

2.3.3. Effects of OA in humans

OA 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 (BEN)". BEN is a bilateral, noninflammatory, chronic nephropathy in which kidneys are extremely reduced in size and weight and show diffuse cortical fibrosis (Vukelic *et al.*, 1991). It has been suggested for several decades that excessive exposure to OA plays a substantive role in the development of BEN (Krogh, 1974). It has been known to be present since the mid-1950s in 14 villages in an endemic area of Croatia, where approximately 10,000 people are at risk. Its prevalence fluctuates between 0.4 and 8.3%, showing a slight decline in recent years, but it has not disappeared from any of the endemic villages. The occurrence of the disease in several ethnic groups contradicts the hypothesis of a primary hereditary basis for BEN (Ceovic *et al.*, 1991). Extremely high incidence of urinary tract tumours was noticed in the endemic areas for BEN, particularly of urothelial tumours of the pelvis and ureter.

2.4. Regulations of mycotoxins in foods and feeds

The occurrence of mycotoxins in foods and feeds is unavoidable due to the environmental conditions. Therefore many countries have attempted to limit exposure to aflatoxins and other selected mycotoxins by imposing legal restrictions or regulatory limits on susceptible commodities in the domestic market and on imports. Limits (maximum levels of mycotoxins in food or feed commodities) are necessary for health protection of consumers and for the industry to produce high quality products. They may range from 0 to 50 μg/kg for aflatoxins in human foods. The rationale for the levels imposed by various countries is based on some type of risk assessment or available scientific information (Stoloff *et al.* 1991). Among the mycotoxins priority is given to the aflatoxins and to ochratoxin A in foods and to relevant analytical methods and sampling plans. The main objectives being consumer protection and to allow trading. Sixty countries in the world at present have imposed legal limits for mycotoxins in foods and animal feed; these limits vary from country to country. Certainly the number of countries setting regulations for aflatoxins is increasing with tolerance levels set from 5 to 20 ppb (5-20 μg/kg). Usually there is a regulatory level set for aflatoxin B1 and/or total aflatoxins (B1, B2, G1 and G2). Japan, the world's largest importer of agricultural products, does not permit any aflatoxin B1 (zero limit) in foodstuffs and 10 to 20ppb for feedstuffs analysed by officially accepted methods (Mycotoxin in human nutrition and health 1994).

There is no truly global consensus on the implementation of standards for mycotoxin levels in foods and feeds. To quote Stoloff *et al.*, (1991) "Although 50 countries have enacted or proposed regulations for control of aflatoxins in food or feed, and 15 of these countries also have regulations for permitted levels of contamination by other mycotoxins, very few countries have formally presented the rationale for the need to regulate, or for the selection of a particular maximum tolerated level."

2.5. Methods for estimation of aflatoxins and ochratoxin A

Many different methods are currently available for the estimation of mycotoxins. Biological methods which are based on the toxic effect of mycotoxins have been used for mycotoxin detection utilizing either the death or pathological lesions,

or biochemical events or the immunotoxic effects as an index for toxicity. These methods are not specific and the sensitivity is generally low as compared to other methods. They are often used as a tool for screening general toxicity. Physicochemical methods such as thin layer chromatography, high performance liquid chromatography, gas chromatography and mass spectrometric methods are laborious and require expensive instrumentation and clean-up of the samples. Thus there are limitations to apply these methods for performing routine safety and quality control screening of mycotoxins in agricultural commodities. To overcome the difficulties encountered with the biological and physicochemical methods, new immunochemical methods have been developed. Immunological methods using specific antibodies are gaining wide acceptance for quantitative estimation of mycotoxins because of their sensitivity and specificity, and are less expensive to perform than the majority of analytical methods.

2.5.1. Thin layer chromatography (TLC)

Thin layer chromatography was the first method to be used for detection of mycotoxins. Since this method is simple and does not need expensive instrumentation, it has remained important in many laboratories. However, extensive sample clean-up is necessary, and experienced personnel are required for quantitative assessment of the data. The TLC conditions for mycotoxin analysis including extraction solvents, clean-up procedures, solvent systems for separation and Rf values of mycotoxins, methods of visualization as well as approaches for multiple detection were documented by a number of investigators (Trucksess and Wood 1994 , Betina *et al*, 1989, Bennet *et al*, 1994, Scott 1981).

2.5.2. High performance liquid chromatography (HPLC)

HPLC can be used to separate many mycotoxins. The detection of mycotoxins containing fluorescent and chromophoric groups has been widely applied since the initial use of this method in the mid-1970s (Chu *et al*, 1978, Scott, 1981). With advances in the chemistry of adsorption materials for column packing, improvement in instrumentation, and enhancement of efficiency of detectors as well as new methods for both pre-column and post-column derivitization, both

sensitivity and reproducibility have improved considerably over the years. Thus HPLC has become one of the most popular methods for mycotoxin detection. However it is laborious, require expensive instrumentation and needs clean-up of the samples.

2.5.3. Mass spectrometry (MS)

Mass spectrometry was not considered as a quantitative analytical tool for mycotoxins in earlier investigations (Dusold *et al.* 1978). They were used as a final step for characterization, identification and confirmation methods for mycotoxins. Only pure compounds were subjected to MS and sensitivity was low. However, recent development of high-resolution mass spectrometry have led to new MS methods for both identification and quantification of mycotoxins (Plattner 1986). However due to the high cost of the instruments, cost of maintenance, and requirement for trained personnel, this method is limited to few research centres and is not widely used.

2.5.4. Immunological methods (polyclonal antibodies, monoclonal antibodies, mimotopes)

Polyclonal antibodies are produced as a result of injecting antigens into an animal. They always contain a mixture of antibodies to various epitopes. The most frequently used method for the production of antibodies to mycotoxins is multiple-site immunization of rabbits with mycotoxin conjugated to a protein, followed by a booster at 5-6 week intervals. Polyclonal antibodies for aflatoxins have been produced by Chu and Ueno (1977) and Pestka *et al.*, (1980) and for ochratoxin A by Morgan *et al.*, (1983) and Lee and Chu (1984).

An alternative approach to the production of polyclonal antibodies is the development of stable hybridoma cell lines that secrete reagent quality **monoclonal antibodies** to mycotoxins. In monoclonal antibody production, the short lived B lymphocytes from the spleen are individually immortalized by fusion with an immortal myeloma cell line to give a range of clones of B lymphocytes (hybridomas), each of which produces its own antibody. This is achieved by isolating splenic lymphocytes that produce a single antibody that reacts with a single epitope, and is of a defined class (IgG) and subclass, from an

immunized mouse and fusing these with a myeloma cell line. Following a series of selection and screening steps an "immortalized" clone that constantly produces antibodies of the desired affinity, specificity, and performance characteristics can be isolated. Monoclonal antibodies have been produced for aflatoxins (Groopman *et al.*, 1982; Lubet *et al.*, 1983; Sun *et al.*, 1983; Candlish *et al.*, 1985) and ochratoxin A (Chiba *et al.*, 1985; Candlish *et al.*, 1986, 1988). Although this approach has disadvantages in terms of cost, effort, time and the requirement for tissue culture facilities, monoclonal antibodies tend to exhibit much lower degrees of inter-assay variation, and yield more highly reproducible results, than do polyclonal antibodies.

To search for an alternative to using mycotoxins as immunochemical reagents, phage-displayed random peptide libraries have been used as a source of peptides that mimic the epitope(s) on mycotoxins (**mimotopes**). In phage display, a peptide or a protein is fused to one of the coat proteins of fd bacteriophage (eg. pIII, pVIII), which means that the fused protein is displayed on the exterior surface of the phage particle (Felici *et al.*, 1991; Parmley and Smith, 1988; Smith, 1991). Libraries consist of phage that contain all possible amino acid sequences in the surface-exposed peptide. These are screened in order to select peptides with specific affinities or activities (Cwirla *et al.*, 1990; Devlin *et al.*, 1990; Scott and Smith, 1990). Using peptide libraries, it is possible to select peptides that bind to an antiprotein antibody. Random peptide libraries have been used in a wide variety of applications (e.g. Cortese *et al.*, 1994,; Scott and Smith, 1990; Katz, 1997). However, very few workers have used this technology to select peptides that mimic non-proteinaceous chemicals, such as biotin (Weber *et al.*, 1992) and carbohydrates (Hoess *et al.*, 1993). To our knowledge only a single report described the use of this technology. This concerned the mycotoxin deoxynivelenol (Yuan *et al.*, 1999).

For the analysis of aflatoxins three types of immuno assays (IA) are commonly employed: the radio immunoassay (RIA), the enzyme-linked immunosorbent assay (ELISA), and the immunoaffinity column assay (Chu, 1990). Radio-labelled aflatoxin is used in the RIA. In the competitive RIA the unlabelled aflatoxin standard or the aflatoxins in the test sample and labelled aflatoxin in the assay

system compete for the limited number of binding sites on the antibodies. The amount of aflatoxins in a sample is inversely related to the amount of radio-labelled aflatoxin in the solution. Only a small amount of antibody is required for RIA. However, the radioisotopes used in the assays present disposal problems. The use of non-isotopic labels such as enzymes has gradually replaced the use of RIA.

In the typical direct ELISA, the binding of aflatoxin-enzyme conjugate by the immobilized antibodies is inhibited by the presence of aflatoxins in the test sample. The bound enzyme catalyses the transformation of a substrate to a coloured complex. The colour intensity formed is inversely related to aflatoxin concentration. Horseradish peroxidase, which catalyses the oxidation of the substrate (tetra methylbenzidine) to form a blue complex, is the most commonly used enzyme for labelling. The assays may be qualitative or quantitative. ELISA techniques have been described for aflatoxin B1 in peanuts, cotton seed and corn (Pestka *et al*., 1980, Ram *et al*., 1986) and for ochratoxin A in barley and wheat (Morgan *et al*., 1983, Lee and Chu, 1984).

Affinity columns are prepared by adsorption of the antibodies onto a gel material contained in a small plastic cartridge. Aflatoxins are captured from test solution by the immunospecific antibodies. After nonbonding impurities have been washed from the cartridge, aflatoxins are desorbed with methanol, then derivatized and quantitated by solution fluorometry or transferred to the liquid chromatographic reversed-phase column for further separation and fluorescence determination (Trucksess *et al*., 1991). The use of affinity columns to capture and concentrate aflatoxins has several advantages, which include increased selectivity, the ability to trap the aflatoxins in large volumes of test samples (biological fluids), and integration with other analytical techniques. However, large amounts of antibodies are needed to prepare immunosorbent columns and the cost is greater than RIA and ELISA.

Chapter 3

Production of Antibodies for Aflatoxins

3.1. PRODUCTION OF POLYCLONAL ANTIBODIES

3.1.1. MATERIALS AND METHODS

3.1.1.1. Immunization

Commercially available aflatoxin B1-bovine serum albumin conjugate (AFB1-BSA, Sigma cat. no. 0-3007) was used. AFB1-BSA (400 µg) in 1.5 ml of sterile 0.01 M phosphate-buffered saline (PBS) was emulsified with an equal volume of complete Freund's adjuvant (Sigma) 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 titre checked by indirect competitive ELISA. Booster injections were given when a drop in the titre was noticed. Serum was lyophilised and stored at −20 °C until utilized.

3.1.1.2. Monitoring antibody titres

An indirect ELISA procedure similar to that reported for aflatoxins (Devi *et al.*, 1999) was used. Microtitre plate wells were coated with 0.1 µg mL^{-1} of AFB1-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 volume were added to 100 µL of AFB1 at concentrations ranging from 100 ng mL^{-1} to 100 pg mL^{-1}. Goat antirabbit immunoglobulins (GAR IgG) conjugated to alkaline phosphatase were used at a 1:1000 dilution to detect rabbit antibodies attached to AFB1. p-nitrophenyl phosphate was used as a substrate at 1 mg mL^{-1} and allowed to develop for 1 h at room temperature. Absorbance was recorded at 405 nm (A_{405}) with an ELISA plate reader (Titretek Multiskan, Labsystems).

3.1.1.3. Characterisation of antibodies

To evaluate the cross reactivity of the antibody with aflatoxin B2, G1, G2, M1 and ochratoxin A, it was essential to determine the optimum conditions for neutralization. These included coating antigen (AFB1-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 AFB1 and B0 is the extinction of the well without toxin derived from the slope of the calibration curves

3.1.2. RESULTS

3.1.2.1. Titre of antibody

The protocol used for immunization gave an antibody titre of 1:100,000, 9 weeks after initiation of immunization (Figure 2).

3.1.2.2. Specificity of antibody

To determine the cross-reaction of the antibody with molecules resembling AFB1, it was decided to test it against aflatoxin B2, G1, G2, M1 and ochratoxin A. Various dilutions of these were used in ELISA and cross reactivity of the antibodies to B2 and G1 was found to be 25%, for G2, 0.4% and did not react with M1 and ochratoxin A (Figure 3).

Figure 2. Standard curves for AFB1 by indirect competitive ELISA at different dilutions of antibody 1:10,000 (•), 1:20,000 (■), 1:40,000 (▲), 1:80,000 (▼), 1:160,000 (♦), 1:320,000 (0)

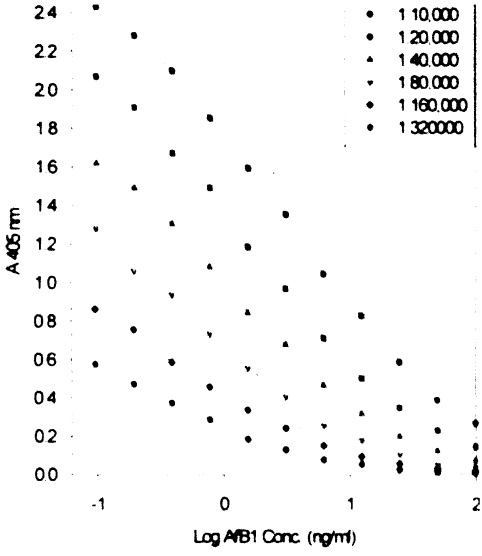
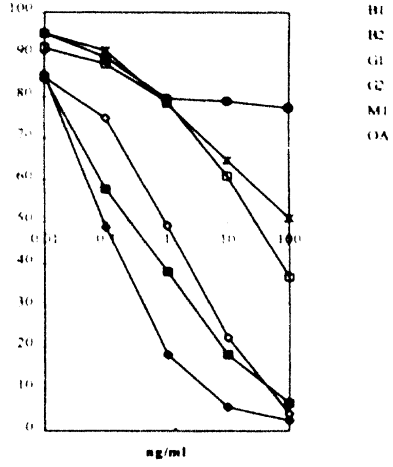


Figure 3. Cross reactivity of AFB1 antiserum (◆) to B2 (◇), G1 (■), G2 (□), M1 (•), and OA (●).



3.2. PRODUCTION OF MONOCLONAL ANTIBODIES

3.2.1. MATERIALS AND METHODS

3.2.1.1. Production of mouse polyclonal antibodies

The immunogen used was AFB1-BSA conjugate (250 µg) dissolved in 250 µl of 0.01 M PBS and emulsified with an equal volume of Freunds adjuvant (Sigma). This was injected intraperitoneally into each of several six-week-old female Balb/c mice. The first injection was given using Freund's complete adjuvant and the rest of the injections except for the booster were given with Freund's incomplete adjuvant. No adjuvant was used for the booster injections. After completing the immunization schedule approximately 5 µl of blood was drawn from each mouse after cutting the tip of the tail.

Initially four injections were given to each of four mice at one-week intervals followed after three weeks by a booster. The mouse that gave the highest titre was used for preparing monoclonal antibodies. This procedure is referred to as protocol "A". The remaining three mice were kept for 8 months without any immunization and a booster injection was given at the end of the eighth month. Sera from the three mice were tested two weeks after the booster injection. Again the mouse that gave the maximum titre was used for antibody preparation (referred to as protocol "B").

3.2.1.2. Media

Tissue culture medium [Iscoves Modified Dulbecco's Medium (IMDM)] was purchased in the form of powder GIBCO BRL (Gibco cat no 12200-036) and the medium was prepared according to the manufacturer's instructions. It was supplemented with foetal bovine serum (FBS) (Gibco cat no 263000-061) to a final concentration of 10% (10% IMDM) for growing myeloma cells and 20% (20% IMDM) for growing hybridoma cells. Glutamine was added at 2 mM (from 200 mM stock, Gibco). As this is unstable it was added to media older than 2 weeks. In addition, the media were supplemented with the antibiotics, penicillin (100U/ml), gentamycin (50 $\mu g\ mL^{-1}$), streptomycin (100 $\mu g\ mL^{-1}$) and nystatin (5U mL^{-1}). Hypoxanthine, aminopterine and thymidine 100X (HAT) and Hypoxanthine, thymidine 100X (HT) were purchased from Gibco (cat no 31062-011).

3.2.1.3. Preparation of myeloma cells

The murine myeloma cell line, Sp2/0-Ag14 in 10% FCS, was grown at 37 °C 25 cm ^{2} or 80 cm^2 tissue culture flasks in an 5% CO_2 incubator The myelo were growing rapidly and appeared healthy before the fusion One day be the fusion, the cells were split into fresh medium supplemented with 1 IMI

3.2.1.4. Polyethylene glycol

One ml of polyethylene glycol, molecular weight 1500 (Boehringer Mannheim cat no 783641) was used PEG solution was pre-warmed to 37 °C before fusion

3.2.1.5. Preparation of macrophage feeder cells

A mouse was killed by cervical dislocation and sprayed with 70% (v/v) alcohol Under aseptic conditions, the skin was cut without penetrating the peritoneum Serum-free medium (10ml) was injected into the peritoneum The body was agitated and as much of the liquid as possible was withdrawn from the peritoneum using a syringe fitted with a needle The liquid containing the macrophage feeder cells was centrifuged at 1500 rev/min for 5 min The pelleted cells were resuspended in 10 ml 20% IMDM and were plated out at 100 µl/well (96-well tissue-culture plates) and incubated overnight in a CO_2 incubator

3.2.1.6. Preparation of spleenocytes

The spleen was removed aseptically from the immunised mouse and placed in a 100-mm tissue culture dish and the contaminating tissue was removed from the spleen Using a syringe, 10 ml of 10% IMDM was injected into the spleen and flushed out such that the spleenocytes came into the medium The process was repeated several times to obtain the maximum number of cells in the medium The cell suspension was centrifuged at 2000 rev/min for 5 min and pelleted cells were washed by resuspension in 10 ml of 10% IMDM The pelleted cells were resuspended in 10 ml lysis buffer (0 017 M Tris-HCl, pH 7.2, 0 084 M

ammonium chloride, filter sterilized through a 0 22 m Millipore filter) The centrifuge step was repeated and cells were resuspended in 10% IMDM, centrifuged and resuspended in 10 ml serum-free medium Spleen cells were counted using a haemocytometer

3.2.1.7. Cell fusion to produce hybridomas

Hybridomas were derived from two fusions between the spleen cells of Balb/c mice immunised against AFB1-BSA and the murine myeloma cell line Sp2/O Ag14 Techniques for the production and culture of hybridomas were essentially same as described by Galfre and Milstein (1981) The spleen cells were mixed with the myeloma cells (previously washed, centrifuged as above and resuspended in 20% IMDM) in a ratio of 10 1 The spleen cell/myeloma cell mixture was centrifuged at 2500 rev/min for 10 min The supernatant fluid was removed and 1 ml of prewarmed PEG was added slowly over a period of 1 min (0 1 ml every 6 sec), while resuspending the cells by stirring with the end of a pipette The cells were suspended in the PEG solution for 1 min Twenty percent IMDM (4 ml) pre-warmed to 37 °C was added gradually during the following minute, swirling the tube as above Medium continued to be added slowly, diluting the cells 1 2 every minute, to a final volume of 20-25 ml The cells suspension was held at 37 °C for 5 min and then centrifuged at 2500 rpm for 5 min The supernatant fluid was removed, the cell pellet was resuspended in 50-80 ml HAT medium (HAT in 20% IMDM) and distributed into 96-well cell culture plates (100 µl/well) containing peritoneal macrophage feeder cell layers The plates were transferred to a CO_2 incubator After two weeks, HAT medium was replaced with HT medium for two weeks and then cells were fed with 20% IMDM On day 12, the supernatant fluids were assayed as described below (section 3 2 1 8)

3.2.1.8. Screening cells for antibody production

After 12 days, culture supernatants from each well were assayed using indirect competitive ELISA Culture supernatants from the cells that gave an absorption value of over 3 as a difference between 0 4 and 400 ng ml^{-1} of toxin were transferred to 24-well microculture plates in IMDM containing 20% FBS, hypoxanthine and thymidine Supernatants from 24-well culture plates were

again tested and only those clones that maintained absorption values over 3 in ELISA tests were chosen for further selection Cell suspensions from each well of the 24-well-cultured plate were diluted to give approximately one cell per well when distributed into a 96-well culture plate The plates were examined for the presence of number of hybridomas Those that contained a single hybridoma in each well were retained and were screened for neutralization titres

3.2.1.9. Cloning of hybridomas by limiting dilution

Cell lines that were positive in the indirect ELISA screening, were transferred to single wells in a 24-well plate Cells were counted with a haemocytometer and diluted to 1 cell/well in 20% IMDM containing HT Cells were plated out in a 96-well plate having a layer of peritoneal macrophage feeder cells prepared the previous day and transferred to the CO_2 incubator for 7-14 days, until growth was apparent Wells were screened for antibody activity by indirect competitive ELISA as described in Section 3 2 1 8 and cells secreting antibody were transferred to single wells in a 24-well plate

3.2.1.10. Multiplication of selected hybridomas

Cells were fed whenever the growth medium turned yellow, by removing the spent medium with a pipette and replacing it with a fresh 20% IMDM Spent medium containing monoclonal antibody was collected, pooled and stored at -20 °C, or at 4 °C with 0 02 % (w/v) sodium azide

3.2.1.11. Preservation of hybridomas

Cells (approximately 8 confluent wells from a 24-well plate) were scraped from the wells, transferred to a Universal tube and centrifuged at 2000 rev/min for 5 min The supernatant fluid was removed and retained Pellets of cells were resuspended in 1 ml of freezing medium (90% (v/v) FBS, 10% (v/v) dimethylsulphoxide) The cell suspension in freezing medium was dispensed at 0 5 ml per freezing vial Vials were placed in a polystyrene box and transferred to a -70 °C freezer for 2 days prior to long term storage in liquid nitrogen

3.2.1.12. Thawing of cells

Cells were removed from liquid nitrogen and thawed quickly at 37 °C and transferred to a sterile tube with 15 ml 20% IMDM. Cells were centrifuged at 2000 rev/min for 5 min. Supernatant fluid was discarded and the cell pellet was resuspended in 1-2 ml 20% IMDM. The resuspended cells in 250 µl to 500 µl volume were transferred to a single well of a 24-well cell-culture plate previously seeded with macrophage feeder cells. Cells were removed after they covered about half of the well, and then distributed to the rest of the wells.

3.2.1.13. Isotyping of monoclonal antibodies (Mabs)

Commercially available ISO-2 kits from Sigma were used to determine the isotypes of the monoclonal antibodies produced by various hybridoma cell lines.

3.2.1.14. Determination of specificity of MAbs

To evaluate the cross reactivity of each of the monoclonal antibodies, it was essential to determine the optimum conditions for neutralization. These included coating antigen (aflatoxin B1-BSA) concentration and the dilution of the antibody required for neutralization. Only known concentrations of IgG's, extracted from tissue culture supernatants (TCS) by sodium sulphate precipitation (Hobbs *et al.* 1987), were used in the experiments. Antibody titres were determined by the indirect competitive ELISA procedure described in the section 3.1.1.2. The optimum concentration required at each step to obtain the maximum sensitivity was determined by 50% displacement values of B/B_0, where B is the extinction of the well containing AFB1 and B_0 is the extinction of the well without toxin, derived from the slope of calibration curves. Using these parameters, various concentrations of the IgG (50 µl/well) from different monoclonal antibodies were added to 100 µl of AFB1 at concentrations ranging from 100 ng to 100 pg ml^{-1}. The protocol used for the characterization of antibody specificity was similar to those employed for the characterisation of polyclonal antibodies (section 3.1.1.3).

3.2.2. RESULTS

3.2.2.1. Immunization

The sera from mice immunized using protocol A showed poor antibody responses. Antibody was used at a dilution of 1:500. In contrast, high antibody titres, exceeding 1:20,000, were recorded from the sera of mice immunized by protocol B (Table 2).

3.2.2.2. Fusion and cloning

The fusion efficiency (number of wells showing cell multiplication in each well of 96-well plate) was 100%. In the first fusion, supernatants from cells derived from 8 wells showed specific binding to AFB1. However, after two successive transfers only 2 retained the antibody activity. In the second fusion, 41 clones that secreted AFB1 specific antibodies were selected. After two successive transfers, 10 clones continued to give high neutralization titres. These clones were transferred to 25 cm^2 flasks and IgGs were extracted from culture supernatants (section 3.2.1.14). IgG concentrations varied from 150 $\mu g\ ml^{-1}$ to 185 $\mu g\ ml^{-1}$ of the culture supernatant.

3.2.2.3. Optimum aflatoxin and antibody activity for ELISA

An aflatoxin B1-BSA conjugate concentration of 125 ng ml^{-1} was found to be optimum for coating the plates. The optimum concentration of antibody for neutralization depended on the titre of antibody and it varied from 5 $\mu g\ ml^{-1}$ to 50 $\mu g\ ml^{-1}$.

3.2.2.4. Characterization of antibodies

The designation, data on isotypes, cross reactivity and the minimal inhibition values for ten monoclonal antibodies are presented in Table 3. The antibodies showed a range of cross reactivities (Figure 4) and could be classified broadly into three distinct groups. Group 1 comprised one monoclonal antibody, 10D5-1A11, that was highly specific for B1 and showed a weak cross reaction to G1. Group 2 (13D1-1D9) contained antibodies that recognized B1, G1, and B2, with a weak cross reaction with G2. The remaining clones that recognized B1 and G1 with equal efficiency were grouped in the category 3. Cell lines produced in

the first fusion (Protocol "A") had a detection range from 10 to 100 ng ml^{-1} in contrast to those of the clones produced in the second fusion (Protocol "B") which gave values ranging from 0.001 to 1 ng ml^{-1}. One clone from the second fusion (10D5-1A11) gave a high titre to aflatoxin B1 with a 50% inhibition at 0.006 ng ml^{-1} (Figure 5).

Table 2. Percentage of antibody binding to the plate in an indirect competitive ELISA from the mice immunized with two different protocols.

Mouse	Protocol A	Protocol B
1	50 %	Used for fusion # 1
2	58 %	Died
3	54 %	67 %
4	49 %	80 % (used for fusion # 2)
5	70 %	70 %

[a]Protocols: A = Three intraperitoneal injections at 1 week intervals followed by a booster three weeks after the last injection. Antibody was used at a dilution of 1:500. B = Same group of mice maintained without immunization for eight months and a booster injection given at the end of the eighth month. Antibody was used at a dilution of 1:20,000

Table 3. Isotypes, cross reactions and minimal inhibition observed with ten monoclonal antibodies

Designation	Isotype[a]	Cross-reaction[b] (%)				Minimum inhibition[c] (ng ml^{-1})		
		B1	B2	G1	G2	B1	B2	G1
1. 10D5-1A11	IgG_1	100	2	12	< 1	0.001	-	-
2. 5D8-2B1	IgG_1	100	-	110	-	1	-	1
3. 13D1-1D9	IgG_1	100	2	100	< 1	0.01	-	0.01
4. 5F2-1E8	IgG_1	100	12	100	3	0.1	-	0.1
5. 3G7-1B8	IgG_1	100	22	100	< 1	0.1	1	0.1
6. 11C8-1A8	IgG_{2a}	100	20	66	1	0.01	-	0.01
7. 3F7-1B9	IgG_1	100	15	60	< 1	< 0.01	-	< 0.01
8. 5H4-1B1	IgG_1	100	13	72	1	< 0.01	-	< 0.01
9. 6G12-2B3	IgG_{2a}	100	7	50	< 1	< 0.01	-	< 0.01
10. 6E12-1E5	IgG_1	100	60	75	5	0.1	0.1	0.1

[a] determined by using a commercial kit.

[b] expressed as fifty percent displacement value of B/B_0 for aflatoxin B1 divided by the fifty percent displacement value for each of the aflatoxin under testing (see text).

[c] Concentration of aflatoxin (ng ml^{-1}) required for first significant inhibition of binding of antibody to AFB1-BSA solid phase.

Figure 4 a-c. Cross reactivity of three groups of monoclonal antibodies (from clones 13D1-1D9, 6E12-1E5 and 10D5-1A11) to four major aflatoxins, B1(◆), B2(◊), G1(■), G2 (□)

Graphs plotted as percentage binding (B/Bo) against mass of toxin per ml (ng ml^{-1}). B is the extinction of the well containing AfB1 and Bo is the extinction of the well without toxin.

(a) Antibody specific for B1with varying degrees of cross reaction to G1, B2 and G2.

(b) Antibody that recognises B1, G1, B2 with a weak cross reaction to G2.

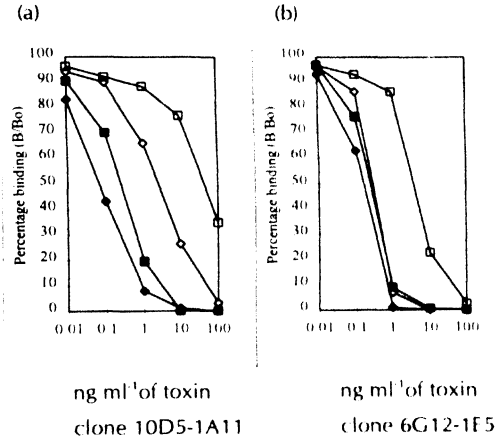
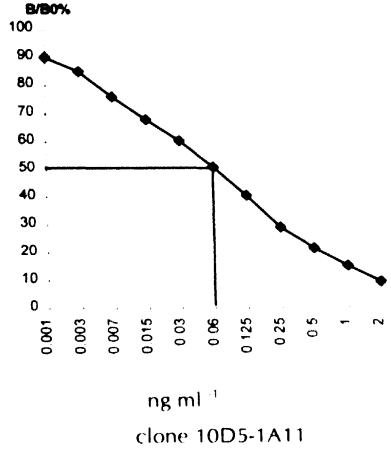


Figure 5. Dosage response curve of aflatoxin B1. Fifty percent inhibition value of AFB1 was 0.06 ng ml^{-1}.



3.3. PRODUCTION OF AFLATOXIN MIMOTOPES

3.3.1. MATERIALS AND METHODS

3.3.1.1. Reagents

Aflatoxin B1, goat anti-rabbit IgG-alkaline phosphatase (ALP) conjugate, goat anti-mouse IgG-ALP conjugate, *p*-nitrophenyl phosphate, bovine serum albumin (BSA), aflatoxin B1-BSA conjugate, tetracycline and polyethylene glycol (PEG), were all purchased from the Sigma Chemical Co., St. Louis, USA. Microtitre plates (Maxi-sorp F96) and immuno-tubes (Maxi-sorp) were obtained from Nalge Nunc International, Denmark. Peptone, yeast extract and agar were obtained from life technologies Gibco BRL. All other chemicals were reagent grade or chemically pure.

3.3.1.2. Monoclonal antibodies for aflatoxin B1

Anti-aflatoxin B1 monoclonal antibodies were those described by Devi *et al.*, (1999). MAb 13D1-1D9 (here coded MAb 24) cross-reacted with aflatoxin G1, but not B2, 6E12-1E5 (here named MAb 13) cross-reacted with aflatoxins B2, G1 and G2, and MAb 10D5-1A11 did not cross-react appreciably with any of the other aflatoxins (Thirumala Devi *et al.*, 1999). Immunoglobulins were concentrated from tissue culture supernatants or ascitic fluid by ammonium sulphate precipitation (Harlow and Lane, 1988).

3.3.1.3. Peptide libraries

The random phage displayed peptide libraries, Cys-4 and Cys-6 used in this study were provided by G. Smith (University of Missouri, Columbia). Each library consists of fd phage that carry a second copy of gene VIII that has at its N-terminus a randomised sequence in which cysteine codons are 4 or 6 codons apart. The cysteines are designed to constrain by cross-linking the conformations that the peptides may adopt (Ziegler *et al.*, 1998).

3.3.1.4. Affinity selection of phage by panning

Panning was as described by Ziegler *et al.*, (1998) using immuno-tubes coated with MAbs at 10 μg ml $^{-1}$. After blocking, phage were bound and eluted, and then used to infect *E. coli* TG-1 cells. After amplification, phages were subjected to two further rounds of panning to enrich the population with binding phage.

3.3.1.5. Immunological assay for affinity-purified phage

Following the third round of panning, single tetracycline-resistant colonies were transferred to 5 ml LB-TET and grown at 37 °C for 18 h. Cells were removed by centrifugation at 17000 g for 5 min and the culture media were tested by ELISA using microtitre plates. At each step the plates were incubated for 1h at 37 °C. Initially plates were coated with IgG at 10 μg ml $^{-1}$ in 0.2 mol l $^{-1}$ sodium carbonate, pH 9.6. In the second step, plates were blocked with 200 μl/well of 3% MPBS. Phage particles in 80 μl were mixed with 20 μl of MPBS. A mixture of rabbit anti-M13 antibody (1:500) and anti-rabbit IgG conjugated to alkaline phosphatase (1:100000 dilution) were used to detect the phage. Substrate (p-nitrophenyl phosphate at 1 mg ml $^{-1}$) was added and absorbance at 405 nm was read in an ELISA plate reader after 1 h at room temperature. Selected individual clones were characterized by DNA sequencing and ELISA.

3.3.1.6. Indirect competitive ELISA with phage-displayed peptides

To determination of optimum number of phage to use in ELISA, different dilutions of phage particles in carbonate coating buffer were added to the ELISA plate and incubated overnight at 4 °C. After blocking, a mixture of MAb and goat anti-mouse-alkaline phosphatase conjugate was added and the ELISA was completed as described. An indirect ELISA procedure similar to that reported for aflatoxins (Devi *et al.*, 1999) was used. Microtitre plate wells were coated with 150 μl of phage at 10^{11} ml $^{-1}$ that carried peptides in 0.2 mol l $^{-1}$ sodium carbonate, pH 9.6 and incubated overnight in a refrigerator. The plates were washed and blocked as above for panning elution selection. MAbs in 50 μl were added to various concentrations of AFB1 (0 to 10 μg ml $^{-1}$ in PBS). The mixtures were added to the phage-coated microtitre plate wells (150 μl/well) and the preparations were incubated at 37 °C for 1 h. After washing four times with PBS-T, the amounts of enzyme bound were determined by incubation with goat anti-mouse-ALP conjugate at 37 °C for 1 h. Amounts of bound enzyme were determined as described above. The results of inhibition tests were analysed using Genstat 5 and logistic curves were fitted to the data (Genstat 5 Committee, 1987)

3.3.1.7. Nucleotide sequencing

Phage particles were recovered by PEG-precipitation and single-stranded DNA was extracted from them by phenol-chloroform treatment (Sambrook *et al.*, 1989). Sequencing was done using an ABI PRISM dye primer cycle sequencing ready reaction kit (Perkin Elemer Applied Biosystems, Warrington, UK). The primer was complementary to DNA encoding the recombinant pVIII gene, downstream of the peptide insert. Sequences were analysed using the Genetics Computer Package (Devereux *et al.*, 1984).

3.3.1.8. UV irradiation of phage particles

Phage particles were uv-irradiated at 254 nm with doses of between 10 and 800 mJ/cm^2 using a UV cross linker (Stratalinker UV crosslinker; Stratagene Ltd., Cambridge, UK). The treated phage particles were then used to infect log-phase TG-1 cells or assayed by ELISA.

3.3.1.9. Sample preparation

Groundnut samples were obtained in 200 g quantities. After a thorough mixing, three 15 g sub-samples were drawn from each 200 g sample. Each sub-sample was finely ground and then extracted in 75 ml 30% methanol containing 0.5% KCl 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 1:10 with PBS-T/BSA for processing by ELISA.

3.3.1.10. Indirect competitive ELISA procedure for processing groundnut samples

The protocol was similar to that for indirect competitive ELISA for phage-displayed peptides (as described above) with the exception that aflatoxin standards in 100 μl volume, ranging from 100 ng ml^{-1} to 100 pg ml^{-1}, were prepared in a diluted extract from groundnuts. Groundnut samples that did not contain aflatoxins were extracted in methanol as described above, filtered and used at a 1:10 dilution prepared in PBS-T BSA. Test samples were also diluted to 1:10 in PBS-T/BSA prepared in aflatoxin-free groundnut extract. One hundred μl of each sample were added to wells containing 50 μl of antiserum. The wells were washed four times with PBS-T incubation with goat anti-mouse ALP-

conjugate at 37 °C for 1 h. The amounts of enzyme bound were determined as described above. Standard curves were obtained by plotting $\log_{10}$ values of aflatoxin B1 standards against optical density at A_{405}.

3.3.2. RESULTS

3.3.2.1. Screening of a random peptide phage libraries for the selection of specific phages by panning

Tubes were coated with MAb 24 or MAb 13 and used for selection using either Cys-4 or Cys-6 libraries. Irrespective of the library used, the proportion of phage that bound (input titre/output titre) increased after each round. After the third round, input and output titres were about 10^{11} ml $^{-1}$. All 20 of 20 clones from the Cys-4 library and 4 of 20 from the Cys-6 library yielded phage that bound to MAb 24 (A_{405} of >0.5 to 2 in 1h using DAS ELISA). From the 21 clones obtained using MAb 13, 11 from the Cys-4 library and 12 from the Cys-6 library gave positive results in ELISA tests. No clones were obtained from either library that bound to MAb 10D5-1A11.

3.3.2.2. Optimum number of phage particles for coating the ELISA plate

ELISA plates were coated with different amounts of phage particles to establish the number of particles that were needed to give a useful response in phage ELISA. Figure 6 shows the results of ELISA of four mimotope clones at different concentrations assayed using either MAb 24 (clones 24-4 and 24-6) or MAb 13 (clones 13-4 and 13-6). The best results were obtained with phage concentrations of 10^{11} ml $^{-1}$. In parallel tests, plates coated with an unrelated Cys-4 phage clone at 10^{11} ml $^{-1}$ gave an A_{405} of <0.1.

3.3.2.3. Specificity tests

The mimotopes 24-4.1, 24-6.1, 13-4.1, 13-6.1 were tested in phage ELISA at 10^{11} ml $^{-1}$ using dilutions of polyclonal antibody raised against an aflatoxin B1-BSA conjugate. The results for different mimotopes were indistinguishable. For example, a 4-fold decrease in antibody concentration decreased the ELISA readings obtained in 1 h from 1.04, 1.07, 1.05 or 1.08 to 0.43, 0.47, 0.45 or 0.43 for each mimotope respectively. In ELISA tests, mimotopes obtained using MAb 24 did not bind to MAb 13, and neither did MAb 13 mimotopes bind to MAb 24.

To test the specificity of the mimotopes, indirect competitive ELISA was done with mimotopes 24-4.1 and 13-4.1 using the corresponding MAbs. Figure 7 shows the results. For both mimotopes, the homologous toxin was the most competitive; other toxins were less competitive in the order G2 < B2 < G1. With 24-4.1 (Figure 7a), B2 and especially G2 were much less competitive whereas with 13-4.1, the differences between toxins were less marked. The median doses (the toxin concentrations that resulted in an estimated 50% inhibition of binding) and the slopes of the curves at the median were calculated from the curves fitted to the data using the formula,

$$A_{405} = A + C / (1 + \exp(-B * (X-M)))$$

where A and C are the asymptotes, M is the median dose and B is the slope at the median dose.

Table 4 shows the results. These confirm the impressions from Figure 7 that the strongest competition as judged by the slopes of the lines was with B1, the 'homologous' antigen and the weakest competition was with G2.

3.3.2.4. Effect of dithiothreitol

The peptide sequences displayed by phage from the Cys-4 and Cys-6 libraries contain cysteine residues that are intended to cross-link so as to create a loop structure of either 4 or 6 amino acids. Mimotope phage were treated with dithiothreitol prior to being used to coat ELISA plates in order to prevent the formation of this cross-link. With all clones tested (24-4.1, 24-4.2, 24-6.1, 13-4.1, 13-4.2, 13-6.1), the effects of dithiothreitol treatment were to increase the binding of the corresponding MAbs by between 2-fold and 5-fold.

3.3.2.5. Effect of UV irradiation on phage infectivity

The infectivity of a dilute sample of mimotope 24-4.1 was decreased from about 4500 colonies ml^{-1} to about 60 colonies ml^{-1} by irradiation at 20 mJ/cm^2 and was abolished by larger doses. At these doses there was little effect on MAb binding as assessed by phage ELISA. Phage irradiated at 50 mJ/cm^2 bound about 90% of the amount of MAb bound by untreated samples. Binding decreased to 70% following irradiation with 100 mJ/cm^2, and further after higher doses.

3.3.2.6. Recovery of aflatoxin B1 from groundnut samples

In order to test the effectiveness of the phage ELISA, 10 to 200 $\mu g\ kg^{-1}$ of aflatoxin B1 were added to finely ground 10 g samples, which were then extracted and assayed. Table 5 lists the results from each of two ELISA plates. The recoveries were between 92% and 110%; the average recoveries on each plate were 101.3% and 98.9%.

To assess the assay method in practice, samples were taken from farmers' fields that were presumed to be at high risk of aflatoxin contamination because of drought. These were extracted and assayed by using competition ELISA. The results (Table 6) show that 5 samples were contaminated to various extents. Results for duplicate samples and duplicate plates were within 10%. The permissible level in India for aflatoxin B1 contamination is currently 30 μg/kg .

3.3.2.7. Mimotope sequences

For mimotope phage from either library, the nucleotide sequences were determined for the section of recombinant pVIII gene that encoded the randomized peptide sequence. These are shown as Figure 7. The sequences of mimotopes to MAb 24 suggested a consensus sequence of '---CYMD-C---'. Those for mimotopes to MAb 13 did not suggest a clear consensus, although the dipeptide 'PW' was present in most, and all were particularly rich in proline residues.

Figure 6. Results of indirect ELISA with four phage mimotopes (24-4.1, 24-6.1, 13-4.1, 13-6.1) at concentrations of 10^{11}, 10^{10} or 10^{9} ml^{-1}.

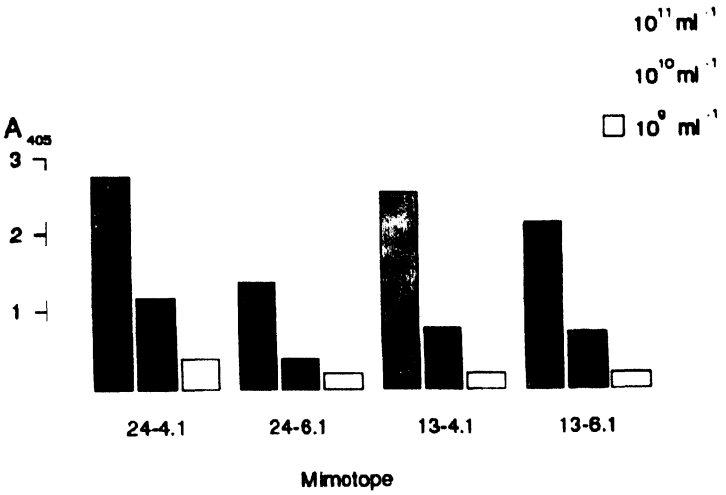


Figure 7. Competition between pure aflatoxins (B1, B2, G1 and G2) in the solution and phage mimotopes (24-4.1 and 13-4.1) coated on ELISA plate surface for binding to MAbs, in indirect competitive ELISA. (a) Mimotope 24-4.1 binding to MAb 24. (b) Mimotope 13-4.1 binding to MAb 13.

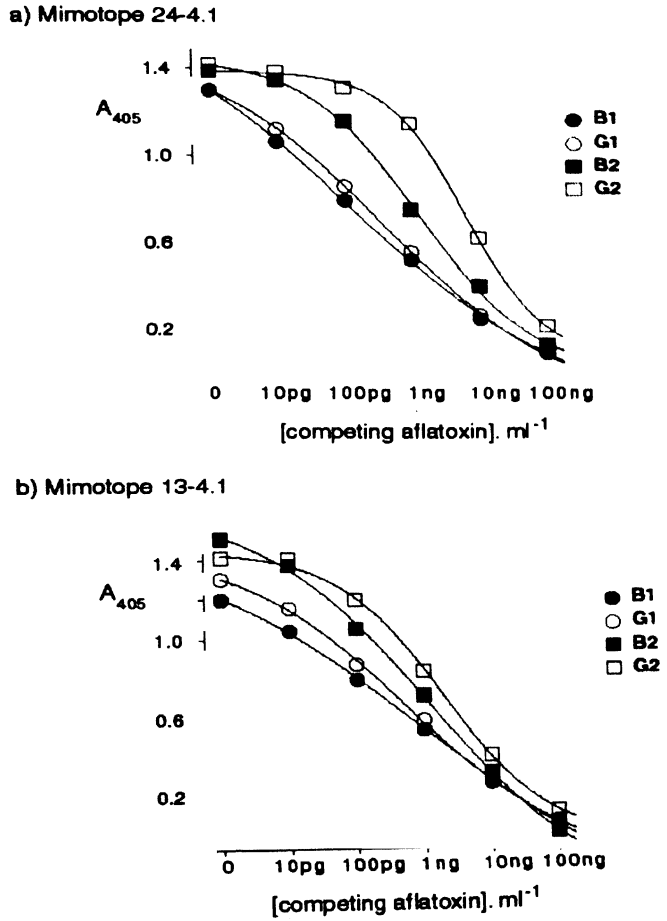


Figure 8. Amino acid sequences of mimotope peptides.
Nucleotide sequences were determined for the part of the recombinant gene VIII that encodes amino acids randomized in the libraries Cys-4 and Cys-6. Where two amino acids are shown, the sequences obtained were ambiguous and either amino acid is possible.

MAb	Library	Mimotope	sequence in recombinant gene VIII
24	Cys-4	24-4.1	A N T W C Y V D E C M R I A
		24-4.2	E T Y G C F M D W C K L V T
		24-4.3	N/D T P V C Y M N W C V E S/T D
		24-4.4	N N/T K R C Y M D L C I Q T P
24	Cys-6	24-6.1	E/M G T I C P M/T D I K G C N Q/H T P
		24-6.2	G C E Q C Y V D Y C Y C S D A G
		24-6.3	G C E Q C Y V D Y C Y C S D A G
		24-6.4	M R G Q C Y M N Q N M C K H P A
13	Cys-4	13-4.1	F H P R C N E M T C H I K P
		13-4.2	N N P T C P W L T C P L P S
		13-4.3	T R W N/D C P/R T T Y C P P S G
		13-4.4	N T N H C Y M D H C I Q T H
		13-4.5	V A N G C E K P W C N T T R
13	Cys-6	13-6.1	R Q S Y C H P W E A I C H Q H K
		13-6.2	R Q S Y C H P W E A I C H Q H K
		13-6.3	V D L W C P P A P W Q C L P S D

Table 4. Parameters of the inhibition dose curves shown in Fig.7

Inhibitor	Mimotope 24-4.1 median dose*	slope	Mimotope 13-4.1 median dose	slope
B1	1.38	-0.6044	1.04	-0.53
B2	1.39	-1.2032	1.22	-0.80
G1	0.45	-0.7495	0.58	-0.79
G2	6.77	-1.7623	1.98	-1.27

ng/ml

Table 5. Efficiencies of extraction

Concentration of AFB1 in spiked sample ($\mu g\ kg^{-1}$)	Recovery*	
	Plate 1	Plate 2
10	98%	102%
20	102.5%	110.4%
40	105%	96%
80	99.9%	94.1%
100	100.7%	99.6%
150	101.1%	91.9%
200	102.2%	98.5%
Mean	101.3%	98.9%

* measured concentration/known concentration x 100

Table 6. Assays of groundnut samples for aflatoxin B1

Sample	[aflatoxin B1] ($\mu g\ kg^{-1}$)				Result
	Plate 1		Plate 2		
	test 1	test 2	test 1	test 2	
1	15.2	14.0	19.8	21.6	+
2	< 10	< 10	< 10	< 10	-
3	101	89.3	101	113	+
4	55.1	49.0	46.8	45.1	+
5	302	276	295	286	+
6	< 10	< 10	< 10	< 10	-
7	< 10	< 10	< 10	< 10	-
8	329	314	331	331	+
9	< 10	< 10	< 10	< 10	-
10	< 10	< 10	< 10	< 10	-

Chapter 4

Production of Antibodies for Ochratoxin A

4. PRODUCTION OF ANTIBODIES FOR OCHRATOXIN A

4.1. MATERIALS AND METHODS

4.1.1. Immunization

Commercially available OA-BSA conjugate (Sigma cat. no. O-3007) was used. Immunization procedure is as described in the section 3.1.1.1 for aflatoxins

4.1.2. Monitoring antibody titres

An indirect ELISA procedure similar to that explained in the section 3.1.1.2 for aflatoxins was employed.

4.1.3. Characterization of antibodies

To evaluate the cross reactivity of OA antibody with ochratoxin B, coumarin, 4-hydroxycoumarin, L-phenylalanine and aflatoxin B1, the procedure adopted is similar to that describd in the section 3.1.1.3 for aflatoxins.

4.2. RESULTS

4.2.1. Production of antibody

The protocol used for immunization gave an antibody titre of 1:132,000, 34 weeks after the initiation of immunization (Figure 9). Standard curves for OA by indirect competitive ELISA at three different dilutions of antibody are shown in Figure 10.

4.2.2. Specificity of antibody

To determine the cross-reaction of antibodies antiserum with molecules resembling OA, they were tested against ochratoxin B, L-phenylalanine, coumarin, 4-hydroxy coumarin, and Aflatoxin B1. Results presented in Figure 11 clearly show that OA antibodies did not react with any of the molecules tested.

Figure 9. Antibody titres against OA-BSA as determined by indirect ELISA. Booster injections were given at 7, 11, 16 and 30 weeks after initial immunization (Details are given in the section 3.1.1.1).

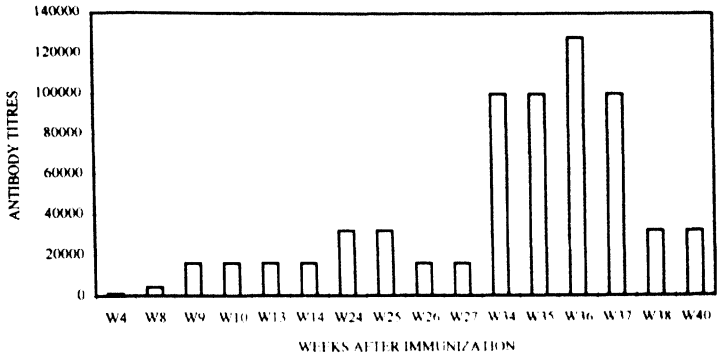


Figure 10. Standard curves for OA by indirect competitive ELISA at three different dilutions of antibody 1:40,000 (●), 1:80,000 (■), 1:160,000(▲) (Details of test procedure given in the section 3.1.1.2).

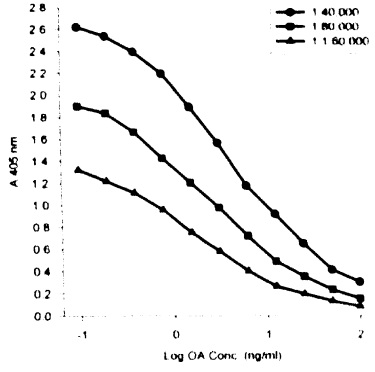
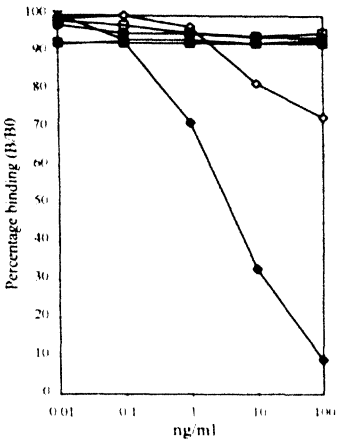


Figure 11. Cross reactivity of OA antibodies (♦) to OB (◊), Coumarin (■), 4-Hydroxy coumarin (□) L-phenylalanine (✱) and AFB1 (●).



Chapter 5

Enzyme-Linked Immunosorbant Assay for Aflatoxins and Ochratoxin A

5.1. MATERIALS AND METHODS

5.1.1. Quantitative Estimation: Indirect Competitive ELISA (IC-ELISA)

The protocol used was similar to that for determining antibody specificity (as described in the section 3.1.1.2). In brief, ELISA plates were coated by incubating toxin-BSA conjugate in 0.2 M sodium carbonate buffer, pH 9.6 (150 µl/well) and incubated overnight in a refrigerator. Subsequent steps were performed at 37°C for 1 h. Antibody dilutions prepared in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and 0.2% BSA (PBS-T BSA) in 50 µL volume were added to 100 µL sample or toxin standards ranging from 100 ng/mL to 100 pg/mL. The amount of antibody bound to the toxin was detected by the addition of goat anti-rabbit or goat anti-mouse immunoglobulins (GAR IgG, Cat # A4914 and GMA IgG, Cat # A, 5153 Sigma) conjugated to alkaline phosphatase. The substrate for ALP, p-nitrophenyl phosphate (Sigma, cat # N 2765) was used at 1 mg/mL. Colour was allowed to develop for 1 h at room temperature and then the absorbance of the hydrolysed product was measured at 405 nm in an ELISA plate reader (Titretek Multiskan ™, Labsystems).

5.1.2. Quantitative Estimation: Direct Competitive ELISA (DC-ELISA)

Preparation of conjugates

Three enzymes, alkaline phosphatase (ALP) (Sigma, cat # P 5521), horseradish peroxidase (HRP) (Sigma cat # P 8375) or penicillinase (PNC) (cat # P 0389) were conjugated to commercially produced aflatoxin-BSA using the single bridge glutaraldehyde method for each of the two enzymes (ALP and PNC), and the periodate oxidation method specifically for HRP. AFB1-BSA (1.0 mg) was reacted with 1.0 mg of ALP or PNC and dialysed for 1h. Glutaraldehyde was added to 0.05% final concentration and the mixture incubated at 22 ° C for 4 h. Glutaraldehyde was then removed by dialysis against several changes of PBS and the conjugate stored with 1 % bovine serum albumin at 4 ° C. Conjugation of aflatoxin-BSA to horseradish peroxidase (HRP) was by periodate oxidation method (Tsang *et al.*, 1995) which links protein to HRP via carbohydrate moieties on the enzyme. HRP (4.0 mg) in 0.2 ml of citric acid/sodium citrate buffer (0.1M, pH 5.0) was warmed to 37°C to which 26µl of sodium periodate (30 mg/ml) was added. After 5 min exposure, 13µl of 1% ethylene glycol was

added, followed immediately by dialysis of the oxidized enzyme against 1L of citric acid/sodium citrate buffer (0.001M, pH 5.0) for 1h. The oxidized HRP was then reacted with AFB1-BSA (1mg in 1mL of 0.1 M sodium carbonate buffer, pH 10.0) at 4° C for 48h, followed by the addition of 10µl of sodium cyanoborohydride solution (10 mg/ml), and reacted for another 2h at 4° C. The reaction mixture was then dialyzed against PBS and stored at 4 ° C after the addition of glycerol (1:1 v/v).

Test procedure

Protocols for the DC-ELISAs were essentially the same as those described for aflatoxin B1 (Chu *et al.*, 1987). The optimal concentration of antibodies and enzyme marker used in the assay were determined using a checker board titration test. Microtitre plates were coated with 100 µl of aflatoxins polyclonal antibodies (1:80,000 dilution). After overnight incubation, the wells were washed four times with PBS-T and then incubated with 200 µl of blocking solution [0.2% (m/v) BSA in PBS-T (BSA-PBS-T)] at 37°C for 30 min. The wells were washed four times with PBST. Toxin standards or samples dissolved in 7% MeOH/PBS in 50µl volume together with 50µl of toxin labelled with the enzyme (diluted in BSA-PBS) were added to each well. Competition was between labelled toxin in BSA-PBS and unlabelled toxin present in the sample. Enzyme labelled toxin attached to the antibody was detected by adding a suitable substrate.

ALP substrate: Para-nitro-phenylphosphate (1mg/ml) added to 10% diethanolamine pH 9.8.

HRP substrate: Kirkegaard and Perry (50-76-05)

PNC substrate: 15 mg bromothymol blue (BTB) in 100 ml of 0.01 M NaOH, alkali neutralised by adding 0.1 N HCL drop wise until the pH of the solution is 7.2. Sodium penicillin-G was incorporated at 0.5 mg/ml (w/v).

Calculations: Standard curves were obtained by plotting $\log_{10}$ values of toxin standards against optical density at A_{405}, for ALP, A_{450} for HRP and A_{620} for PNC system.

5.1.3. Qualitative Estimation: Dip-Strip ELISA

Preparation of membranes

To prepare test strips 1cm x 1.5cm sections of the Immunodyne Immunoaffinity membranes were mounted onto 1cm x 6 cm plastic supports with a double sided adhesive layer. An area of the membrane (approximately 3 mm in diameter) was coated with 3µl of the respective polyclonal or monoclonal antibody in an appropriate dilution in PBS, and then the test strips were dried at 35°C for 30 min. To block free protein-binding sites on the membrane, the strips were immersed in 2% milk powder prepared in BSA-PBS-T for 20 min. Finally, the strips were dried again, and stored at room temperature, protected from light and humidity.

Test procedure

To perform the test strip assay, a solution of pure toxin standard in PBS containing 7% methanol was mixed with toxin-BSA-conjugated to the enzyme in a plastic test tube. Solutions not containing toxin were prepared accordingly to serve as negative control. Test strips were incubated for 30 min at room temperature, with gentle shaking. Then each strip was washed and the enzyme-labelled toxin attached to the antibody was detected by adding an insoluble chromogenic substrate. The substrate for HRP was TMB membrane peroxidase (Kirkegaard and Perry 50-77-00) and for ALP, Sigma Fast ™ Fast Red TR/Naphthol AS-MX ™ insoluble alkaline phosphatase substrate with 1mM levamisole (Sigma F 4523). Substrates were prepared according to the manufacturer's instructions.

5.2. RESULTS

5.2.1. Efficacy of DC-ELISA for quantitative estimation of aflatoxins utilizing three enzyme systems (ALP, PNC and HRP)

Among the three enzyme systems tested, HRP conjugates prepared by periodate-oxidation method and PNC conjugates prepared by single bridge gluteraldehyde method were more effective and could be used at dilutions exceeding 1:15,000. ALP conjugate could be used at 1:2000. Methanol present in the sample did not influence the reaction. This is the first time that PNC system has successfully been applied successfully for aflatoxin estimation by ELISA. PNC system offers opportunities to develop an inexpensive method for aflatoxin estimation.

5.2.2. Test Strips

Non-specific covalent binding of the toxin-enzyme conjugate to the membrane, not bound by the antibody, was successfully blocked with 2% milk powder. An incubation time of 30 min was sufficient to achieve the optimum results. A reduction of intervals of incubation to 5 min resulted in weak reaction. Intervals longer than 30 min did not improve colour intensity. Due to the inverse relationship between toxin content and colour development, it was essential to determine the lowest concentration of toxin required to give a reaction with out any colour development. Samples without toxin gave the maximum colour intensity.

Table 7. Estimates of within-assay and inter-assay variability of AFB1 standard curves by DC-ELISA.

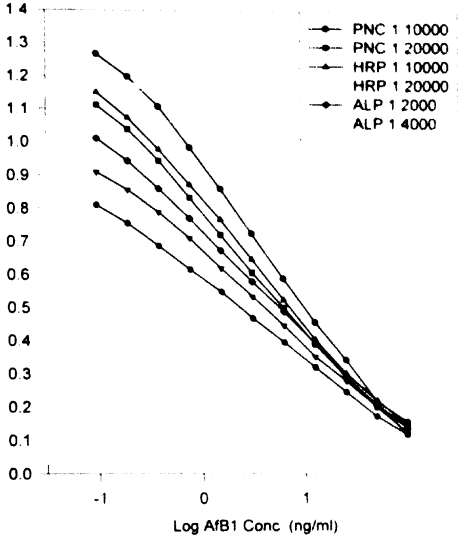
No.	Concentration of	Mean ± SD, ng/ml (% CV)[a] Within-assay	Inter-assay
PNC.AFB1-BSA (1:15000)			
1	10	9.5 ± 0.3 (3.2)	10.0 ± 0.8 (7.8)
2	20	19.9 ± 1.4 (7.1)	21.6 ± 1.0 (4.6)
3	40	36.7 ± 2.0 (5.3)	39.9 ± 3.2 (8.1)
4	80	78.8 ± 7.5 (9.5)	78.1 ± 7.5 (9.6)
5	100	103.5 ± 9.6 (9.3)	101.2 ± 6.8 (6.7)
6	150	169.1 ± 5.8 (3.4)	149.2 ± 4.7 (3.2)
7	200	211.1 ± 9.9 (4.7)	202.1 ± 1.6 (0.8)
HRP.AFB1-BSA (1:15000)			
1	10	10.3 ± 1.1 (10.6)	9.4 ± 0.8 (8.0)
2	20	19.1 ± 1.8 (9.5)	21.1 ± 1.8 (8.3)
3	40	31.3 ± 1.3 (4.1)	35.6 ± 3.4 (9.6)
4	80	74.9 ± 7.1 (9.4)	80.9 ± 3.0 (3.7)
5	100	93.0 ± 6.0 (6.5)	93.0 ± 8.0 (8.6)
6	150	142.9 ± 4.7 (3.3)	154.9 ± 3.9 (2.5)
7	200	208.4 ± 8.6 (4.1)	199.7 ± 6.2 (3.1)
ALP.AFB1-BSA (1:2000)			
1	10	9.1 ± 0.5 (5.0)	9.5 ± 0.9 (9.7)
2	20	20.0 ± 1.3 (6.3)	21.8 ± 1.0 (4.6)
3	40	36.4 ± 1.4 (3.8)	40.8 ± 2.6 (6.3)
4	80	81.5 ± 5.6 (6.9)	79.2 ± 6.0 (7.6)
5	100	108.4 ± 9.0 (8.3)	101.9 ± 5.7 (5.6)
6	150	160.0 ± 6.2 (3.6)	153.9 ± 8.5 (5.5)
7	200	210.4 ± 9.0 (4.3)	202.2 ± 1.6 (0.8)

[a] Values are means ± SD (%CV)

Table 8. Comparison of efficiency and cost-effectiveness of DC-ELISA with the thin layer chromatography and high performance liquid chromatography.

Property	TLC	HPLC	ELISA
Extraction of samples per day	8	4 in two days	60
Analysis of samples per day	36	24	160
Cost/sample (Rs.)	88	1000	50
Equipment (Rs.)	0.5 lakhs	4.5 lakhs	2.0
Analysis of more than one toxin at a time	Not very efficient	Possible	Possible
Throughput	Not possible	Possible	Possible

Figure 12. Standard curves for AFB1 by DC-ELISA at different dilutions of AFB1-BSA-HRP, AFB1-BSA-PNC and AFB1-BSA-ALP conjugates.



Chapter 6

Analysis of Foods and Feeds for Aflatoxins and Ochratoxin A

6.1. MATERIALS AND METHODS

6.1.1. Collection of samples

A total of 226 samples of the most widely used spices in Indian cooking, black pepper, chillies, coriander, ginger and turmeric were selected for the estimation of OA. Dried chilli pods were collected from market yards and cold storage units of the two major chilli growing areas in the state of Andhra Pradesh in India, namely Guntur and Khammam districts. From 50 kg bags, approximately one kg sample was drawn from several points. A total of 80 samples (70 from market yards and 10 from cold storage units) were collected and graded to three types, grade-1, grade-2 and grade-3, based on the quality of the pods. Grade-1 samples were of good quality and contained fully matured pods, mostly from the first plucking, that were unbroken, bright red coloured and absolutely free from pod diseases, insect damage and moulds. Grade-2 had samples of medium quality and size and only a few pods were discoloured. Grade-3 samples contained mouldy, damaged and discoloured pods left over when sound pods were mechanically separated from the produce. In addition, 20 different brands of chilli powders, 25 turmeric, 50 coriander, 25 ginger and 26 Black pepper samples were purchased from retail shops in 200 g quantities. Black pepper and coriander were purchased as seeds and turmeric and ginger were bought as powders.

A total of 216 poultry feed samples, groundnut cake, maize, millets, mixed feeds, rice bran, sorghum, soybean and sunflower were also collected from the poultry feed manufacturing companies.

6.1.2. Preparation of samples for ELISA.

Samples were thoroughly mixed, three aliquots of 50 g were drawn and ground to a fine powder, of which 15 g was processed for ELISA. 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 give two-fold to ten-fold step-wise diltutions in PBS-T-BSA for processing by ELISA.

6.1.3. Indirect competitive ELISA procedure for processing the samples

An indirect ELISA procedure was used that was similar to that described in the section 5.1.1. for estimating aflatoxins. Standard curves were obtained by plotting $\log_{10}$ values of toxin standards against optical density at A_{405}. Concentration of toxin in the sample extract was determined from the standard curves and expressed in μg/kg using the formula: toxin concentration (ng/ml) in sample extract × dilution with buffer × extraction solvent volume used (ml) /sample weight (g). In order to test the recovery of toxin from 15 g quantity samples, they were spiked with toxin concentrations ranging from 1 to 100 μg/kg. Spiked samples were extracted with methanol as described and then diluted in PBS-T BSA.

6.2. RESULTS

6.2.1. Effect of sample extract on ELISA

The ELISA procedure used for the estimation of OA in spices did not at first give accurate results presumably due to interference of substances present in the extracts of spices. To confirm that interference in ELISA was due to substances in spice samples, OA standards prepared in PBS-T BSA were compared with those prepared in extracts of spices free from OA. Curves were found to be influenced by substances present in different spice extracts (Figure 13, 14). As a result it was essential to prepare the standard solutions in extracts from toxin free samples. Presumably, the sample extracts contained substances that bind non specifically to IgG's and thus interfere in ELISA so as to cause non specific reaction.

6.2.2. Recovery of aflatoxins and OA from samples

Extraction of samples in 70 % methanol followed by 10 times dilution in PBS-T BSA gave > 90 % recoveries for aflatoxins in poultry feed ingredients. Of four OA extraction procedures tried (Sungsoo *et al.*, 1984; Candlish *et al.*, 1988; Ramakrishna *et al.*, 1990; Barna-vetro et al., 1996) for processing chilli samples for ELISA, extraction in 70% methanol followed by 4 times dilution in PBS-T gave 90% recoveries in spiked samples. The mean recoveries from chilli samples devoid of OA, spiked with 1 to 100 µg/kg OA, were 93 to110% (Table 9).

6.2.3. OA contamination in selected spice samples and aflatoxins and OA contamination in poultry feeds

A total of 226 selected samples of black pepper, chillies, coriander, ginger and turmeric were analyzed for the presence of OA. OA was found to exceed 10 µg/kg in 14 (in the range of 15 to 69 µg/kg) of 26 black pepper samples; 26 (in the range of 10 to 120 µg/kg) of 100 chilli samples; 20 (in the range of 10 to 51 µg/kg) of 50 coriander samples; 2 (2 µg/kg and 80 µg/kg) of 25 ginger samples and 9 (in the range of 11 to 102 µg/kg) of 25 turmeric samples. This is the first record in India of the occurrence of OA in the most widely used spices in Indian cooking.

A total of 216 poultry feed samples (groundnut cake, maize, millets, mixed feeds, rice bran, sorghum, soybean and sunflower) were analysed for the presence of aflatoxins and OA. Aflatoxins were found to exceed 10 µg/kg in 10 (in the range of 50 to 2700 µg/kg) of 27 groundnut cake samples; 41 (in the range of 10 to 300 µg/kg) of 95 maize samples; 1 (20 µg/kg) of 8 millet samples; 18 (10 to 1500 µg/kg) of 30 mixed feeds; 3 (in the range of 10 to 100 µg/kg) of 14 rice bran samples; 6 (in the range of 10 to 3500 µg/kg) of 29 sorghum samples and 5 (in the range of 30 to 50 µg/kg) of 10 sunflower samples. No aflatoxin contamination was detected in 3 soybean samples. OA was found to exceed 10 µg/kg in 1 (73 µg/kg) of 27 groundnut cake samples; 2 (120 µg/kg and 145 µg/kg) of 8 millet samples; 1 (26 µg/kg) of 14 rice bran samples; 9 (in the range of 10 to 400 µg/kg) of 29 sorghum samples; 1 (30 µg/kg) of 10 sunflower samples. No OA contamination was found in 95 maize samples, 30 mixed feeds and 3 soybean samples.

Table 9. Recovery of OA from artificially contaminated spice samples as determined by ELISA.

No.	Concentration of OA used for spiking (μg/kg)	Concentration of OA estimated (μg/kg)[a]	Percent recoveries of OA in spiked samples[b]
Black pepper			
1	5	4.80 ± 0.39	96.0 ± 8.07
2	10	9.60 ± 0.82	96.0 ± 8.57
3	50	49.2 ± 0.88	98.4 ± 1.78
4	100	103.9 ± 4.08	102.6 ± 3.98
Chillies			
1	5	4.6 ± 0.37	92.0 ± 7.4
2	10	9.36 ± 0.94	94.0 ± 8.9
3	50	52.1 ± 0.87	103.0 ± 1.6
4	100	125 ± 3.9	103.0 ± 3.3
Coriander			
1	5	5.90 ± 0.57	118.0 ± 9.72
2	10	10.53 ± 0.82	105.3 ± 7.75
3	50	52.47 ± 2.1	104.9 ± 4.03
4	100	108.1 ± 7.9	108.1 ± 7.38
Ginger			
1	5	4.73 ± 0.29	94.7 ± 6.22
2	10	9.30 ± 0.73	93.0 ± 7.82
3	50	49.5 ± 0.72	99.1 ± 1.45
4	100	103.9 ± 5.46	103.9 ± 5.26
Turmeric			
1	5	4.97 ± 0.05	99.3 ± 1.0
2	10	10.0 ± 0.08	100.0 ± 0.82
3	50	52.77 ± 3.11	105.5 ± 5.90
4	100	98.73 ± 1.01	98.7 ± 1.03

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

Table 10. Incidence and range of OA in chillies as determined by indirect competitive ELISA.

Sample type [a]	OA contamination					
	Incidence	% contamination	No of samples with OA (µg/kg) contents in the ranges of			
			10-29	30-49	50-100	120
Black pepper	14/26	54	8	5	1	0
Chillies						
Chillies grade-1 (Market yards)	2/32	6	0	1	1	0
Chillies grade-2 (Market yards)	2/14	14	1	0	1	0
Chillies grade-3 (Market yards)	8/23	35	3	5	0	0
Chillies (Cold storage)	0/10	0	0	0	0	0
Chilli powders (From super markets)	14/21	66	8	4	1	1
Coriander	20/50	40	16	3	1	0
Ginger	2/25	8	1	0	1	0
Turmeric	9/25	36	3	2	3	1

[a] see text for details.

Table 11. Incidence and range of aflatoxin (AF) and OA in poultry feed samples as determined by indirect competitive ELISA.

Sample type [a]	Incidence		AF and OA contamination % contamination		No of samples with aflatoxin & OA (μg/kg) contents in the ranges of							
					10-29		30-49		50-100		> 100	
	AF	OA	AF	OA	AF	OA	AF	OA	AF	OA	AF	OA
GN cake	10/27	1/27	37	50	2	0	1	0	3	1	4	0
Maize	41/95	0/95	43	0	15	0	9	0	8	0	9	0
Millets	1/8	2/8	12	25	1	0	0	0	0	0	0	2
Mixed feeds	18/30	0/30	60	0	11	0	3	0	1	0	3	0
Rice bran	3/14	1/14	21	7	1	1	0	0	2	0	0	0
Sorghum	6/29	9/29	20	31	2	2	2	1	0	1	2	5
Soybean	0/3	0/3	0	0	0	0	0	0	0	0	0	0
Sunflower	5/10	1/10	50	10	0	0	5	1	0	0	0	0

[a] See text for details

Figure 13. Effect of chilli 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 chilli extract (▲).

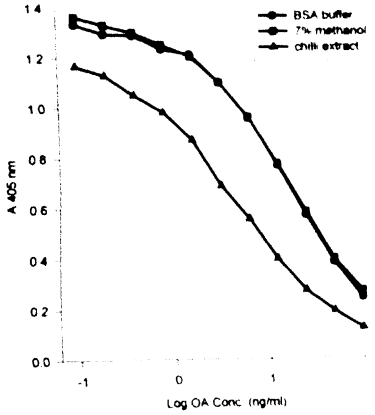
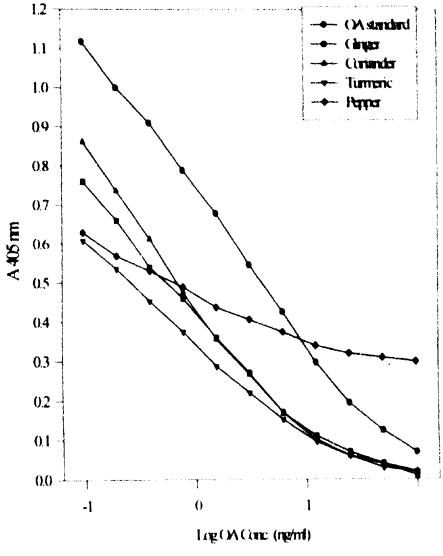


Figure 14. Effect of spices extract on the standard curve of OA by indirect competitive ELISA. OA standard curves prepared in 7% methanol ( ), Black pepper(♦), Coriander ( ), Ginger ( ), and Turmeric ( ),extracts.



Chapter 7

7. GENERAL DISCUSSION

7.1. Production of antibodies against aflatoxins and ochratoxin A

Mycotoxins are low molecular weight compounds (i.e haptens, 300-400 MW) and by themselves are ineffective to stimulate the immune system to produce antibodies. Therefore, they must be linked to a higher molecular weight carrier antigens, e.g., a protein, in order to induce antibody production. Several mycotoxins do not have a free reactive site for coupling to a protein and therefore require to be derivatized. The conjugation techniques for mycotoxins have been extensively reviewed by Chu (1986), all using the standard approaches for low molecular weight haptens, such as drugs and hormones. Aflatoxin B1 was derivatized to a carboxymethyl-oxime at cyclopentene position. Ochratoxin A has a free carboxylic group (Figure 1) and therefore it could be directly linked to a protein. In this study haptens were not conjugated to carrier molecules and instead commercially available aflatoxin B1 and ochratoxin A conjugated to bovine serum albumin were employed for antibody production.

Polyclonal antibodies

The frequently used method for the production of polyclonal antibodies comprise multiple-site intramuscular injections of rabbits with mycotoxin conjugated to a protein, followed by a booster normally 6 weeks after the initiation of immunization schedule. This procedure was tried in this communication as well as in a report (Reddy *et al.*, 1988) and the antibody titre for AFB1 did not exceed 1:10 000 by ELISA. As a result, a modified procedure was adopted that involved several subcutaneous injections at multiple sites followed by a non-immunization rest period of six to eight months and a booster subcutaneous injection soon after the rest period. This procedure resulted in the production of high titered antibodies for aflatoxins and ochratoxin A. Polyclonal antibodies produced for aflatoxin B1 recognized all the four aflatoxins, reacting

relatively well with B1, B2, G1 and weakly with G2. In contrast, polyclonal antibodies raised against ochratoxins were found to be specific to ochratoxin A.

Monoclonal antibodies

The main aim of generating monoclonal antibodies was to obtain antibodies with appropriate antigen affinity, negligible cross reactivity and high sensitivity. The production of monoclonal antibodies is dependent largely on the immunization schemes, fusion procedures, and stabilities of the hybrid clones. The first step is to obtain as many antigen-specific monoclonal antibodies as possible. When immunizations were done by conventional methods (Candlish *et al.*, 1990, Wang *et al.*, 1995), very few monoclonal antibodies were generated. Interestingly, the same group of mice gave better immune response after a long resting period, and a booster injection. This presumably resulted in the accumulation of B memory lymphocytes with high affinity to Ig receptors in the spleen, thereby enhancing the chances for selecting hybridomas. Booster injections have been given normally 3 days prior to removal of spleen for fusion (Candlish *et al.*, 1990, Ward *et al.*, 1990). In contrast, boosters were given 18 days before fusion. This was attempted because booster injections given 3 days before fusion (protocol 1) resulted in the selection of only a few monoclonal antibodies. This is the first time that a rest period after initial immunization has been shown to contribute to enhanced generation of monoclonal antibodies. It is currently not known if this protocol will consistently lead to the generation of antibodies with wide variation in affinity in particular with haptens. Monoclonal antibodies produced by Candlish *et al.*, (1990) were largely specific to B1 with poor cross reaction to B2, G1, and G2 aflatoxins. However antibodies reported by Kawamura *et al.*, (1988) were highly specific to B1 and showed partial reaction to G1. On the other hand, Hefle and Chu (1990) reported equal cross reactivities with all the four aflatoxins. The wide variation in the specificities of monoclonal antibodies obtained in this study for four aflatoxins has so far not been reported to occur in the products of a single fusion. The antibodies produced can be used either to estimate B1 alone or B1, B2 and G1. Since G2 is the least toxic of all the four aflatoxins, and is not known to occur widely (FAO Food and nutrition paper 14/10, 1990) in foods and feeds, this deficiency is unlikely to contribute to inaccurate estimation of total aflatoxin content. The

clone 10D5-1A11 (Figure 5) could detect as little as 1pg/ml of AFB1. This is in contrast to the limits of 200 pg ml^{-1} reported by Candlish et *al.*, (1985) and 10 pg ml^{-1} reported by Ward et *al.*, (1990).

Mimotopes

The results of panning of random phage-displayed peptide libraries showed that it is possible to obtain peptides that mimic the binding of aflatoxin B1 to antibodies raised against it. However, with one of the three MAbs tried no mimotopes were obtained from either library. This MAb does not cross-react with other aflatoxins (Thirumala-Devi et *al.*, 1999) and it is possible that the epitope involved in this highly specific binding cannot be mimicked by peptides. However, mimotopes were obtained to the other two MAbs that these had similar properties to the toxin in ELISA tests. All mimotopes reacted equally well with polyclonal antibodies. In a competitive ELISA, aflatoxin B1 could compete with the selected peptides for binding to MAb, while the synthetic peptide also competed with aflatoxin for binding to the same antibody. This strongly suggests that these clones bind to the same antigen-binding site of the MAb. However, in competition ELISA with aflatoxins B2, G1 and G2, the mimotope from the set binding to MAb 24 was differently affected by competition from the one binding to MAb 13 (Figure 2). The differences among the slopes reflected the extent of cross-reactivity each MAb had with heterologous aflatoxins. For MAb 24 these were B1 = G1 >> B2 and G2 and for MAb 13 B1>G1 = B2 >> G2 (Thirumala-Devi et *al.*, 1999). Thus in these tests, G1 showed the same affinities. This suggests that the change from a five-membered ring to a six-membered ring containing an extra oxygen atom that distinguishes B1 and G1 from B2 and G2 alters the accuracy of the mimicry by the peptides more than does the change from a single bond to a double bond, in a different ring, that distinguishes B aflatoxins from G aflatoxins.

The amino acid sequences of the mimotope peptides differed among the clones isolated, although there some residues, especially aromatic amino acids were abundant. MAb 24 mimotopes contained one or more tyrosine residues and MAb 13 mimotopes contained tryptophan residues. This may reflect a degree of molecular mimicry by the ring structures in the aromatic amino acids for the ring

structures in the aflatoxin molecules. In sequence, the MAb 24 mimotope peptides were unrelated to those for MAb 13. Presumably the binding pockets in the two MAbs differ appreciably despite the simplicity of the common immunogen to which they were raised.

By its nature, the phage display method tends preferentially to isolate strongly binding phage. The results show that aflatoxin MAbs select several approximately equally strongly binding peptides. This contrasts with the results obtained by Yuan *et al.*, (1999) with mimotopes isolated from an unconstrained library for MAbs specific to the toxin deoxynivenolol. Possibly, the mimotope these authors obtained is a much stronger binder than any others present in the library used for the panning.

Aflatoxins are important human and animal toxins, and so care must be taken to avoid direct contact during analysis. The possibility of replacing toxins in diagnostic procedures with presumably non-toxic peptides should therefore result in enhanced laboratory and environmental safety. This advantage could be even more marked when diagnostic test kits are used in "the field" by relatively unskilled operators unused to laboratory discipline.

Although many mimotope peptides for protein antigens have been isolated from phage-displayed peptide libraries (e.g. Hoess *et al.*, 1993, Scott *et al.*, 1992), this is only the second report of mimotopes for a non-proteinaceous, low molecular weight mycotoxin and the first report of aflatoxin mimotopes. Sixteen somewhat related mimotope peptides that mimic the binding of aflatoxin to specific antibodies were identified and the potential for using these peptides in aflatoxin immunoassays was demonstrated. This use of peptide libraries as sources of reagents in immunoassays could well be applicable in ELISA tests for other mycotoxins.

7.2. Development of quantitative and qualitative ELISA for aflatoxins and ochratoxin A estimation.

Once an appropriate antibody has been produced and characterized, the next step will be to develop an immunoassay for specific mycotoxin determination.

The ELISA techniques used were based on the competition between mycotoxin-BSA conjugate and the mycotoxin in the sample extract or between toxin present in the sample and specific antibody. Both the direct and indirect competitive assays developed exhibited similar sensitivities with a detection limit of 10 μg/kg using polyclonal antibodies and 1 μg/kg using MAbs. The sensitivity of AFB1 and OA ELISA are comparable to those previously reported using either polyclonal antibodies (Morgan *et al.*, 1983; Lee *et al.*, 1984; Ram *et al.*, 1986; Chu *et al.*, 1987) or MAbs (Chiba *et al.*, 1985 and Ramakrishna *et al.*, 1990). Mean recovery of AFB1 or OA from spiked samples were > 90% by both direct and indirect competitive ELISA procedures. The means within-assay, between assay and coefficients of variation by either ELISA of both artificially and naturally contaminated samples did not exceed 10%. It is less than those reported previously using polyclonal antibodies (Ram *et al.*, 1986) and MAbs (Kawamura *et al.*, 1988).

A simple **dip strip method** was developed for the rapid qualitative estimation of aflatoxins and OA by immobilizing antibodies onto a nitrocellulose membrane. The sensitivity and reproducibility of a solid support immuno-assay for a specific antigen is limited by the ability of the antibodies to remain attached to the membrane matrix during the assay procedure. If antibodies detach during the handling, it can result in a weak colour reaction, thus posing problems, especially for visual evaluation. By using a pre-treated, commercially available nylon membrane (Marlow and Handa, 1987), covalent binding of the antibodies to the membrane, during the processing was achieved.

7.3. Analysis of aflatoxins and ochratoxin A in foods and feeds

The results of the analysis of spices (black pepper, chillies, coriander, ginger and turmeric) collected from retail shops showed that contamination occurred at higher levels in chillies, black pepper, turmeric and coriander than that in ginger. It is noteworthy that high levels of OA (110 μg/kg) were noticed in the turmeric which is one of the most widely used spices in Indian cooking. Analysis of poultry feed samples (groundnut cake, maize, millets, mixed feeds, rice bran, sorghum soybean and sun flower) for aflatoxin and OA showed that the aflatoxin contamination occurred at high levels (3000 μg/kg of aflatoxin in maize and

groundnut and 400 and 150 μg/kg of OA in sorghum and millets). Soybean samples were found to be free from aflatoxins and OA contamination. OA was not detected in maize samples as well as in mixed feeds.

Growth of the mould and the production of mycotoxins is dependent upon a number of factors such as temperature, humidity, handling during harvesting, and conditions during storage. The majority of foods and feeds are produced in countries with tropical climates, which favour the growth of moulds. It is likely that the lack of sufficient surveillance data on ochratoxins in spices in India can be attributed to the unavailability of reliable analytical techniques. The present study clearly showed that the important spices and poultry feed ingredients could harbour aflatoxin and OA at levels, above those permitted by public health authorities. The enzyme immunoassays developed for aflatoxins and ochratoxin A are suitable for toxin detection in naturally contaminated samples. The methods employed have the advantage of being rapid, simple and inexpensive and thus, highly contaminated commodities can be prevented from entering the food as well as feed chain if adequate surveillance is done. The development of strategies that can minimize their contamination will lead to availability of safer foods and feeds and enhanced income to producers as a result opportunities to export.

Chapter 8

Summary and Conclusions

8. SUMMARY AND CONCLUSIONS

8.1. Summary

Aflatoxins and **ochratoxin A** are secondary metabolites of fungi (therefore called **mycotoxins**) and have assumed economic importance, because they are harmful to human beings, poultry and livestock and this exerts substantial influence on the suitability of commodities for export. Aflatoxins are potent carcinogens and ochratoxin A is a nephrotoxin. Mycotoxin presence in agricultural commodities is particularly unavoidable due to the uncontrollable environmental conditions that favour mycotoxin contamination. In order to protect human and animal health, it is important to be able to detect and quantity mycotoxins in foods and feeds, so that the contaminated materials can be handled in a way to reduce their levels. Many different methods are available currently for the estimation of mycotoxins. **Biological detection methods** are not specific and their sensitivity is generally low compared to those of other methods. **Physicochemical assay methods** are laborious and require expensive instrumentation and clean-up of the samples. To overcome the difficulties with biological and physicochemical methods, new immunological methods have been developed.

In most developing countries, limited or no facilities exist for monitoring aflatoxins or ochratoxins in foods and feeds. Commercial kits utilising immunological methods are expensive, in addition to the problem related to importing them. The Food and Agricultural Organization (FAO) of the United Nations estimates that 25% of the worlds food crops are effected by mycotoxins. Therefore for consumption as well as for export, monitoring the foods and feeds for mycotoxin content is essential. Immunological methods that use specific antibodies and suitable assay systems that are sensitive and cost effective are therefore vital to estimate mycotoxins. The main aim of this study was to develop highly sensitive and cost-effective ELISA-based methods for quantitative and qualitative estimation of aflatoxins and ochratoxin A in foods and feeds, a step towards reducing the risk posed by the possible presence of these two mycotoxins.

I. Production of antibodies for aflatoxins and ochratoxin A

Polyclonal antibodies were produced for aflatoxins and ochratoxin A by injecting the toxin haptens conjugated to bovine serum albumin (BSA) at multiple sites into rabbits. A modified immunization procedure resulted in the production of high-titre antibodies for aflatoxins and ochratoxin A. Polyclonal antibodies produced for aflatoxin B1 recognized all the four aflatoxins, B1, B2, G1 and G2 and polyclonal antibodies raised against ochratoxins were found to be specific to ochratoxin A.

Monoclonal antibodies: The novel immunization schedule adopted in this study resulted in the generation of ten hybridomas that secreted aflatoxin-specific antibodies from a single fusion. They were highly sensitive and showed a wide range of differing specificities recognizing B1 alone or B1 and G1 or B1, G1 and B2. Interestingly all the ten antibodies showed little or no cross reaction with G2.

Mimotopes: For the first time peptides that mimic aflatoxins B1, B2 and G1 were selected from two random phage displayed peptide libraries, using two of the MAbs that differed in the specificity. These peptide mimotopes were tested in indirect competitive ELISA for quantitative estimation of aflatoxins. The results showed that the mimotope preparations are an effective substitute for pure toxin in these ELISA procedures and suggest that their use will contribute significantly to enhance the safety of the tests.

II. Enzyme-linked immunosorbant assays for quantitative and qualitative estimation of aflatoxins and ochratoxin A

An indirect competitive ELISA was used for the estimation of aflatoxins in foods and feeds. In this procedure, toxin-BSA conjugate is coated onto the microtitre plate. Sample or standard toxin, along with specific antibody was added simultaneously to the wells. The amount of antibody not neutralized by the toxin, will bind to the toxin immobilized to the plate surface and was detected by the addition of goat anti-mouse or anti-rabbit IgG conjugated to alkaline

phosphatase (ALP). The substrate for ALP was p-nitrophenyl phosphate. The hydrolyzed product (p-nitrophenol) was measured at 405 nm in an ELISA reader.

A rapid and cost effective **direct competitive ELISA** was developed for the analysis of aflatoxins in various agricultural commodities. Three enzymes, alkaline phosphatase (ALP), horseradish peroxidase (HRP), and penicillinase (PNC) were conjugated to commercially produced aflatoxin-BSA using the single bridge glutaraldehyde procedure for ALP and PNC, and periodate oxidation method for HRP. ELISA plates were coated with polyclonal antibodies, followed by competition between toxins present in the sample with the enzyme-labelled BSA-toxin. Labelled toxin attached to the antibody, immobilized to the plate surface, was detected by adding a suitable substrate. The PNC and HRP conjugates could be used at dilutions exceeding 1:15,000 and ALP conjugate at 1:2000 dilution. Methanol present in the sample did not influence the reaction. This is the first time PNC enzyme has been shown to be effective in aflatoxin ELISA, thus offering an inexpensive method for aflatoxin estimation.

A simple **dip strip** method was developed for the **rapid qualitative estimation** of aflatoxins and OA. Antibodies were immobilized onto a nitrocellulose membrane. Toxin-BSA conjugated to the enzyme and samples containing the toxin were used for competition. Enzyme-labelled toxin attached to antibody was detected by an insoluble chromogenic substrate.

III. Analysis of foods and feeds for aflatoxins and ochratoxin A

Aflatoxins and ochratoxin A contamination in selected **spice samples** and **poultry feeds** was estimated using an indirect competitive ELISA. Samples were extracted with 70% methanol (1:5 w/v) and diluted subsequently to give two- to ten-fold step wise dilutions in phosphate buffered saline containing 0.05% Tween 20 and 0.2% bovine serum albumin (PBS-T BSA). Each of the diluted sample was spiked with 5 to 100 µg/kg toxin and tested for recovery. Ninety to hundred percent of the spiked toxin could be recovered. The extent of the dilution influenced the recoveries. Extracts of toxin-free samples interfered with ELISA presumably due to substances which contributed to non-specific reaction.

This effect could be avoided by preparing all the test solutions in toxin-free extracts of samples.

A total of 226 selected samples of black pepper, chillies, coriander, ginger and turmeric were analyzed for the presence of OA. OA was found to exceed 10 µg/kg in 54% of black pepper, 26% of chillies, 40% of coriander, 8% of ginger and 36% of turmeric obtained from supermarkets. This is the first report in India of the occurrence of OA in the most widely used spices in Indian cooking.

A total of 216 samples which included groundnut cake, maize, millets, mixed feeds, rice bran, sorghum, soybean and sunflower, intended for incorporation into chicken feed were analysed for aflatoxin and OA contamination. Aflatoxin exceeding (10 µg/kg) was recorded in 37% of the groundnut samples, 4% of maize, 12% of millet, 60% of mixed feeds, 21% of rice bran, 20% of sorghum and 50% of sunflower. OA contamination was found in 4% of groundnut, 25% of millet, 7% of rice bran, 31% of sorghum and 10% of sunflower samples. Maize and mixed feeds were found to be free from OA and soybean was free from both the toxins.

8.2. Conclusions

The objectives of this thesis were:

1. Production of polyclonal and monoclonal antibodies for aflatoxins,
2. Production of polyclonal antibodies for ochratoxin A,
3. Development of ELISA-based test procedures for the estimation of aflatoxins and ochratoxin A,
4. Analysis of foods and feeds for aflatoxins and ochratoxin A contamination

These have been successfully accomplished. In addition, aflatoxin mimotopes were selected from the random phage-displayed peptide libraries. The following conclusions are drawn from this study.

- As a result of application of a modified immunization schedule high titreed polyclonal antibodies were produced for aflatoxins and ochratoxin A that

could be used at dilutions exceeding 1: 80,000 in an indirect and direct competitive ELISA.

Application of a novel immunization schedule resulted in the selection of 10 hybridomas from a single fusion, of which one antibody was highly specific for B1, four antibodies reacted equally strongly with B1, G1 and weakly with B2. Another four reacted strongly with B1, G1 and B2. Interestingly all the 10 antibodies showed little or no cross-reaction with G2. This wide variation in the specificities and the number of clones produced in a single fusion has so far not been reported to occur.

Using phage-display technology, twenty four clones were selected that produced phages that bound specifically to MAbs. This is the first report on the selection of mimotopes for aflatoxins and these mimotope phages were used in an ELISA format for assaying aflatoxin concentrations. The results demonstrated the potential for using these peptides in aflatoxin immunoassays.

The immunological assays developed are simple and safe to perform and are robust and cost-effective to carry out on a wide scale in a variety of laboratory circumstances and on a range of agricultural commodities. This is the first time PNC enzyme could be used in aflatoxin ELISA, thus making available an inexpensive method for aflatoxin estimation.

Analysis of selected spices in India for ochratoxin A contamination showed that the contamination occurred at non permissible levels in chillies, black pepper, turmeric and coriander. It is noteworthy that the chillies and turmeric, the most widely used spices in Indian cooking, contained high levels of OA. This is the first record in India for the occurrence of OA in these spices.

Analysis of agricultural commodities intended for incorporation into chicken feed showed that 38% and 6% of the samples contained aflatoxins and

ochratoxin A, respectively. This results confirming the importance of ingredients prior to incorporating them in mixed feeds.

8.3. Scope for further work

- Use of peptide libraries as sources of reagents in immunoassays could be extended to other mycotoxins.

- The study on aflatoxins and ochratoxin A contamination in spices and poultry feeds clearly showed that they could harbour aflatoxins and ochratoxin A at levels, beyond those permitted by public health authorities and therefore emphasizes the need for surveillance of these toxins in foods and feeds. Development of strategies which can lead to minimize their contamination are vital to reduce risks due to these toxins.

- Immense potential exists for attracting funds for research aimed at reducing of mycotoxin contamination in foods and feeds.

Chapter 9

References

REFERENCES

Adhikari, M., Ramjee, G., and Berjak, P. (1994). Aflatoxin, kwashirokar and morbidity. *Natural Toxins* 2(1): 1-3.

Anon (1962). Progress in cancer research: Potent liver toxin and carcinogen produced in groundnuts as a result of infection with the fungus *Aspergillus flavus*. *British Medical Journal* 2 (5298), 172-174.

Austwick, P. K. C. (1978). Mycotoxicoses in poultry in mycotoxic fungi mycotoxins, mycotoxicoses. An encyclopaedic handbook Volume 2 *'Mycotoxicoses of domestic laboratory animals, poultry and aquatic invertebrates and vertebrates*. Wyllie T D and More house L G, eds. 279-301. Marcel-Bekker Inc., New York and Basel.

Bennet, G. A., Richard, J. L. (1994). Fate and distribution of Fusarium Mycotoxins during processing of contaminated grains. *Proceedings of Distillers Feed Research Conference*, New Orleans, LA, pp 23-29.

Betina, V. (1989). Review: Chromatographic methods as tools in the field of mycotoxins. *Journal of Chromatography* 477:187.

Bhat, R. V., and Nageshwara Rao. (1989). Human and animal mycotoxicoses. *Advances in Toxicology and Environmental Health* 159-175.

Blount, W. P. (1961). Turkey "X" disease. Turkeys (Journal of British Turkey Federation), 9 (2): 52, 55-58, 61.

Bosch, F. X. and Peers, F. (1991). Aflatoxins: data on human carcinogenic risk. In relevance to human cancer of N-Nitroso compounds. In *Tobacco and Mycotoxins*, ed. J.K. O'Neill, J. Chen. and H. Barstch, pp. 48-53. International Agency for Research on Cancer, Lyon, France.

Bressac, B., Kew, M., Wands, J., and Ozturk M. (1991). Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* 350: 429-431.

Busby, W. F. Jr. and Wogan, G. N. (1979). Food-borne mycotoxins and alimentary mycotoxicoses. In *Food-borne infections and Intoxications*, 2nd ed., H. Riemann and F. L. Bryan, pp. 519-610. Academic Press, New York.

Butler, W. H. (1974). Aflatoxins. In *Mycotoxins*, ed I.F.H. Purchase, pp. 1-28. Elsevier Scientific Publishing Co., New York.

Campbell, T. C., and Stoloff, L. (1974). Implications of mycotoxins for human Health. *Journal of Agricultural Food Chemistry* 22, 1006-1015.

Campbell, T. C., Davies, N. D. (1969). Aflatoxin formation by Aspergillus flavus. In L.L Goldblatt (Ed), *Aflatoxin Academic Press*, New York, pp 13-54.

Candlish, A. A. G., Smith, J. E. and Stimson, W. H. (1990). Aflatoxin monoclonals: academic development to commercial production. *Letters in Applied Microbiology* 10, 167-169.

Candlish, A. A. G., Stimson, W. H., Smith, J. E. (1988). Determination of Ochratoxin A by monoclonal antibody based enzyme immunoassay. *Journal of the Association of Official Analytical Chemists* 71, 961-964.

Candlish, A.A.G., Stimson, W. H., Smith, J. E. (1986). A monoclonal antibody to ochratoxin A. *Letters in Applied Microbiology* 3: 9-11.

Candlish, A. A. G., Stimson, W. H. and Smith, J. E. (1985). A monoclonal antibody to aflatoxin B1: detection of the mycotoxin by enzyme immunoassay. *Letters in Applied Microbiology* 1, 57-61.

Ceovic, S., Plestina, R., Miletic-Medved, M., Stavljenic, A., Mitar, J., and Vukelic, M. (1991). Epidemiological aspects of Balkan endemic nephropathy in a typical focus in Yugoslavia. IARC Sci. Publ. (115): 5-10.

Chiba, J., Kanji, H., Kawamura O., Ohi, K., Moroora, Y., and Ueno,Y. (1985). *Proceedings of the Japanese Association of Mycotoxicologists* 21, 28-29.

Chu, F. S. and Uneo I. (1977). Production of antibody against aflatoxin B1. *Applied and Environmental Microbiology* 33, 1125-1128.

Chu, F.S. (1978). Mycotoxin problems in indigenous fermented foods and new methods for mycotoxin analysis. Handbook of indigenous fermented foods (K H Stein Kraus, ed.) Marcel Dekker, New York pp 637-656.

Chu, F. S. (1986). Immunoassays for mycotoxins. In "Modern methods in the analysis and structural elucidation of mycotoxins", pp 207-237. Edited by R. J. Cole. Academic Press: New York.

Chu, F. S., Fan, T. S. L., Zhang, Guang-Shi, Xu, Yi-Chun, Faust, S., and McMohan, P. L. (1987). Improved enzyme-linked immunosorbant assay for aflatoxin B1 in agricultural commodities. *Journal of the Association of Official Analytical Chemists* 70, 854-857.

Chu, F.S. (1995). Mycotoxin analysis, In Analysing food for nutrition labelling and hazardous contaminats edited by Ike J. Jeon and William , Marcel Dekker, Inc.

Cortese, R., Felici, F., Galfre, G., Luzzago, A., Monaci, P. and Nicosia, A. (1994). Epitope discovery using peptide libraries displayed on phage. *Trends in Biotechnology* 12:262-267.

Cwirla, S. E., Peters, E. A., Barrett, R. W. and Dower, W. J. (1990). Peptides on phage: a vast library of peptides for identifying ligands. *Proceedings of the*

National Academy of Sciences of the United States of America 87:6378-6382.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of nucleotide sequence analysis program for the VAX. *Nucleic Acids Research* 12, 387-395.

Devi, K. T., Mayo, M. A., Reddy, K. L. N., Delfosse, P., Gopal Reddy., Reddy, S. V., Reddy, D. V. R. (1999). Production and characterization of monoclonal antibodies for aflatoxin B1. *Letters in Applied Microbiology* 29, 284-288.

Devlin, J. J., Panganiban, L. C., and Devlin, P. E. (1990). Random peptide libraries: A source of specific protein binding molecules. *Science* 249:404-406.

Diener, U. L., and Davis, N. D. (1969). Aflatoxin formation by Aspergillus flavus in L.L Goldblatt (Ed) *Aflatoxin, Academic Press,* New York, pp 13-54.

Doster, R. C., Sinnhuber, R. O. (1972). Comparative rates of hydrolysis of ochratoxins A and B in vitro. *Food and Cosmetics Toxicology* 10(3):389-94.

Dusold, L. R., Pohland, A. E., Dreifuss, P. A., and Sphon, J. A. (1978). Mycotoxins Mass spectral Data Bank, *Association of Official Analytical Chemists,* Arlington, VA.

Dvorackova, I., Kusak, V., Vesley, D., Vesela, J., and Nesmidal, P. (1977). Aflatoxin and encephalopathy with fatty degeneration of viscera (Reye). *Annales de la Nutrition et de L' Alimentation* 31, 977-990.

Galfre, G., Milstein, C. (1981). Preparation of monoclonal antibodies: strategies and procedures. *Methods in Enzymology* 73(Pt B): 3-46.

Genstat 5 Committee (1987). Genstat 5 Reference Manual. Clarendon Press, Oxford. 796 pp.

Goldblatt, L. A. (1969). ed. Aflatoxins, Scientific background, control and implication. Academic press, New York and London.

Groopman, J D., Haugen, A., Goodrich, G.R., Wogan, G.N., and Harris, C.C. (1982). Quantitation of aflatoxin B1 modified DNA using monoclonal antibodies. *Cancer Research* 42, 3120-3124.

Hald, B., and Krogh, P. (1972). Symp. Intern. Union of Pure and applied Chemistry, Aug 21-22, Kungalr, Sweden (Abstract)

Harris, A. L. (1991). Cancer genes. Telling changes of base. *Nature* (6317): 377-378.

Hayes, A. W., Hood, R. D., Lee. H. L. (1974). Teratogenic effects of ochratoxin A in mice. *Teratology* 9 (1): 93-7.

Heathcote, J.G., and Hibbert, J. R. (1978). *Aflatoxins. chemical and biological aspects developments in food Science-* I Elsevier Scientific Publishing company, Amsterdam, Oxford, New York pp 30-52.

Hefle, S. L., and Chu, F. S. (1990). Production and characterization of monoclonal antibodies cross-reactive with major aflatoxins. *Food and Agricultural Immunology* 2, 181-188.

Hesseltine, C. W., Shotwell, O. L., Ellis, J. J. and Stubblefield, R. D. (1966). Aflatoxin formation by *Aspergillus flavus*. *Bacteriological Review* 30(4): 795-805.

Hesseltine, C. W., Vandergraft, E. E., Fennel, D. I., Smith, M. I., Shotwell, O. L. (1972). *Aspergillus* as ochratoxin producers. *Mycologia* 64 (3): 539-550.

Hobbs, H. A., Reddy, D. V. R., Rajeshwari, R and Reddy, A. S. (1987). Use of direct antigen coating and protein A coating ELISA procedures for detection of three peanut viruses. *Plant Disease* 71, 747-749.

Hoess, R., Brinkmann, U., Handel, T. and Pastan, I. (1993). Identification of a peptide, which binds to the carbohydrate-specific monoclonal antibody B3. *Gene* 128, 43-49.

Hohler, D. (1998). Ochratoxin A in food and feed: Occurrence, legislation and mode of action. *Z Ernahrungswiss* 37, 2-12.

Hsu, I. C., Metcalf, R. A., Sun, T., Welsh, J. A., Wang, N. J., and Harris, C. C. (1991). Mutational hot spot in the P53 gene in human hepatocellular carcinomas. *Nature* 350: 427-428.

IARC (1993). IARC Monographs on the evaluation of carcinogenic risks to humans. V.56. Some naturally occurring substances: Food items and constituents, Heterocyclic aromatic amines and mycotoxins. Lyon, France.

Joffe, A. Z. (1971). *Alimentary toxic Aleukia.* In microbial toxins, vol VII Edited by S. Kadis, A. Ceigler and S.J Ail. pp 139-189. New York. Academic press.

Katiyar, S., Thakur, V., Gupta, R. C., Sarin, S. K. and Das, B.C. (2000). P53 tumor suppressor gene mutations in hepatocellular carcinoma patients in India. *Cancer* 88: 1565-73.

Katz, B. A. (1997). Structural and mechanistic determinants of affinity and specificity of ligands discovered or engineered by phage display. *Annual Review Biophys. Biomol. Struct.* 26:27-45.

Kawamura, O., Nagayama, S., Sato, S., Ahtani, K., Uneo, I., and Ueno, Y. (1988). A monoclonal antibody-based enzyme-linked immunosorbant assay for aflatoxin B1 in peanut products. *Mycotoxin Research* 4, 71-87.

Krishnamachari, K. A. V. R., Bhat, R. V., Nagarajan, V., Tilak, T. B. G. (1975). Hepatitis due to aflatoxicosis – An outbreak in Western India. *Lancet* 1061 – 1062.

Krogh, P., Hald, B., Pederson, E. J. (1973). Occurrence of ochratoxin A and citrinin in cereals associated with mycotoxin porcine nephropathy. *Acta Pathol. Microbiol. Scand.*, [B] 81(6). 689-695.

Krogh, P. (1974). Abstracts of the 2nd International Union of Pure and Applied chemistry Sponsored International symposium Mycotoxins in Food, Pulaway, Poland, July 23-25th.

Kuiper-Goodman, T., Scott, P. M. (1989). Risk assessment of the mycotoxin ochratoxin A. *Biomedical and Environmental Sciences* 2, 179-248.

Lai, M., Semeniuk, G., Hesseltine, C. W. (1970). Conditions for producing ochratoxin A by Aspergillus species in a synthetic medium. *Applied Microbiology* 19(3): 542-544.

Lee, S. C., and Chu, F. S. (1984). Enzyme linked immunosorbent assay of ochratoxin A in wheat. *Journal of the Association of Official Analytical Chemists* 67: 45-49.

Lubet, M. T., Olson, D. F., Yang, G., Ting, R and Steuen, A. (1983). Use of a monoclonal antibody to detect aflatoxin B1 and M1 in enzyme immunoassay. *Abstracts of the AOAC Annual Meeting* 97th. Washington, D.C.p 71.

Mackenzie, D. W. R. (1988). Aspergillus in man. *In Aspergillus and Aspergillosis* (eds H. Vanden Bosche DWR Mackenzie and G Canwengergh) pp 1-8 Plenum Press, New York.

Madhavan, T. V., and Gopalan, C. (1968). The effect of dietary protein on carcinogenesis of aflatoxin. *Arch. Pathol* 85: 133-137.

Madhyastha, S. (1985). Mycotoxic fungi and mycotoxins in major spices with special reference to *Piper nigrum* L. *Ph.D. thesis, University of Mysore, Mysore, India.*

Marlow, S. J., Handa, A. K., (1987). Immuno slot-blot assay using a membrane which covalently binds protein, *Journal of Immunological Methods* 101, 133-139.

Morgan, M. R. A., McNerney, R., Chan, H. W. S. (1983). Enzyme-linked immunosorbant assay of ochratoxin A in barley. *Journal of the Association of Official Analytical Chemists* 66, 1481-1484.

Munro, C., Scott, P. M., Moodie, C. A., Willes, R. F. (1973). Ochratoxin A-occurrence and toxicity. *J. Am. Vet. Med. Assoc.* 163(11):1269-1273.

Mycotoxins in human nutrition and health (1994). Published by agro-industrial research division; science, research and development, EUR 16048 EN (XII)

Nesheim, S., Hardin, N., Francis, O., Langham, W. (1971). 85th *Annual meeting. Association of Official Analytical Chemists* Oct 11-14th (Abst).

Parmley, S. F., and Smith, G. P. (1988). Antibody-selectable filamentous fd phage vectors: Affinity purification of target genes. *Gene* 73:305-318.

Patterson, D. S. P., and Allcroft, R. (1970). Metabolism of aflatoxin in susceptible and resistant animal species. *Food and Cosmetics Toxicology* 8: 43-53.

Peers, F. G., and Linsell, C. A., (1973). Dietary aflatoxins and liver cancer – A population based study in Kenya. *Britain Journal of Cancer* 27: 473-484.

Pestka, J. J. (1980). Enhanced surveillance of food-borne mycotoxins by immunochemical assay. *Journal of the Association of Official Analytical Chemists* 71, 1075-1081.

Plattner, R. D. (1986). Detection of fumonisins production in *Fusarium moniliforme* cultures by HPLC with electro spray M S and evaporative light scattering detectors. *Natural Toxins* 3: 294.

Purchase, I. F., and Theron, J. J. (1968). The acute toxicity of ochratoxin A to rats *Food and Cosmetics Toxicology* 6(4): 479-483.

Raisuddin, S. Singh K. P., Zaidi, S. I., Paul, B. N., Ray, P. K. (1993). Immunosuppressive effects of aflatoxin in growing rats. *Mycopathologia* 124 (3): 198-194.

Ram, B. P., Hart, L. P., Shotwell, O. L., and Pestka J. J. (1986). Enzyme linked immunosorbent assay of aflatoxin B1 in naturally contaminated corn and cottonseed. *Journal of the Association of Official Analytical Chemists* 69, 904-907.

Ramakrishna, N., Lacey, J., Candlish, A. A. G., Smith, J. E., Goodbrand, I. A. (1990). Monoclonal antibody based enzyme linked immunosorbant assay of aflatoxin B1, T-2 toxin and ochratoxin A in barley. *Journal of the Association of Official Analytical Chemists* 73, 71-76.

Reddy, S. V., Thirumala-Devi, K., Uma Reddy., Delfosse, P., Reddy, D. V. R. (1998). Quantitative estimation of aflatoxins in chilli samples using indirect competitive enzyme linked immunosorbant assay (ELISA), paper presented at the 1st workshop on "Natural Toxins" organized by Dr Tu at Colorado State University, fort Collins, Colorado, USA and Dr Tatsuo Higa at the University of Ryukens, Okinawa, Japan, held in Bangkok, Thailand from 27 Sept to 1 October.

Rodricks, J. V., and Eppley, R. M. (1974). *Stachybotrys and Stachybotrytoxicosis.* In mycotoxins edited by IFH Purchase pp 181-197. Amsterdam: Elsevier.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sargeant, K., O'Kelly, J., Carnaghan, R. B. A and Allcroft, R. (1961). *Veterinary Record* 73:1219-1223.

Scott, J. K. and Smith, G. P. (1990). Searching for peptide ligands with an epitope library. *Science* 249, 386-390.

Scott, J. K., Loganathan, D., Eusley, R. B., Gong, X. and Goldstein, I. J. (1992). A family of concanavalin A-binding peptides from a hexapeptide epitope library. *Proceedings of the National Academy of Sciences (USA)* 89, 5398-5402.

Scott, P. M. (1981). Analysis of mycotoxins other than aflatoxins in food stuffs. Thin layer chromatography: Quantitative Environment and clinical applications (J. C. Touchstone and D. Rogers, eds). Wiley Interscience, New York .

Scott, P. M., Lawrence, G. A., Van Walbeek, W. (1970). Detection of mycotoxins by thin-layer chromatography: application to screening of fungal extracts. *Applied Microbiology* 20(5): 839-842.

Scott, P. M., van Walbeek, W., Kennedy, B., Anyeti, D. (1972). Mycotoxins (ochratoxin A, citrinin, and sterigmatocystin) and toxigenic fungi in grains and other agricultural products. *Journal of Agricultural Food Chemistry* 20(6): 1103-1109.

Serck-Hanssen, A., (1970). Aflatoxin induced fatal hepatitis. A case report from Uganda. *Archives of Environment Health* 20:729-731.

Shank R. C, Bhamarapravati, N, Gordon J. E., and Wogan, G. N. (1972a). Dietary aflatoxins and human liver cancer. IV Incidence of primary liver cancer in two municipal populations of Thailand. *Food and Cosmetics Toxicology* 10: 171-179.

Shank, R. C., Gordon, J. E., Wogan, G. N., Nondasuta, A., and Subhamani, B. (1972b). Dietary aflatoxins and human liver cancer. III Field survey of rural Thai families for ingested aflatoxins. *Food and Cosmetics Toxicology* 10: 71-84.

Shank, R. C., Wogan, G. N., Gibson, J. B., and Nondasuta, A. (1972c). Dietary aflatoxin and human liver cancer II Aflatoxin in market food and food stuffs in Thailand and Hongkong. *Food and Cosmetics Toxicology* 10: 61-69.

Sinnhuber, R. O., Lee, D. J., Wales, J. H., Ayres, J. L. (1968). Dietary factors and hepatoma in rainbow trout (Salmo gairdneri). II. Cocarcinogenes by cyclopropenoid fatty acids and the effect of gossypol and altered lipids on aflatoxin-induced liver cancer. *J. Natl Cancer Inst.* 41 (6): 1293-1301.

Smith J. E. and Moss, M. O. (1985). Mycotoxins: Formation, Analysis and Significance. John Wiley & Sons, London.

Smith, G. P. (1991). Surface presentation of protein epitopes using bacteriophage expression systems. *Current Opinions in Biotechnology* 2, 668-673.

Stoloff, L., Van Egmond, H. P., and Park, D. L. (1991). Rationales for the establishment of limits and regulations for mycotoxins. *Food additives and contaminants.* 8: 213-222.

Sun, T., Wu, Y and Wu, S., (1983). Monoclonal antibody against aflatoxin B1 and its potential applications. *Chinese Journal of Oncology* 5, 401-405.

Torrance, L. (1999). Antibodies as diagnostic tools in plant pathology: summary and outlook. In "Recombinant Antibodies: applications in plant science and plant pathology" pp. 107-110. Edited by K. Harper and A. Ziegler: Taylor and Francis: London and Philadelphia.

Trucksess, M. W., Stack, M. E., Nesheim, S., Page, S. W., Albert, R .H., Hansen, T. J., and Donahue (1991). Immunoaffinity column coupled with solution flouorometry or liquid chromatography- post column derivitization for determination of aflatoxins in corn, peanuts, and peanut butter: Collaborative study. *Journal of the Association of Official Analytical Chemists* 74: 81-88.

Trucksess, M. W., Wood, G. E. (1994). Recent methods of analysis for aflatoxins in foods and feeds. In: *The Toxicology of Aflatoxins: Human Health, Veterinary and Agricultural Significance*. DL Eaton, JD Groopman, eds., San Diego: Academic Press. p409.

Tsang, V. C. W., Greene, R. M., Pilcher, J. B. (1995). Optimization of the covalent conjugating procedure ($NaIO_4$) of horseradish peroxidase to antibodies for use in enzyme-linked immunosorbant assay. *Journal of Immunoassay* 16, 395-418.

Tung, T. C., and Ling, K. H. (1968). Study on aflatoxin in food stuffs in Taiwan. *Journal of Vitaminol.* 14: 48-52.

Van der Merwe, K. J., Steyn, P. S., Fourie. L. (1965). Mycotoxins. II. The constitution of ochratoxins A, B and C, metabolites of *Aspergillus ochraceous* Wilh. *Journal of Chemical Society.* 7083-7088.

Van Egmond, H. P. (1991). Worldwide regulations for ochratoxin A. In *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours* ed. Castegnard, M., Plestina, R., Dirheimer, G., Chernozansky, J. N. and Bartsch, H. Lyon: International Agency for Research on Cancer. 331-336.

Van Rensberg, S. J, Vander Watt, J. J., Purchase, I. F. H., Coutinho, Z. P., and Markham, R. (1974). Primary liver cancer rate and aflatoxin intake in a high cancer area. *South African Medical Journal* 48: 2508 a –2508 d

Van Walbeek., Scott, P. M., Thatcher, F. S. (1968). *Canadian Journal of Microbiology* 14, 131.

Verret M. J. (1974). unpublished data.

Vesonder, R., (1991). Aspergillus flavus aflatoxins B1, B2 and M1 in corn associated with equine death. *Arch Environ.Contam Toxicol* 20: 151-153.

Vukelic, M., Sostaric, B., and Fuchs, R. (1991). Balkan endemic nephropathy in Croatia. In: Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours. M. Castegnaro, R. Plestina, G. Dirheimer, I.N. Chernozemsky, and H. Bartch (Eds). IARC, Lyon, pp. 37-42.

Wang, G., Mu, J., Wang, D., Lu, C., and Ye, M. (1995). Production and characterization of monoclonal antibody against aflatoxin B1. *Hua His I Ko Ta Hsueh Hsueh Pao* 26 (3): 275-8.

Ward, C. M., Wilkinson, A. P., Bramham, S., Lee, H. A., Chan, H. W., Butcher, G. W., Hutchings, H., and Morgan, M. R. A. (1990). Production and characterisation of polyclonal and monoclonal antibodies against aflatoxin B1 oxime-BSA in an enzyme-linked immunosorbant assay. *Mycotoxin Research* 6, 73-83.

Weber, P. C., Pantoliao, M. W., and Thompson, L.D. (1992). Crystal structure and ligand-binding studies of a screened peptide complexed with streptavidin. *Biochemistry* 31:9350-9354.

Wogan, G. N. (1977). Mycotoxins and naturally occurring carcinogens in environmental cancer. *Advances in modern toxicology.* Vol. 3. Kray bill, H

F and Mehlman M A eds. P263-290. Hemisphere publishing corp. Washington, London, John Wiley and Sons, New York.

Yuan, Q., Pestka, J. J., Hespenheide, B. M., Kuhn, K. A., Linz, J. E. and Hart, L. P. (1999). Identification of mimotope peptides which bind to the mycotoxins deoxynivalenol-specific monoclonal antibody. *Applied and Environmental Microbiology* 65, 3279-3286.

Ziegler, A., Mayo, M.A. and Torrance, L. (1998). Synthetic antigen from a peptide library can be an effective positive control in immunoassays for the detection and identification of two Gemini viruses. *Phytopathology* 88, 1302-1305.

Publications During PhD Research

Journal articles published or submitted:

1. **Thirumala Devi K.,** M A Mayo., K L N Reddy., P Delfosse., Gopal Reddy., S V Reddy and D V R Reddy (**1999**) Production and characterization of monoclonal antibodies for aflatoxin B1, *Letters in Applied Microbiology* 29, 284-288

2. **Thirumala-Devi K.,** M A Mayo., P Delfosse., Gopal Reddy., S V Reddy and DVR Reddy (**2000**) Production of polyclonal antibodies for ochratoxin A and its detection in chillies by ELISA, *Journal of Agricultural and Food Chemistry* 48, 5079-5082

3. **Thirumala-Devi K** and D V R Reddy (**2000**) Safe to eat or why chickens die and human beings suffer from liver cancer: Towards development of cost effective technologies for the estimation of mycotoxins in foods and feeds. www.cgiar.org/icrisat (GREP Website)

4. **Thirumala-Devi K.,** J Miller., Gopal Reddy., D V R Reddy., and M A Mayo (**2001**) Identification of aflatoxin mimotopes from phage displayed peptide libraries. *Journal of Applied Microbiology* (In press)

5. **Thirumala-Devi K.,** M A Mayo., Gopal Reddy and D V R Reddy (**2001**) Occurrence of ochratoxin A in black pepper, coriander, ginger and turmeric, *Food Additives and Contaminants* (In press)

6. Reddy S V., D Kiran Mayi., M Uma Reddy., **K Thirumala-Devi** and D V R Reddy (**2001**) Aflatoxins in different grades of chillies (*Capsicum annum* L.) in India as determined by indirect competitive ELISA, *Food Additives and Contaminants* (In press)

7. **Thirumala-Devi K.,** J.S. Miller., Gopal Reddy., D V R Reddy and M A Mayo(**2001**) Aflatoxin mimotopes from phage displayed peptide libraries. *Indian Journal of plant protection* (In press)

Journal articles in preparation:

8. **Thirumala-Devi K.,** M A Mayo., Gopal Reddy and D V R Reddy (**2001**) Rapid and cost effective direct competitive ELISA for the quantitative estimation of aflatoxins and ochratoxin A

9. **Thirumala-Devi K.,** M A Mayo., Gopal Reddy and D V R Reddy (**2001**) Development of one step dip strip test for the detection of aflatoxins

10. **Thirumala-Devi K.,** M A Mayo., Gopal Reddy and D V R Reddy (**2001**) Occurrence of aflatoxins and ochratoxin A in poultry feeds (In preparation)

Conference proceedings:

11. **Thirumala-Devi K.,** S V Reddy., K L N Reddy., P Delfosse., M A Mayo., and D V R Reddy (**1998**). Production of monoclonal antibodies for aflatoxin B, paper presented at the 1st workshop on "Natural Toxins" organized by Dr Tu at Colorado State University, fort Collins, Colorado, USA and Dr Tatsuo Higa at the University of Ryukens, Okinawa, Japan, held in Bangkok, Thailand from 27 Sept to 1 October.

12. Reddy S V., **K Thirumala-Devi.,** Uma Reddy., P Delfosse and D V R Reddy (**1998**). Quantitative estimation of aflatoxins in chilli samples using indirect competitive enzyme linked immunosorbant assay (ELISA), paper presented at the 1st workshop on "Natural Toxins" organized by Dr Tu at Colorado State University, fort Collins, Colorado, USA and Dr Tatsuo Higa at the University of Ryukens, Okinawa, Japan, held in Bangkok, Thailand from 27 Sept to 1 October.

13. DVR Reddy., K K Sharma, R C Nageswara Rao., S V Reddy., **K Thirumala-Devi.,** H D Upadhyay, S N Nigam, N Mallikarjuna, M A Mayo., K L N Reddy and P J Bramel Cox (**1999**) Current research on aflatoxin detection and genetic transformation in peanut at ICRISAT. *Australian centre for international agricultural research (ACIAR proceedings No.89* Elimination of aflatoxin contamination in peanut (Eds. Ralf G. Dietzgen) pp 32-33.

14. **Thirumala-Devi K.,** M A Mayo., Gopal Reddy., S V Reddy and D V R Reddy (**2000**). Recent advances in mycotoxin diagnostics. *Proceedings of consultative group meeting on technical and institutional options for sorghum grain mold management* 18-19 May 2000, ICRISAT, Patancheru.

15. **Thirumala-Devi K.,** M A Mayo., Gopal Reddy., S V Reddy and D V R Reddy (**2000**) Immunodiagnostics for the estimation of aflatoxins and ochratoxin A in foods and feeds, In the abstracts of International Conference on Microbial Biotechnology, Trade and Public Policy, organized by the department of microbiology, Osmania University, Hyderabad, July 15-17.

16. **Thirumala-Devi K.,** M A Mayo., Gopal Reddy., S V Reddy and D V R Reddy (**2000**) Advances in immunological methods for quantitative estimation of mycotoxins, paper presented at the International Conference on Microbial Biotechnology, Trade and Public Policy, organized by the department of microbiology, Osmania University, Hyderabad, July 15-17.

17. JS Miller., **K Thirumala Devi.,** DVR Reddy and MA Mayo (**2000**) Mimotopes for use in ELISA to detect aflatoxin contamination AAB meeting, Dundee, Scotland, UK.

18. D V R Reddy[1], **K Thirumala-Devi**, S Veera Reddy, Farid Waliyar, M A Mayo, K Rama Devi, R Ortiz and J M Lenné (**2000**) Estimation of aflatoxin

levels in selected foods and feeds in India: A step towards the assessment of risk to the health of humans and livestock. Paper presented in the FAO-CIRAD workshop held at Montpellier in December 11-16.

Letters in Applied Microbiology 1999 29 264–268

Production and characterization of monoclonal antibodies for aflatoxin B1

K. T. Devi[1], M.A. Mayo[2], K.L.N. Reddy[2], P. Delfosse[3], G. Reddy[1], S.V. Reddy[1] and D.V.R. Reddy[1]

[1]*International Crops Research Institute for the Semi Arid Tropics Patancheru India* [2]*Scottish Crop Research Institute Dundee UK and* [3]*Osmania University Hyderabad India*

2248/99 Received 28 June 1999 accepted 30 June 1999

K T DEVI M A MAYO K L N REDDY P DELFOSSE G REDDY S V REDDY AND D V R REDDY 1999 Hybridomas that secreted antibodies for aflatoxin B1 were selected using two immunization protocols referred to as A and B Protocol A is a standard immunization method and resulted in the selection of only two clones that produced monoclonal antibodies against aflatoxin B1 In protocol B a unique immunization schedule which resulted in the generation of 10 hybridomas is described Of the 10, one antibody was highly specific to B1, four antibodies reacted equally strongly with B1, G1 and weakly with B2 Another four reacted strongly with B1 and weakly with B2 and G1 One clone reacted equally strongly with B1, G1 and B2 Interestingly all the 10 antibodies showed little or no cross-reaction with G2

INTRODUCTION

Agricultural products are often contaminated with fungi that can produce toxic metabolites referred to as mycotoxins Among these, aflatoxins have assumed economic importance because of their influence on the health of human beings and livestock, and on the marketability of agricultural products Aflatoxins are potent carcinogenic and immunosuppressive agents In most developing countries limited or no facilities exist for monitoring these toxins in foods or feeds They are based on physicochemical methods such as TLC and to a limited extent high performance liquid chromatography (HPLC) These methods are laborious and require expensive instrumentation and clean-up of the samples Immunological methods which are cost effective and adaptable to the situation in developing countries have been reported for the estimation of aflatoxins using polyclonal antibodies (Chu and Uneo 1977, Anjaiah *et al* 1989, Zhang and Chu 1989) However, commercial kits using immunological methods are expensive, and in many countries can be problematic to import Therefore, the main aim of this investigation was to generate highly sensitive antibodies for the precise analysis of samples using hybridoma technology They are preferred over polyclonal antibodies because their affinity and specificity do not vary from bleed to bleed and additionally, unlike polyclonal antibodies, do not react with other aflatoxin analogues Most of the reports that have appeared so far on the production of monoclonal antibodies for aflatoxins have involved standard immunization protocols which include four intraperitoneal injections at weekly intervals (Candlish, Stimson and Smith 1985) or monthly intervals (Ward *et al* 1990) with a final immunization 3 days prior to fusion The numbers of clones obtained in each fusion have varied from one to a maximum of seven This paper describes the production and characterization of monoclonal antibodies with high sensitivity and varying specificities for aflatoxins using a novel immunization schedule

Correspondence Dr D V R Reddy Principal Scientist GREP International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Patancheru 502324, India (e-mail d.reddy@cgiar org)

MATERIALS AND METHODS

Production of monoclonal antibodies

Immunization The immunogen used was AFB1 BSA conjugate (250 μg) dissolved in 250 μl of 0 01 mol/L PBS and emulsified with an equal volume of Freund's adjuvant (Sigma) This was injected intraperitoneally into each of several 6-week-old female Balb/c mice The first injection was given using Freund's complete adjuvant and the rest of the injections, except for the booster, were given with Freund's incomplete adjuvant No adjuvant was used for the booster injections After completing the immunization schedule,

approximately 5 μl of blood was drawn from each mouse after cutting the tip of the tail.

Initially four injections were given to each of four mice at 1-week intervals, followed after 3 weeks by a booster. The mouse that gave the highest titre was used for preparing monoclonal antibodies. This procedure is referred to as protocol 'A'. The remaining three mice were kept for 8 months without any immunization and a booster injection was given at the end of the eighth month. Sera from the three mice were tested two weeks after the booster injection. Again the mouse that gave the maximum titre was used for antibody preparation (referred to as protocol 'B').

Fusion and cloning. The spleen was removed aseptically and splenocytes were fused with cells of the Sp2/0-Ag14 murine myeloma cell line at a ratio of 1 : 10 in the presence of polyethylene glycol, molecular weight 1500 (Boehringer Mannheim cat.no.783641, Mannheim, Germany). After fusion, cells were suspended in Iscove's Modified Dulbecco's medium (IMDM) (Gibco cat. no. 12200-036) containing 20% foetal bovine serum (FBS) (Gibco cat. no. 263000-061), hypoxanthine, aminopterine and thymidine medium (Gibco cat. no. 31062-011) and added to 96-well microculture plates. After 12 days, culture supernatants from each well were assayed using indirect competitive ELISA (see below). Culture supernatants from the cells that gave an absorption value of over 3 as a difference between 0·4 and 400 ng ml^{-1} were transferred to 24-well microculture plates in IMDM containing 20% FBS, hypoxanthine and thymidine. Supernatants from 24-well culture plates were tested again and only those clones that maintained absorption values over 3 in ELISA tests were chosen for further selection. Cell suspensions from each well of the 24-well-cultured plate were diluted to give approximately one cell per well when distributed into a 96-well culture plate. The plates were examined for the presence of number of hybridomas. Those that contained a single hybridoma in each well were retained and were screened for neutralization titres.

Indirect competitive ELISA procedure for screening hybridomas. Microtitre plates (Maxi-sorp-Nunc) were used and at each step the plates were incubated for 1 h at 37 °C. Initially plates were coated with 150 ng ml^{-1} of AFB1-BSA in 0·2 mol/L carbonate coating buffer (150 μl/well, Hobbs *et al.* 1987). In the second step plates were filled with a suspension of 4% dried milk prepared in phosphate-buffered saline containing 0·05% Tween 20 (PBS-T). Aflatoxin B_1 (0·4 or 400 ng ml^{-1}) in 100 μl, were added to each well and mixed with 50 μl of tissue culture supernatant (TCS). Goat antimouse IgG conjugated to alkaline phosphatase (1 : 1000 dilution) was used to detect mouse antibodies. Substrate, *p*-nitrophenyl phosphate at 1 mg ml^{-1}, was allowed to develop for 1 h at room temperature and absorbance at 405 nm was read in an ELISA plate reader.

Characterization of antibodies

Determination of isotype and cross-reactivity. Commercially available ISO-2 kits from Sigma were used to determine the isotypes of the monoclonal antibodies produced by various hybridoma cell lines.

To evaluate the cross-reactivity of each of the monoclonal antibodies, it was essential to determine the optimum conditions for neutralization. These included coating antigen (aflatoxin B1-BSA) concentration and the dilution of the antibody required for neutralization. IgGs were extracted from tissue culture supernatants using 18% sodium sulphate (Hobbs *et al.* 1987). IgG concentration was determined spectrophotometrically and antibody titres were determined by the indirect competitive ELISA procedure as described. The optimum concentration required at each step to obtain the maximum sensitivity was determined by 50% displacement values of B/B_m, where B is the extinction of the well containing AFB1 and B_m is the extinction of the well without toxin, derived from the slope of calibration curves. Using these parameters various concentrations of the IgG (50 μl/well) from different monoclonal antibodies were added to 100 μl of AFB1 at concentrations ranging from 100 ng to 100 pg ml^{-1}. The protocols used for the characterization of antibody specificity were similar to those used to determine antibody titres except that in addition to B1, aflatoxin B2, G1 and G2 were also included for determining neutralization titres.

RESULTS

Immunization

The sera of mice immunized using protocol A showed poor antibody responses. Antibody was used at a dilution of 1 : 500. In contrast, high antibody titres, exceeding 1 : 20 000, were recorded from the sera of mice immunized by protocol B.

Fusion and cloning

The fusion efficiency (number of wells showing cell multiplication in each well of the 96-well plates) was 100%. In the first fusion, supernatants from cells derived from eight wells showed specific binding to AFB1. However, after two successive transfers only two retained the antibody activity. In the second fusion, 41 clones that secreted AFB1-specific antibodies were selected. After two successive transfers, 10 clones continued to give high neutralization titres. These clones were transferred to 25 cm^2 flasks and IgGs were

extracted from culture supernatants. IgG concentrations varied from 150 to 185 μg ml^{-1} of the culture supernatant.

Optimum aflatoxin and antibody activity for ELISA

An aflatoxin B1-BSA conjugate concentration of 125 ng ml^{-1} was found to be optimum for coating the plates. The optimum concentration of antibody for neutralization depended on the titre of antibody and it varied from 5 to 50 μg ml^{-1}.

Characterization of antibodies

The designation, data on isotypes, cross-reactivity and the minimal inhibition values for 10 monoclonal antibodies are presented in Table 1. The antibodies showed a range of cross-reactivity (Fig. 1) and could be classified broadly into three distinct groups. Group 1 comprised one monoclonal antibody, 10D5-1A11, that was highly specific for B1 and showed a weak cross-reaction to G1. Group 2 (13D1-1D9) contained antibodies that recognized B1, G1 and B2, with a weak cross-reaction with G2. The remaining clones that recognized B1 and G1 with equal efficiency were grouped in the category 3. Cell lines produced in the first fusion (protocol 'A') had a detection range from 10 to 100 ng ml^{-1} in contrast with that of the clones produced in the second fusion (protocol 'B') which gave values ranging from 0·001 to 1 ng ml^{-1}. One clone from the second fusion (10D5-1A11) is highly sensitive to aflatoxin B1 with a 50% inhibition at 0·0006 ng ml^{-1} (Fig. 2).

DISCUSSION

The main aim of generating monoclonal antibodies was to obtain antibodies with appropriate antigen affinity, cross-reactivity and sensitivity. The generation of monoclonal antibodies is largely dependent on the immunization schemes, fusion procedures, and stabilities of the hybrid clones. The first step is to obtain as many antigen-specific monoclonal antibodies as possible. When immunizations were done by conventional methods (Candlish, Smith and Stimson 1990; Wang *et al.* 1995), very few monoclonal antibodies were generated. Interestingly the same group of mice gave better immune response following a long resting period. This presumably resulted in the accumulation of B memory lymphocytes with high affinity to Ig receptors in the spleen, thereby enhancing the chance of selecting hybridomas. Booster injections have been given normally 3 days prior to removal of spleen for fusion (Candlish *et al.* 1990; Ward *et al.* 1990). In contrast, we gave boosters 18 days before fusion. This was attempted because booster injections given 3 days before fusion (protocol 1) resulted in the selection of only a few monoclonal antibodies. To our knowledge, this is the first time that a rest after initial immunization has been shown to contribute to enhanced generation of monoclonal antibodies. It is yet to be tested if this protocol will consistently lead to generation of antibodies with wide variation in affinity, when different antigens are used for immunization. Results are not presented in this communication because the affinity of these antibodies to the four aflatoxins are yet to be evaluated.

Table 1 Isotypes, cross-reactions and minimal inhibition observed with 10 monoclonal antibodies

Designation	Isotype*	Cross-reaction† (%)				Minimal inhibition‡ (ng ml^{-1})		
		B1	B2	G1	G2	B1	B2	G1
10D5-1A11	IgG_1	100	2	12	< 1	0·001		
5D8-2B1	IgG_1	100		110		1		1
13D1-1D9	IgG_1	100	2	100	< 1	0·01		0·01
5F2-1E8	IgG_1	100	12	100	3	0·1		0·1
3G7-1B8	IgG_1	100	22	100	< 1	0·1	1	0·1
11C8-1A8	IgG_{2b}	100	20	66	1	0·01		0·01
3F7-1B9	IgG_1	100	15	60	< 1	< 0·01		< 0·01
5H4-1B1	IgG_1	100	13	72	1	< 0·01		< 0·01
6G12-2B3	IgG_{2b}	100	7	80	< 1	< 0·01		< 0·01
6E12-1E5	IgG_1	100	60	75	5	0·1	0·1	0·1

*Determined using a commercial kit.

†Expressed as 50% displacement value of B/B_0 for aflatoxin B1 divided by the 50% displacement value for each of the aflatoxin under testing (see text).

‡Concentration of aflatoxin (ng ml^{-1}) required for first significant inhibition of binding of antibody to AFB1-BSA solid phase.

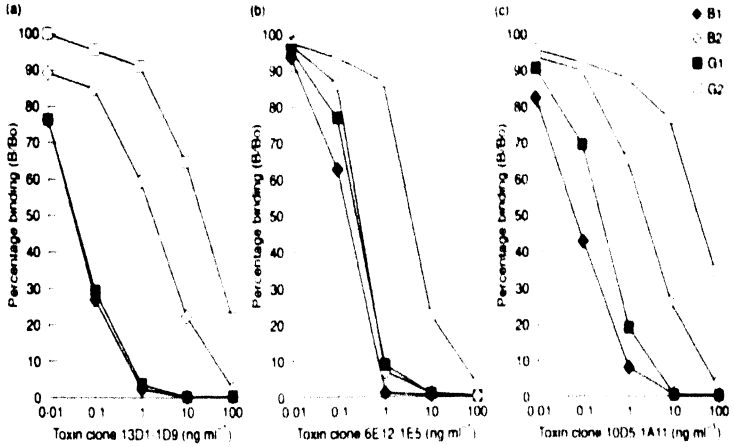


Fig. 1 Cross-reactivity of three groups of monoclonal antibodies to four major aflatoxins. Graphs plotted as percentage binding (B/Bo) against mass of toxin per millilitre (ng ml^{-1}). (a) Antibodies that recognize B1 and G1 with equal efficiency. (b) Antibodies that recognize B1, G1 and B2, with a weak cross-reaction to G2. (c) Antibody specific for B1 with varying degrees of cross-reaction to G1, B2 and G2.

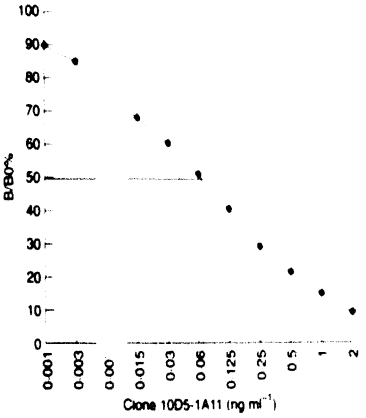


Fig. 2 Dose response curve of aflatoxin B1. Fifty percent inhibition value of AFB1 was 0·06 ng ml^{-1}.

Monoclonal antibodies produced by Candlish *et al.* (1990) were largely specific to B1 with poor cross-reaction to B2, G1 and G2 aflatoxins. However, antibodies reported by Kawamura *et al.* (1988) were highly specific to B1 and showed partial reaction to G1. On the other hand Hefle and Chu (1990) reported equal cross-reactivities with all four aflatoxins. The wide variation in the specificities of monoclonal antibodies obtained in this study for four aflatoxins has so far not been reported to occur in the products of a single fusion. The antibodies produced can be used either to estimate B1 alone or B1, B2 and G1. Since G2 is the least toxic of all the four aflatoxins, and is not known to occur widely (FAO 1990) in foods and feeds, this deficiency is unlikely to contribute to inaccurate estimation of total aflatoxin content. The clone 10D5 1A11 (Fig. 2) could detect as little as 1 pg ml^{-1} of AFB1. This is in contrast with the limits of 200 pg ml^{-1} reported by Candlish *et al.* (1985) and 10 pg ml^{-1} reported by Ward *et al.* (1990).

ACKNOWLEDGEMENTS

The financial support of the UK Department For International Development ([DFID] project number R7083) is gratefully acknowledged. We are grateful to Dr Anjali Karande, Department of Biochemistry, Indian Institute of Sciences, Bangalore, for her advice in the production of monoclonal antibodies. M. Mayo is supported by the Scottish Office Agriculture, Environment and Fisheries Department, UK. This paper was submitted as journal article number 2323 by ICRISAT.

REFERENCES

Anjaiah, V., Mehan, V.K., Jayanthi, S., Reddy, D.V.R. and

McDonald, D. (1989) Enzyme-linked immunosorbent assay (ELISA) for aflatoxin B1 estimation in groundnuts. ICRISAT (International Crops Research Institute for the Semi-Arid Tropics) 1989, pp. 183–189. In *Aflatoxin Contamination of Groundnut: Proceedings of the International Workshop*, 6–9 October 1987, ICRISAT Center, India. Patancheru, A.P. 502 324, India: ICRISAT.

Candlish, A.A.G., Smith, J.L. and Stimson, W.H. (1990) Aflatoxin monoclonals: academic development to commercial production. *Letters in Applied Microbiology* **10**, 167–169.

Candlish, A.A.G., Stimson, W.H. and Smith, J.E. (1985) A monoclonal antibody to aflatoxin B1: detection of the mycotoxin by enzyme immunoassay. *Letters in Applied Microbiology* **1**, 57–61.

Chu, F.S. and Ueno, I. (1977) Production of antibody against aflatoxin B1. *Applied and Environmental Microbiology* **33**, 1125–1128.

Food and Agriculture Organisation of United Nations (1990) *FAO Food and Nutrition Paper* **14/10**, 108.

Hefle, S.L. and Chu, F.S. (1990) Production and characterization of monoclonal antibodies cross-reactive with major aflatoxins. *Food and Agricultural Immunology* **2**, 181–188.

Hobbs, H.A., Reddy, D.V.R., Rajeshwari, R. and Reddy, A.S. (1987) Use of direct antigen coating and protein A coating ELISA procedures for detection of three peanut viruses. *Plant Disease* **71**, 747–749.

Kawamura, O., Nagayama, S., Sato, S., Ahtani, K., Ueno, I. and Ueno, Y. (1988) A monoclonal antibody-based enzyme-linked immunosorbent assay for aflatoxin B1 in peanut products. *Mycotoxin Research* **4**, 71–87.

Wang, G., Mu, L., Wang, D., Liu, C. and Ye, M. (1985) Production and characterization of monoclonal antibody against aflatoxin B1. *Hua Hsi I Ke Ta Hsueh Hsueh Pao* **26** (3), 275–278.

Ward, C.M., Wilkinson, A.P., Bramham, S., Lee, H.A., Chan, H.W., Butcher, G.W., Hutchings, H. and Morghan, M.R.A. (1990) Production and characterization of polyclonal and monoclonal antibodies against aflatoxin B1 oxime-BSA in an enzyme-linked immunosorbent assay. *Mycotoxin Research* **6**, 73–83.

Zhang, G. and Chu, F.S. (1989) Production and characterization of antibodies cross reactive with major aflatoxins. *Experientia* **45** (2), 182–184.

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

Journal of Agricultural and Food Chemistry®

Reprinted from
Volume 48, Number 10, Pages 5079–5082

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 to 100 ng 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 to 100 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 (Madhvastha, 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-AP 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. O-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.

10.1021/jf000049o CCC: $19.00
Published on Web 09/07/2000

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 anti-rabbit 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*/*B*0, where *B* is the extinction of the well containing OA and *B*0 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.

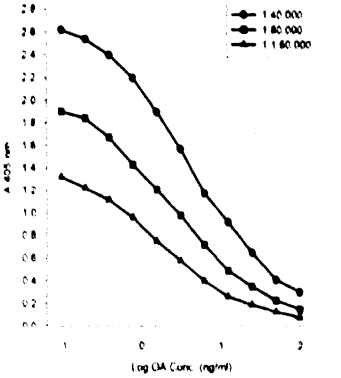


Figure 1. Standard curves for OA by indirect competitive ELISA at three different dilutions of antibody: 1:40,000 (●), 1:80,000 (■), 1:160,000 (▲).

RESULTS AND DISCUSSION

Production of Antibody. The protocol used for immunization gave an antibody titer of 1:132,000 at 4 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

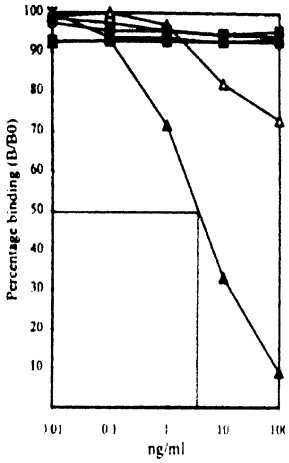


Figure 2. Cross reactivity of OA antiserum (▲) to OB (△), coumarin (■), 4-hydroxy coumarin (□), L-phenylalanine (•) and AFB1 (●).

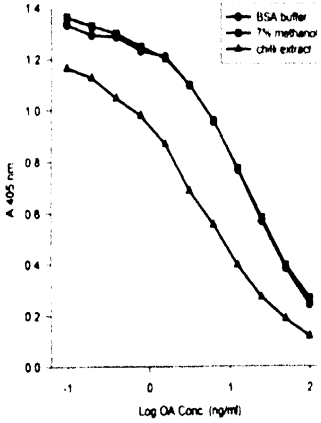


Figure 3. Effect of chili extract on the standard curve of OA by indirect competitive ELISA. Standard curves were prepared for OA standards, either by diluting in BSA PBS-T (●), or in BSA PBS-T containing 7%methanol (■), or in BSA PBS-T containing chili extract (▲).

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)[a]	concn. of OA estimated (μg/kg)[b]	percent recoveries of OA in spiked samples[c]
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	104 ± 1.6
5	100	1.75 ± 3.9	103 ± 5.3

[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

sample type[a]	OA contamination: incidence	no. of samples with OA contents (μg/kg) in the ranges of
chilies grade 1 (market yards)	2/42	
chilies grade 2 (market yards)	2/14	
chilies grade 3 (market yards)	8/24	
chilies (cold storage)	0/10	
chili powders (from super markets)	14/21	

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

estimating aflatoxins in chilies (Kiranmayi D., Reddy S. V., Reddy D., Durumala Devi K., and Reddy, D. V. R., unpublished). Therefore we presume that chili extracts contain substances which can bind non specifically to IgGs.

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.

ACKNOWLEDGMENT

We thank D. Kiranmayi for sample collection and Y. Ramachandraiah for technical assistance. We are especially grateful to Dr. Duncan McDonald for reviewing the manuscript.

LITERATURE CITED

Atanda, O. O., Akano, D. A., Atolabi, J. F. Mycoflora of dry 'tatase' pepper (*Capsicum annum* L.) stored for sale in Ibadan markets. *Lett. Appl. Microbiol.* **1990**, *10*, 35−37.

Balzer, I., Boddanic, C., Pepljujak, S. Rapid thin layer chromatographic method for determining aflatoxin B1, ochratoxin A, and zearalenone in corn. *J. Assoc. Off. Anal. Chem.* **1978**, *61*, 584−585.

Barna-Vetro, I., Solti, L. Sensitive ELISA test for determination of ochratoxin A. *J. Agric. Food Chem.* **1996**, *44*, 4071−4074.

Candlish, A. A. G., Stimson, W. H., Smith, J. F. Determination of ochratoxin A by monoclonal antibody based enzyme immunoassay. *J. − Assoc. Off. Anal. Chem.* **1988**, *71*, 961−964.

Devi, K. T., Mayo, M. A., Reddy, K. L. N., Delfosse, P., Reddy, G., Reddy, S. V., Reddy, D. V. R. Production and characterization of monoclonal antibodies for aflatoxin B1. *Lett. in Appl. Microbiol.* **1999**, *29*, 284−288.

Flannigan, B., Hui, S. C. The occurrence of aflatoxin producing strains of *Aspergillus flavus* in the mold floras of ground spices. *J. Appl. Bacteriol.* **1976**, *41*, 411−418.

Hobbs, D. A., Reddy, D. V. R., Rajeswari, R., Reddy, A. S. Use of direct antigen coating and protein coating ELISA procedures for detection of three peanut viruses. *Plant Dis.* **1987**, *71*, 747−749.

Holder, D. Ochratoxin A in food and feed: Occurrence, legislation and mode of action. *Z. Ernaehrungswiss.* **1998**, *37*, 2−12.

Josefsson, E., Moller, T. High pressure liquid chromatographic determination of ochratoxin A and zearalenone in cereals. *J. − Assoc. Off. Anal. Chem.* **1979**, *64*, 1165−1168.

Kuiper-Goodman, T., Scott, P. M. Risk assessment of the mycotoxin ochratoxin A. *Biomed. Environ. Sci.* **1989**, *2*, 179−248.

Lee, S. C., Chu, F. S. Enzyme linked immunosorbent assay of ochratoxin A in wheat. *J. − Assoc. Off. Anal. Chem.* **1984**, *67*, 45−49.

Madhvastha, S. Mycotoxic fungi and mycotoxins in major spices with special reference to *Piper nigrum* L. *Ph.D. Thesis*, University of Mysore, Mysore, India, 1985.

Morgan, M. R. A., McSweeney, K., Chan, H. W. S. Enzyme-linked immunosorbant assay of ochratoxin A in barley. *J. − Assoc. Off. Anal. Chem.* **1983**, *66*, 1481−1484.

Munro, I. C., Moodie, C. A., Kuiper-Goodman, T., Scott, P. M., Grice, H. C. Toxicologic changes in rats fed graded dietary levels of ochratoxin A. *Toxicol. Appl. Pharmacol.* **1974**, *28*, 180−188.

Osborne, B. G. Reversed-phase high-performance liquid chromatography determination of ochratoxin A in flour and bakery products. *J. Sci. Food Agric.* **1979**, *30*, 1065−1070.

Ramakrishna, N., Lacey, J., Candlish, A. A. G., Smith, J. E., Goodbrand, I. A. Monoclonal antibody based enzyme linked immunosorbant assay of aflatoxin B1, T-2 toxin and ochratoxin A in barley. *J. − Assoc. Off. Anal. Chem.* **1990**, *73*, 71−76.

Shantha, T. Critical evaluation of methods available for the estimation of aflatoxin B1 in chili powder. *J. Food Sci. Technol.* **1999**, *36*, 163−165.

Van Egmond, H. P. World wide regulations for ochratoxin A. In *Mycotoxins, Endemic Nephropathy and Urinary Tract Tumours*; Castegnaro, M., Plestina, R., Dirheimer, G., Chernozemsky, I. N., Bartsch, H., Eds.; IARC: Lyon, France, 1991; pp 331−336.

Received for review January 6, 2000. Revised manuscript received June 20, 2000. Accepted July 24, 2000. This document is an output from a project (R7084) funded by the UK Department for International Development (DFID) for the benefit of developing countries. The views expressed are not necessarily those of DFID. ICD and DVRR are supported by ICRISAT and MAM is supported by Scottish Executive Rural Affairs Department, UK.

JF0000490

B 0 5 7 1 2 4 9 B
Journal No. Manuscript No.
Dispatch Journal
Author Received No. of pages

Journal of Applied Microbiology 2001, 00, 000–000

Phage-displayed peptides that mimic aflatoxin B1 in serological reactivity

K. Thirumala-Devi[1], J.S. Miller[2], G. Reddy[3], D.V.R. Reddy[1] and M.A. Mayo[2]

[1]International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh, India, [2]Scottish Crop Research Institute, Dundee, UK, and [3]Osmania University, Hyderabad, Andhra Pradesh, India

513/8/00 received 18 August 2000, revised 20 October 2000 and accepted ?????? 2000

K. THIRUMALA-DEVI, J.S. MILLER, G. REDDY, D.V.R. REDDY AND M.A. MAYO. 2000

Aim: To test phage-displayed random peptide libraries as sources of peptides that mimic the binding of aflatoxin B1 to monoclonal antibodies raised against the toxin.

Methods and Results: For two of the three MAbs tested, clones were obtained by panning, producing phage that bound specifically to MAb 13D1–1D9 (MAb 24, specific for aflatoxins B1 and G1) and MAb 6E12–1E9 (MAb 13, specific for aflatoxins B1, G1 and B2) in ELISA. The amino acid sequences of the binding peptides varied. Those binding to MAb 24 contained the sequence of '...YMD...', and those that bound to MAb 13 contained the dipeptide 'PW'. Mimotope phage was used in a competition ELISA format for assaying aflatoxin concentrations.

Conclusions: The results show that mimotope preparations are effective substitutes for pure toxin in these ELISA procedures.

Significance and Impact of the Study: These results should contribute significantly to enhancing the safety of aflatoxin assays.

INTRODUCTION

Aflatoxins are potent carcinogenic, mutagenic, teratogenic and immuno-suppressive agents. They are produced as secondary metabolites by the fungi *Aspergillus flavus* and *A. parasiticus* (Busby and Wogan 1979) and can contaminate a variety of agricultural commodities (Butler 1974). A positive correlation has been shown between exposure to aflatoxin and the incidence of liver cancer in humans (Anon 1962, Bosch and Peers 1991, IARC 1993). To reduce this risk, governments in many countries have set limits for permissible levels of aflatoxins in foods and feeds.

A variety of analytical methods for aflatoxin detection has been devised to ensure compliance with these standards (Chu *et al.* 1987). ELISA is by far the most widely used serological test for the detection of mycotoxins because of its simplicity, adaptability and sensitivity (Pestka 1988). The immunogens used to raise antibodies to aflatoxins are conjugates made to a carrier protein. This conjugation involves structural modifications to aflatoxin molecules, as well as blocking stages, either of which may lead to substantial bridge group interference and unwanted cross reactions (Xiao *et al.* 1995). As the toxin antigen is essentially monovalent, assay procedures are based on competition and necessarily involve the use in the tests of pure toxin. This is hazardous and therefore, an alternative, less toxic form of aflatoxin would reduce the risks while carrying out the assays and thereby make immunoassays more widely and safely applicable.

In a search for this alternative, an attempt was made to obtain peptides that mimic aflatoxin by selection from phage-displayed random peptide libraries. Random peptide libraries displayed on phage have been shown to be powerful tools for identifying the peptide and non-peptide epitopes recognized by monoclonal antibodies (MAbs) (Smith 1991). In phage display, a peptide or a protein is fused to a coat protein of M13 or fd bacteriophage (e.g. pIII or a second recombinant copy of pVIII; Felici *et al.* 1991, Smith 1991), such that phage carrying the peptide also carry the encoding DNA. Determination of the recombinant pVIII gene sequences reveals the sequence of the binding peptide (Scott and Smith 1990). Phage borne peptides that mimic antigens in binding to antibodies are termed mimotopes (Geysen *et al.* 1987). There are few reports of the selection of mimotopes for antibodies

Correspondence to: Dr Mike Mayo, Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK (e-mail mmayo@scri.sari.ac.uk)

to non-proteinaceous chemicals, other than biotin (Weber *et al.* 1992) and carbohydrates (Hoess *et al.* 1993). Recently, Yuan *et al.* (1999) showed that it was possible to obtain mimotopes to MAbs raised against the mycotoxin deoxynivelenol. The mimotopes obtained were essentially of a single amino acid sequence.

Aflatoxins differ slightly in their chemical formulae; B1 is the principal hazard from fungal contamination of peanuts. MAbs raised to an aflatoxin B1-bovine serum albumin conjugate (Thirumala-Devi *et al.* 1999) cross-reacted with the other main aflatoxin types (B2, G1 and G2) to different extents. In this paper, the selection of mimotopes for aflatoxins, using two of these MAbs that differed in specificity, and the application of these mimotopes in ELISA for quantitative estimation of aflatoxins, is reported for the first time.

MATERIALS AND METHODS

Reagents

Aflatoxin B1 (AFB1), goat anti-rabbit IgG-alkaline phosphatase (ALP) conjugate, goat anti-mouse IgG-ALP conjugate, *p*-nitrophenyl phosphate, bovine serum albumin (BSA), aflatoxin B1-BSA conjugate, tetracycline and polyethylene glycol (PEG), were all purchased from the Sigma Chemical Co. Microtitre plates (Maxi-sorp F96) and immuno-tubes (Maxi-sorp) were obtained from Nalge Nunc International, Denmark. Peptone, yeast extract and agar were obtained from life technologies (Gibco BRL). All other chemicals were reagent grade or chemically pure.

Monoclonal antibodies for aflatoxin B1

Anti-aflatoxin B1 monoclonal antibodies were those described by Thirumala-Devi *et al.* (1999). MAb 13D1–1D9 (here coded MAb 24) cross-reacted with aflatoxin G1, but not B2, 6E12–1E5 (here named MAb 13) cross-reacted with aflatoxins B2, G1 and G2, and MAb 10D5–1A11 did not cross-react appreciably with any of the other aflatoxins (Thirumala-Devi *et al.* 1999). Immunoglobulins were concentrated from tissue culture supernatant or ascitic fluid by ammonium sulphate precipitation (Harlow and Lane 1988).

Peptide libraries

The random phage displayed peptide libraries, Cys-4 and Cys-6, used in this study were provided by G. Smith (University of Missouri, Columbia, USA). Each library consists of fd phage carrying a second copy of gene VIII that has at its N-terminus a randomized sequence in which cysteine codons are four or six codons apart. The cysteines are designed to constrain by cross-linking the conformations that the peptides may adopt (Ziegler *et al.* 1998).

Affinity selection of phage by panning

Panning was as described by Ziegler *et al.* (1998), using immuno-tubes coated with MAbs at 10 μg ml^{-1}. After blocking, phage were bound and eluted, and then used to infect *Escherichia coli* TG-1 cells. After amplification, phage were subjected to two further rounds of panning to enrich the population with binding phage.

Immunological assay for affinity-purified phage

Following the third round of panning, single tetracycline-resistant colonies were transferred to 5 ml LB-TET and grown at 37°C for 18 h. Cells were removed by centrifugation at 17 000 *g* for 5 min, and the culture media were tested by ELISA using microtitre plates. At each step, the plates were incubated for 1 h at 37°C. Initially, plates were coated with IgG at 10 μg ml^{-1} in 0·2 mol l^{-1} sodium carbonate, pH 9·6. In the second step, plates were blocked with 200 μl well^{-1} of 3% MPBS. Phage particles in 80 μl were mixed with 20 μl of MPBS. A mixture of rabbit anti-M13 antibody (1 : 500) and anti-rabbit IgG conjugated to alkaline phosphatase (1 : 100 000 dilution) was used to detect the phage. Substrate (*p*-nitrophenyl phosphate at 1 mg ml^{-1}) was added and absorbance at 405 nm was read in an ELISA plate reader after 1 h at room temperature. Selected individual clones were characterized by DNA sequencing and ELISA.

Indirect competitive ELISA with phage-displayed peptides

To determine the optimum number of phage to use in ELISA, different dilutions of phage particles in carbonate coating buffer were added to the ELISA plate and incubated overnight at 4°C. After blocking, a mixture of MAb and goat anti-mouse-alkaline phosphatase conjugate was added and the ELISA was completed as described. An indirect ELISA procedure similar to that reported for aflatoxins (Thirumala-Devi *et al.* 1999) was used. Microtitre plate wells were coated with 150 μl of phage at 10^{11} ml^{-1} that carried peptides in 0·2 mol l^{-1} sodium carbonate, pH 9·6, and incubated overnight at 4°C. The plates were washed and blocked as above for panning elution selection. MAbs in 50 μl were added to various concentrations of AFB1 (0–10 μg ml^{-1} in PBS). The mixtures were added to the phage-coated microtitre plate wells (150 μl well^{-1}) and the preparations were incubated at 37°C for 1 h. After washing four times with PBS-T, the plates were incubated with goat anti-

mouse-ALP conjugate at 37°C for 1 h. The amounts of bound enzyme were determined as described above. The results of inhibition tests were analysed using Genstat 5 and logistic curves were fitted to the data (Genstat 5 Committee 1987).

Nucleotide sequencing

Phage particles were recovered by PEG-precipitation and single-stranded DNA was extracted from them by phenol-chloroform treatment (Sambrook *et al.* 1989). Sequencing was done using an ABI PRISM dye primer cycle sequencing ready reaction kit (Perkin Elemer Applied Biosystems, Warrington, UK). The primer was complementary to DNA encoding the recombinant pVIII gene, downstream of the peptide insert. Sequences were analysed using the Genetics Computer Package (Devereux *et al.* 1984).

Ultraviolet irradiation of phage particles

Phage particles were u.v.-irradiated at 254 nm, with doses of between 10 and 800 mJ cm^{-2}, using a u.v. cross linker (Stratalinker UV crosslinker, Stratagene Ltd, Cambridge, UK). The treated phage particles were then used to infect log-phase TG-1 cells, or assayed by ELISA.

Sample preparation

Groundnut samples were obtained in 200 g quantities. After a thorough mixing, three 15 g sub-samples were drawn from each 200 g sample. Each sub-sample was finely ground and then extracted in 75 ml 30% methanol, containing 0·5% KCl, 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 : 10 with PBS-T/BSA for processing by ELISA.

Indirect competitive ELISA procedure for processing groundnut samples

The protocol was similar to that for indirect competitive ELISA for phage-displayed peptides (as described above), with the exception that aflatoxin standards in 100 µl volumes, ranging from 100 ng ml^{-1} to 100 pg ml^{-1}, were prepared in a diluted extract from groundnuts. Groundnut samples that did not contain aflatoxins were extracted in methanol as described above, filtered, and used at a 1 : 10 dilution prepared in PBS-T/BSA. Test samples were also diluted to 1 : 10 in PBS-T/BSA. A 100 µl aliquot of each sample was added to wells containing 50 µl of antiserum. The wells were washed four times with PBS-T and incubated with goat anti-mouse ALP-conjugate at 37°C for 1 h. The amounts of enzyme bound were determined as described above. Standard curves were obtained by plotting $\log_{10}$ values of aflatoxin B1 standards against optical density (O.D.) at A_{405}.

RESULTS

Screening of random peptide phage libraries for the selection of specific phages by panning

Tubes were coated with MAb 24 or MAb 13 and used for selection using either Cys 4 or Cys 6 libraries. Irrespective of the library used, the proportion of phage that bound (input titre/output titre) increased after each round. After the third round, input and output titres were about 10^{11} ml^{-1}. All 20 of 20 clones from the Cys 4 library, and four of 20 from the Cys 6 library, yielded phage that bound to MAb 24 (A_{405} of >0·5 to 2 in 1 h using DAS ELISA). From the 21 clones obtained using MAb 13, 11 from the Cys 4 library and 12 from the Cys 6 library gave positive results in ELISA tests. No clones were obtained from either library yielding phage that bound to MAb 10D5-1A11.

Optimum number of phage particles for coating the ELISA plate

ELISA plates were coated with different amounts of phage particles to establish the number of particles that were needed to give a useful response in phage ELISA. Figure 1 shows the results of ELISA of four mimotope clones, at different concentrations, assayed using either MAb 24 (clones 24·4 and 24·6) or MAb 13 (clones 13·4 and 13·6). The best results were obtained with phage concentrations of 10^{11} ml^{-1}. In parallel tests, plates coated with an unrelated Cys 4 phage clone at 10^{11} ml^{-1} gave an A_{405} of < 0·1.

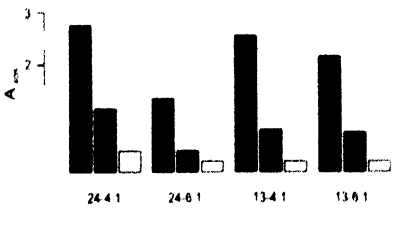


Fig. 1 Results of indirect ELISA with four phage mimotopes (24·4·1, 24·6·1, 13·4·1, 13·6·1) at concentrations of (■) 10^{11}, (■) 10^{10} or (□) 10^{9} ml^{-1}.

Specificity tests

The mimotopes 24-4-1, 24-6-1, 13-4-1 and 13-6-1 were tested in phage ELISA at 10^{11} ml^{-1} using dilutions of polyclonal antibody raised against an aflatoxin B1 BSA conjugate. The results for different mimotopes were indistinguishable. For example, a fourfold decrease in antibody concentration decreased the ELISA readings obtained in 1 h from 1·04, 1·07, 1·05 or 1·08 to 0·43, 0·47, 0·45 or 0·43 for each mimotope, respectively. In ELISA tests, mimotopes obtained using MAb 24 did not bind to MAb 13, and neither did MAb 13 mimotopes bind to MAb 24.

To test the specificity of the mimotopes, indirect competitive ELISA was done with mimotopes 24-4-1 and 13-4-1 using the corresponding MAbs. Figure 2 shows the results. For both mimotopes, the homologous toxin was the most competitive, other toxins were less competitive in the order G2 < B2 < G1. With 24-4-1 (Fig. 2a), B2, and especially G2, were much less competitive whereas with 13-4-1, the differences between toxins were less marked. The median doses (the toxin concentrations that resulted in an estimated 50% inhibition of binding) and the slopes of the curves at the median were calculated from the curves fitted to the data using the formula

$$A_{405} = A + C/(1 + \exp[-B \cdot (X - M)])$$

where A and C are the asymptotes, M is the median dose and B is the slope at the median dose.

Table 1 shows the results. These confirm the impressions from Fig. 2 that the strongest competition, as judged by the slopes of the lines, was with B1, the 'homologous' antigen, and the weakest competition was with G2.

Effect of dithiothreitol

The peptide sequences displayed by phage from the Cys-4 and Cys-6 libraries contain cysteine residues that are intended to cross-link, so as to create a loop structure of either four or six amino acids. Mimotope phage were treated with dithiothreitol, prior to being used to coat ELISA plates, in order to prevent the formation of this cross-link. With all clones tested (24-4-1, 24-4-2, 24-6-1, 13-4-1, 13-4-2, 13-6-1), the effects of dithiothreitol treatment were to increase the binding of the corresponding MAbs by between twofold and fivefold.

Effect of u.v. irradiation on phage infectivity

The infectivity of a dilute sample of mimotope 24-4-1 was decreased from about 4500 colonies ml^{-1} to about 60 colonies ml^{-1} by irradiation at 20 mJ cm^{-2}, and was abolished by larger doses. At these doses, there was little effect on MAb binding as assessed by phage ELISA. Phage irradiated at 50 mJ cm^{-2} bound about 90% of the amount of MAb bound by untreated samples. Binding decreased to

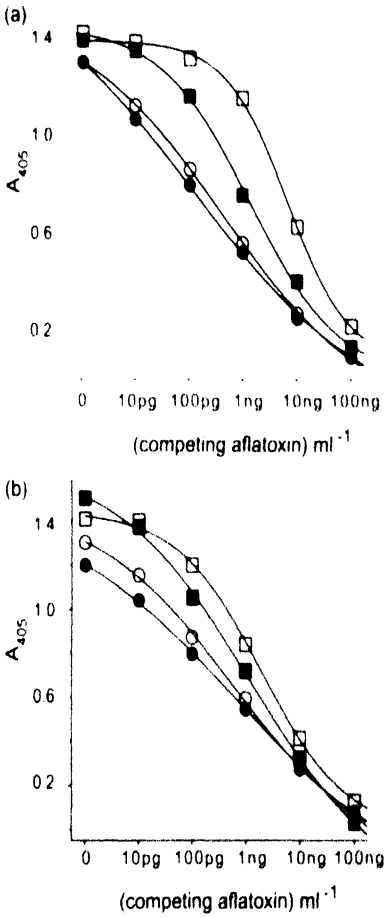


Fig. 2 Competition between pure aflatoxins (●) B1, (○) B2, (■) G1 and (□) G2, in the solution and phage mimotopes (24-4-1 and 13-4-1) coated on ELISA plate surface for binding to MAbs, in indirect competitive ELISA. (a) Mimotope 24-4-1 binding to MAb 24. (b) Mimotope 13-4-1 binding to MAb 13.

1 Parameters of the inhibition dose curves shown in Fig. 2

	Mimotope 24·4·1		Mimotope 13·4·1	
Inhibitor	Median dose*	Slope	Median dose	Slope
B1	1·38	−0·6044	1·04	−0·53
B2	1·39	−1·2032	1·22	−0·80
G1	0·45	−0·7495	0·58	−0·79
G2	6·77	−1·7623	1·98	−1·27

*ng ml^{-1}

70% following irradiation with 100 mJ cm^{-2}, and further after higher doses.

Recovery of aflatoxin B1 from groundnut samples

In order to test the effectiveness of the phage ELISA, 10–200 μg kg^{-1} of aflatoxin B1 were added to finely ground 10 g samples, which were then extracted and assayed. Table 2 lists the results from each of two ELISA plates. The recoveries were between 92 and 110%, the average recoveries on each plate were 101·3% and 98·9%.

To assess the assay method in practice, samples were taken from farmers' fields that were presumed to be at high risk of aflatoxin contamination because of drought. These were extracted and assayed by using competition ELISA. The results (Table 3) show that five samples were contaminated to various extents. Results for duplicate samples and duplicate plates were within 10%. The permissible level in India for aflatoxin B1 contamination is currently 30 μg kg^{-1}.

Mimotope sequences

For mimotope phage from either library, the nucleotide sequences were determined for the section of recombinant pVIII gene that encoded the randomized peptide sequence. These are shown in Fig. 3. Each sample was sequenced twice. All sequences were confirmed except where shown in Fig. 3. The altered sequences presumably arose during phage propagation between the two sequencing experiments, either from the appearance of point mutations, or from the sample being a mixture of phages that changed in preponderance.

The sequences of mimotopes to MAb 24 suggested a consensus sequence of 'C(Y/M)D(C)'. Those for mimotopes to MAb 13 did not suggest a clear consensus, although the dipeptide 'PW' was present in most, and all were particularly rich in proline residues.

Table 2 Efficiencies of extraction

Concentration in sample (μg kg^{-1})	Recovery*	
	Plate 1	Plate 2
10	98%	102%
20	102·5%	110·4%
40	105%	96%
80	99·9%	94·1%
100	100·7%	99·6%
150	101·1%	91·9%
200	102·2%	98·5%
Mean	101·3%	98·9%

*Measured concentration/known concentration × 100

Table 3 Assays of groundnut samples for aflatoxin B1

	[Aflatoxin B1] (μg kg^{-1})				
	Plate 1		Plate 2		
Sample	Test 1	Test 2	Test 1	Test 2	Result
1	15·2	14·0	19·8	21·6	+
2	< 10	< 10	< 10	< 10	
3	101	89·5	101	115	+
4	55·1	49·0	46·8	45·1	+
5	302	276	295	286	+
6	< 10	< 10	< 10	< 10	
7	< 10	< 10	< 10	< 10	
8	329	314	331	331	+
9	< 10	< 10	< 10	< 10	
10	< 10	< 10	< 10	< 10	

DISCUSSION

The results show that by panning phage displayed peptide libraries, it is possible to obtain peptides that mimic the binding of aflatoxin B1 to antibodies raised against it. However, with one of the three MAbs tried, no mimotopes were obtained from either library. This MAb does not cross-react with other aflatoxins (Thirumala Devi *et al.* 1999) and it is possible that the epitope involved in this highly specific binding cannot be mimicked by peptides. However, mimotopes were obtained to the other two MAbs and these had similar properties to the toxin in ELISA tests. All mimotopes reacted equally well with polyclonal antibodies. In a competitive ELISA, aflatoxin B1 could compete with the selected peptides for binding to MAb, while the synthetic peptide also competed with aflatoxin for binding to the same antibody. This strongly suggests that these clones bind to the same antigen-binding site of the MAb. However, in competition ELISA with aflatoxins B2, G1 and G2, the mimotope from the set binding to MAb 24 was differently affected by competition from the one binding to MAb 13

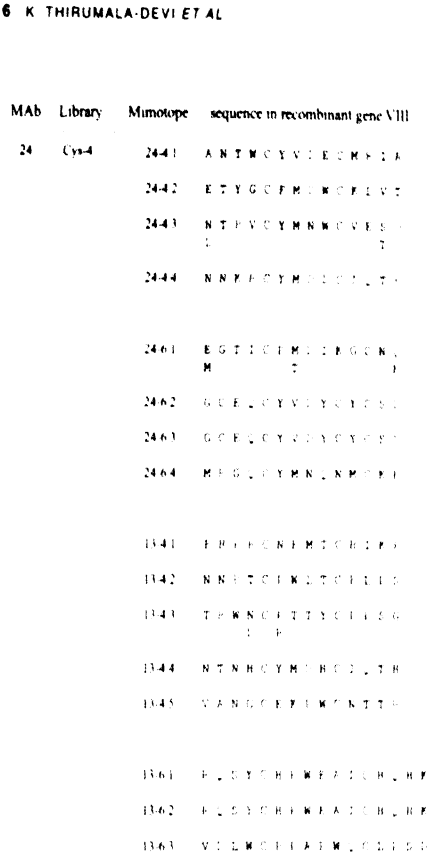


Fig. 3 Amino acid sequences of mimotope peptides. Nucleotide sequences were determined for the part of the recombinant gene VIII that encodes amino acids randomized in the libraries Cys 4 and Cys 6. Where two amino acids are shown, a second sequencing experiment yielded different sequences. The contiguous sequence is that obtained first. differences are shown below the altered amino acid

(Fig. 2). The differences among the slopes (Table 1) reflected the different extents of cross-reactivity each MAb had with heterologous aflatoxins (Thirumala-Devi *et al* 1999). These were, B1 = G1 ≥ B2 and G2 for MAb 24, and B1 > G1 = B2 ≥ G2 for MAb 13. Thus, in these tests, G1 was the most similar aflatoxin to B1. This suggests that the change from a five-membered ring to a six-membered ring containing an extra oxygen atom, which distinguishes B aflatoxins from G aflatoxins, alters the accuracy of the mimicry by the peptides less than does the change from a double bond to a single bond, in a different ring, which distinguishes aflatoxins B1 and G1 from B2 and G2.

The amino acid sequences of the mimotope peptides differed among the clones isolated, although some residues, especially aromatic amino acids, were abundant. MAb 24 mimotopes contained one or more tyrosine residues and MAb 13 mimotopes contained tryptophan residues. This may reflect a degree of molecular mimicry by the ring structures in the aromatic amino acids for the ring structures in the aflatoxin molecules. In sequence, the MAb 24 mimotope peptides were unrelated to those for MAb 13. Presumably, the binding pockets in the two MAbs differ appreciably despite the simplicity of the common immunogen to which they were raised.

By its nature, the phage display method tends preferentially to isolate strongly binding phage. The present results show that aflatoxin MAbs select several approximately equally strongly binding peptides. This contrasts with the results obtained by Yuan *et al* (1999) with mimotopes isolated from an unconstrained library for MAbs specific to the toxin deoxynivalenol. Possibly, the mimotope these authors obtained is a much stronger binder than any others present in the library used for the panning.

Aflatoxins are important human and animal toxins, and so care must be taken to avoid direct contact during analysis. The possibility of replacing toxins in diagnostic procedures

with presumably non-toxic peptides should therefore result in enhanced laboratory and environmental safety. This advantage could be even more marked when diagnostic test kits are used in 'the field' by relatively unskilled operators unused to laboratory discipline.

Although many mimotope peptides for protein antigens have been isolated from phage-displayed peptide libraries (e.g. Scott *et al.* 1992, Hoess *et al.* 1993), this is only the second report of mimotopes for a non-proteinaceous, low molecular weight mycotoxin, and the first report of aflatoxin mimotopes. Sixteen somewhat related mimotope peptides have been identified that mimic the binding of aflatoxin to specific antibodies, and the potential for using these peptides in aflatoxin immunoassays has been demonstrated. This use of peptide libraries as sources of reagents in immunoassays could well be applicable in ELISA tests for other mycotoxins.

ACKNOWLEDGEMENTS

This document is an output from a project (R7083) funded by the UK Department for International Development (DFID) for the benefit of developing countries. The views expressed are not necessarily those of DFID. The authors are grateful to Dr Angelika Zeigler and Michele Laney for helpful discussions during this work, and to Jim McNicol, BioSS, for statistical guidance. MAM acknowledges financial support from the Scottish Executive Rural Affairs Department. KT-D and DVRR are supported by ICRISAT.

REFERENCES

Anon. (1962) Progress in cancer research. Potent liver toxin and carcinogen produced in groundnuts as a result of infection with the fungus *Aspergillus flavus*. *British Medical Journal* 2(5298), 172–174.

Bosch, F.X. and Peers, F. (1991) Aflatoxins: data on human carcinogenic risk. In relevance to human cancer of N-Nitroso compounds. In *Tobacco and Mycotoxins* ed. O'Neill, I.K., Chen, J. and Bartsch, H. pp. 48–53. Lyon, France: International Agency for Research on Cancer.

Busby, W.F. Jr and Wogan, G.N. (1979) Food-borne mycotoxins and alimentary mycotoxicoses. In *Food-Borne Infections and Intoxications* 2nd edn, ed. Riemann, H. and Bryan, F.L. pp. 519–610. New York: Academic Press.

Butler, W.H. (1974) Aflatoxins. In *Mycotoxins* ed. Purchase, I.F.H. pp. 1–28. New York: Elsevier Scientific Publishing Co.

Chu, F.S., Fan, T.S.L., Zhang, G.-S., Xu, Y.C., Faust, S. and McMohan, P.L. (1987) Improved enzyme-linked immunosorbant assay for aflatoxin B1 in agricultural commodities. *Journal of the Association of Official Analytical Chemists* 70, 854–857.

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of nucleotide sequence analysis program for the VAX. *Nucleic Acids Research* 12, 387–395.

Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R. and Cesareni, G. (1991) Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *Journal of Molecular Biology* 222, 301–310.

Genstat 5 Committee (1987) *Genstat 5 Reference Manual*. Oxford: Clarendon Press.

Greven, H.M., Rodda, S.J., Mason, T.L., Tribbick, G. and Schoofs, P.G. (1987) Strategies for epitope analysis using peptide synthesis. *Journal of Immunological Methods* 102, 259–274.

Harlow, E. and Lane, D. (1988) *Antibodies: a Laboratory Manual*. New York: Cold Spring Harbor Laboratory.

Hoess, R., Brinkmann, U., Handel, T. and Pastan, I. (1993) Identification of a peptide which binds to the carbohydrate specific monoclonal antibody B3. *Gene* 128, 43–49.

IARC (1993) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins*. Lyon, France.

Pestka, J.J. (1988) Enhanced surveillance of food-borne mycotoxins by immunochemical assay. *Journal of the Association of Analytical Chemists* 71, 1073–1081.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Scott, J.K., Loganathan, D., Easley, R.B., Gong, X. and Goldstein, I.J. (1992) A family of concanavalin A-binding peptides from a hexapeptide epitope library. *Proceedings of the National Academy of Sciences USA* 89, 5398–5402.

Scott, J.K. and Smith, G.P. (1990) Searching for peptide ligands with an epitope library. *Science* 249, 386–390.

Smith, G.P. (1991) Surface presentation of protein epitopes using bacteriophage expression systems. *Current Opinions in Biotechnology* 2, 668–673.

Thirumala-Devi, K., Mayo, M.A. *et al.* (1990) Production and characterization of monoclonal antibodies for aflatoxin B1. *Letters in Applied Microbiology* 29, 284–288.

Weber, P.C., Pantoliano, M.W. and Thompson, L.D. (1992) Crystal structure and ligand binding studies of a screened peptide complexed with streptavidin. *Biochemistry* 31, 9350–9354.

Xiao, H., Clarke, J.R., Marquardt, R.R. and Frohlich, A.A. (1995) Improved methods for conjugating selected mycotoxins to carrier proteins and dextran for immunoassays. *Journal of Agricultural Food Chemistry* 43, 2092–2097.

Yuan, Q., Pestka, J.J., Hespenheide, B.M., Kuhn, K.A., Lanz, J.L. and Hart, L.P. (1990) Identification of mimotope peptides which bind to the mycotoxins deoxynivalenol-specific monoclonal antibody. *Applied and Environmental Microbiology* 65, 3279–3286.

Ziegler, A., Mayo, M.A. and Torrance, L. (1998) Synthetic antigen from a peptide library can be an effective positive control in immunoassays for the detection and identification of two germinivirus species. *Phytopathology* 88, 1302–1305.

Occurrence of ochratoxin A in black pepper, coriander, ginger and turmeric in India

K. Thirumala-Devi†, M. A. Mayo‡, Gopal Reddy§, Emmanuel K. TANGNI , Yvan Larondelle 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; § Osmania University, Hyderabad, Andhra Pradesh 500 007, India; Unité de Biochimie de la Nutrition, Faculté des Sciences Agronomiques, Université catholique de Louvain, Place Croix du Sud 2 Bte 3, B-1348 Louvain-la-Neuve, Belgium

• To whom correspondence should be addressed:

DR. D. V. R. Reddy

Principal Scientist

GREP

International Crops Research Institute for the Semi-Arid Tropics (ICRISAT)

Patancheru 502 324, India

Tel: +91-40-3296161 ext.2669

Fax: +91-40-3296182

E-mail: D.REDDY@CGIAR.ORG

Abstract

Ochratoxin A (OA) contamination of black pepper, coriander seeds, powdered ginger and turmeric powder was estimated using indirect competitive ELISA. Samples (1g) were extracted with 0.5% potassium chloride (KCl) in 70% methanol (5 ml) and diluted subsequently to give two-fold to ten-fold step wise dilutions in phosphate-buffered saline containing 0.05% Tween 20 and 0.2% bovine serum albumin (PBS-T-BSA). For extracts from the spices analyzed, ELISA estimates of OA concentrations were compared with those made by HPLC. All estimates were within 1-2 standard deviation of the ELISA values. More than 90% of OA added to spice samples was recovered from samples containing between 5 and 100 μg/kg OA. Extracts of OA-free spice samples contained substances that interfered with ELISA presumably because of non-specific reactions. This effect was avoided by preparing all the test solutions in extracts of OA-free spice samples. In 126 samples obtained from retail shops, OA was found to exceed 10 μg/kg in 14 (in the range of 15 to 69 μg/kg) of 26 black pepper samples, 20 (in the range of 10 to 51 μg/kg) of 50 coriander samples, 2 (23 μg/kg and 80 μg/kg) of 25 ginger samples and 9 (in the range of 11 to 102 μg/kg) of 25 turmeric samples. This is the first record in India of the occurrence of OA in what are some of the most widely used spices in Indian cooking.

Keywords: ochratoxin A, indirect competitive ELISA, HPLC, black pepper, coriander, ginger, turmeric

Introduction

Ochratoxins are a group of toxic secondary metabolites produced by certain fungi in the genera *Aspergillus* and *Penicillium* (Applegate *et al.* 1973, Chu 1974, Harwig 1974, Steyn 1971). Among them, ochratoxin A is the most toxic. It has been shown to be nephrotoxic, hepatotoxic, teratogenic, carcinogenic, mutagenic and to be an immunosuppressive agent (Kuiper-Goodman and Scott 1989). Of greatest concern for human health is its implication in an irreversible and fatal kidney disease referred to as "Balkan Endemic Nephropathy" (Krogh 1974). OA has been found to occur in foods of plant origin, in edible animal tissues, and in human milk, blood sera and tissues (Creppy *et al.* 1995).

OA has been detected in diverse food and feed commodities (Veldman *et al.* 1992, Oyelami *et al.* 1996, Zimmerli *et al.* 1996) including spices. Spices found by HPLC to contain relatively high (>5 μg/kg) levels of OA were Chinese red pepper (Akiyama *et al.* 1998), paparica and nutmeg (Vrabcheva *et al.* 1998). The moulds isolated from spices are predominantly *Aspergillus* and *Penicillium* species (Flannigan and Hui, 1976) and these are implicated in the production of several mycotoxins.

Spices and condiments are extensively used in Oriental and Indian cooking. The only record of ochratoxin A contamination in spices from India is its detection by ELISA in chillies (Thirumala-Devi *et al.* 2000). In this paper, we report the occurrence of OA in

68 many samples of four of the most commonly used spices in India, namely black pepper,
69 coriander, ginger and turmeric by both ELISA and HPLC methods.
70
71 ## Materials and methods
72
73 ### *Materials*
74
75 Ochratoxin A, ochratoxin A-BSA conjugate, goat anti-rabbit IgG-ALP conjugate, *p*-
76 nitrophenyl phosphate, and bovine serum albumin (BSA), were all purchased from the
77 Sigma Chemical Co., St. Louis, USA. Microtiter plates (Maxi-sorp F96) were obtained
78 from Nunc (Nalge Nunc International, Denmark). Immunoaffinity coloumn from Vicam
79 L.P., Watertown, MA, USA. Chloroform, acid acetic and orthophosphoric acid were
80 analytical grade; acetonitrile, methanol and toluene were HPLC grade. All other
81 chemicals were reagent grade or chemically pure.
82
83 ### *Collection of samples and preparation for ELISA*
84
85 Black pepper (26 samples), coriander (50 samples), ginger (25 samples), and turmeric (25
86 samples) were purchased from retail shops in 200 g quantities. Black pepper and
87 coriander were purchased as seeds and turmeric and ginger were bought as powders.
88 Black pepper and coriander were ground to a fine powder in a Waring blender and ginger
89 and turmeric powders were used without any further grinding. Samples of 200 g were
90 thoroughly mixed and three 15 g sub-samples were taken. Each sub-sample was extracted

with 75 ml of a mixture of 0.5% KCl in 70% methanol by blending in a Waring blender. Extraction was followed by shaking for 30 min and filtration through Whatman No. 41 filter paper. The filtrate was diluted two-to ten-fold in phosphate-buffered saline containing 0.05% Tween 20 and 0.2% bovine serum albumin (PBS-T BSA) prior to ELISA.

Antibodies

OA polyclonal antibodies were those described by Thirumala-Devi *et al.* (2000). Antibodies were highly specific for OA and did not cross react with ochratoxin B, coumarin, 4-hydroxy coumarin or L-phenylalanine.

Indirect competitive ELISA procedure for processing the various spice samples

An indirect ELISA procedure similar to that reported for aflatoxins (Thirumala-Devi *et al.* 1999) and ochratoxin A (Thirumala-Devi *et al.* 2000) 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) overnight at 4°C. Subsequent steps were at 37°C for 1 h. Antiserum was diluted in PBS-T BSA, held for 45 min at 37°C and added in 50 μl volumes to 100 μl of OA standards ranging from 100 ng/ml to 100 pg/ml, prepared in a diluted extract from spices that did not contain OA (verified by HPLC). Spice samples were extracted with methanol-KCl as described above, filtered and used at a 1:10 dilution in PBS-T BSA, except for ginger which was diluted to 1:8. One hundred μl of each sample were added to

wells containing 50 μl of antiserum diluted to 1:100,000. Goat antirabbit immunoglobulins (GAR IgG) conjugated to alkaline phosphatase were used at a 1:1000 dilution to detect rabbit antibodies attached to OA-BSA. *p*-nitrophenyl phosphate was used as a substrate at 1 mg/ml. Absorbance was recorded at 405 nm (A_{405}) with an ELISA plate reader (Titertek Multiskan, Lab systems, Finland) after incubation at room temperature for 1h.

Standard curves were obtained by plotting $\log_{10}$ values of OA concentration against optical density at A_{405}. The concentrations of OA in samples were determined from the standard curves and expressed in μg/kg using the formula: OA concentration (ng/ml) in sample extract × dilution with buffer × extraction solvent volume used (ml) / sample weight (g). In order to test the recovery of OA, 15 g spices were mixed with pure OA to give concentrations ranging from 1 to 100 μg/kg. Spiked samples were extracted and assayed as for unknown samples.

Determination of OA by HPLC

Ochratoxin A was extracted by using an adaptation of the extraction procedure of the Technical Committee of European Committee for Standardization (CEN/TC 275, 1998). Spice powder samples (50 g) were transferred into a 500 ml PTFE (polytetrafluoro ethylene) container mixed with 200 ml chloroform and 20 ml 0.1 M orthophosphoric acid. This mixture was triturated for 3 minutes with the Ultra-Turrax (A1 x 620 (Staufen, Germany) was fitted with a T17-V shaft at 13500 rpm to produce a slurry. After

centrifugation for 10 min under 820 g at 5-10°C, the chloroform phase was transferred to a 500 ml beaker. The remaining part was extracted again with 200 ml chloroform and 20 ml of 0.1 M orthophosphoric acid. The combined chloroform phases (c.a. 350 ml) were evaporated to dryness by rotary evaporation at 30-40°C. The residue was dissolved in 100 ml of 0.5 M $NaHCO_3$ and transferred to a 120 ml PTFE container. After 10 minutes centrifugation at 820 g at 5-10°C, 20 ml of samples were passed through an Ochratest™ immunoaffinity column (Vicam L.P., Watertown, MA, USA) at about 1-2 ml/minute. Before loading the extract, the Ochratest ™ column was conditioned with PBS (20 ml). Twenty ml of de-ionized water was used to wash the loaded immunoaffinity column and OA was then eluted with 2 ml methanol and 2 ml de-ionized water. Atmospheric air (c.a. 20 ml) was passed through the column to collect all the eluate. One ml sample of the elute was then filtered through a 0.45 μm microfilter (Millex®-HV) for HLPC analysis. For HPLC, 50 μl samples were injected by full loop injection. The chromatographic system consisted of a Perkin-Elmer LC049 isocratic pump (Norwalk, CO, USA) equipped with a Rheodyne model 7125 NS injection valve (50 μl) (Rheodyne, Cotati, CA, USA), a RF-551 fluorescence spectrophotometer detector (Shimadzu, Kyoto, Japan) equipped with a 150 W xenon lamp ($\lambda_{excitation}$ = 332 nm and $\lambda_{emission}$ = 462 nm) and a Spectra-Physics SP4290 chromato-integrator (San José, CA, USA). The analytical column was an Hypersil™ BDS reversed-phase C_{18} (150 x 4.0 mm i.d., 3 μm particles size) (Tracer Analytical, Barcelona, Spain). The column was left at ambient temperature. The mobile phase was acetonitrile-water-acetic acid (45:54:1 v/v/v) eluted at a flow rate of 1.0 ml/min. OA was assayed by measuring peak height at the ochratoxin A retention time and comparing it with the relevant calibration curve (5 points, in the range 1 - 5 ng

of ochratoxin A/ml, r-squared=0.9992). Standard solutions for the calibration curve were prepared in the mobile phase from a stock solution containing 20 μg ml of OA in toluene-acetic acid (99:1).

Within the tested range of spiking samples (0.4-10 μg OA kg), the procedure showed good precision (RSD = 3.1%) and accuracy with an overall recovery of 89 ± 1.6 %. The detection limit of ochratoxin A was 10 ppt (based on the ratio signal noise = 3:1) and the quantitation limit was 35 ppt (based on the ratio signal noise = 10:1).

Results and Discussion

Comparison of estimates of OA concentration by ELISA and HPLC

The OA contents of samples spiked with pure OA were estimated by ELISA and HPLC. The results show good correspondence (Table 1). HPLC estimates were within 2 SD of ELISA estimates, although always less.

Effect of extracts from various spices in ELISA

The ELISA procedure reported for OA estimation in chillies (Thirumala-Devi *et al* 2000) gave a non-specific reaction, presumably due to interference by substances present in the spice extracts. To confirm that interference in ELISA was due to substances in

spice samples, we compared OA standards prepared in PBS-1 BSA with those prepared in spice extract shown by ELISA to contain the equivalent of less than 10 μg kg OA. Curves were found to be influenced by substances present in different spice extracts (Figure 1). As a result, it was essential to prepare the standard solutions of OA in extracts of spices. Chillies have been shown to contain substances that bind to aflatoxins (Shantha, 1999) and also that interfere in the estimation by indirect ELISA of OA (Thirumala-Devi *et al.* 2000). Thus, toxin standards were prepared in spice extracts shown to be substantially free of OA.

Recovery of OA from spiked spice samples

To confirm that the extraction procedures were effective, pure OA was added to powdered spice samples and extracted in 70 % methanol-KCl. Recoveries from samples of black pepper, coriander and turmeric samples were greater than 90 % (Table 2). However, recovery from ginger samples was ~ 90 % only when extracts were diluted 8-fold.

Analysis of spice samples collected from retail shops

The results of OA analysis of spices collected from retail shops are shown in Table 3. Three replicates of 126 samples of black pepper, coriander, ginger or turmeric were measured. The results showed that 45 samples contained more than 10 μg/kg OA in amounts that ranged up to 110 μg/kg (Table 3).

206 OA contamination occurred at higher levels in black pepper, turmeric and coriander than
207 in ginger. High levels of OA (110 μg kg) were detected in turmeric, which is one of the
208 most widely used spice in Indian cooking.
209
210 The production of OA and the growth of the fungi responsible are dependent upon factors
211 such as temperature, humidity, handling during harvesting and conditions during storage.
212 The majority of spices are produced in countries with tropical climates, which favor
213 growth of the fungi. Unfortunately, methods that would be suitable for particular spices
214 have not been developed. We also presume that lack of sufficient surveillance data on the
215 occurrence of OA in spices in India can be attributed to the non-availability of
216 inexpensive diagnostic tools. The present study clearly showed that four important spices
217 can harbor OA at levels beyond those permitted by public health authorities. Our results
218 emphasize the need for surveillance of OA contamination in spices and suggest that
219 strategies should be developed that can lead to minimize their contamination.
220
221 **Acknowledgments**
222
223 This document is an output from a project (R7083) funded by the UK Department for
224 International Development (DFID) for the benefit of developing countries. The views
225 expressed are not necessarily those of DFID. KT-D and DVRR are supported by
226 ICRISAT. MAM acknowledges financial support from the Scottish Executive Rural
227 Affairs Department.
228

References

AKIYAMA, H., KIKUCHI, Y., CHEN, D., GODA, Y., TAKATORI, K., ICHINOE, M., TOYODA, M., 1998, A rapid analytical method of ochratoxin A (OTA) in foods and natural contamination of OTA in red pepper. *Revue Med Vet* **149**, p. 502.

APPLEGATE, K. L., and CHIPLEY, J. R., 1973, Ochratoxins. *Advances in Applied Microbiology*, **16**, 97-109.

CEN/TC 275, 1998, Foodstuffs-Determination of ochratoxin A in cereals and cereal products-part 2: High performance liquid chromatographic method with bicarbonate clean up (ISO FDIS 15141-2:1998). Brussels, Belgium, 12 p.

CHU, F. S., 1974, Studies on ochratoxins. *Critical Reviews in Toxicology*, **2**, 499-524.

CREPPY, E. E., BAUDRIMONT, I., and BETBEDER, A. M., 1995, Prevention of nephrotoxicity of ochratoxin A, a food contaminant. *Toxicology Letters*, Dec., **82-83**: 869-77.

FLANNINGAN, B., and HUI, S. C., 1976, The occurrence of aflatoxin-producing strains of *Aspergillus flavus* in the mould floras of ground spices. *Journal of Applied Bacteriology*, **41**, 411-418.

HARWIG, J., 1974, Ochratoxin A and related metabolites, edited by I. F. H. Purchase *Mycotoxins*. Elsevier Scientific Publishing Co., Amsterdam, The Netherlands, p. 345-367.

KROGH, P. B., HALD, P., ENGLUND, I., RUTQVIST and SWAHN, O., 1974, Contamination of Swedish cereals with ochratoxin A. *Acta Pathologica et Microbiologica Scandinavia*, **32**: 301-302.

KUIPER-GOODMAN, T., and SCOTT, P. M., 1989, Risk assessment of the mycotoxin ochratoxin A. *Biomedical and Environmental Sciences*, **2**, 179-248.

NESHEIM, S., STACK, M. E., TRUCKSESS, M. W., EPPLEY, R. M., 1992, rapid solvent-efficient method for liquid chromatographic determination of ochratoxin A in corn, barley, and kidney: collaborative study. *Journal of Association of Analytical Chemists*, **75**, 481-487.

OYELAMI, O. A., MAXWELL, S. M., and ADEOBA, F., 1996, Aflatoxins and ochratoxin A in the weaning food of Nigerian children. *Annals of Tropical Pediatrics*, **16:2**, 137-40.

SHANTHA, T., 1999, Critical evaluation of methods available for the estimation of aflatoxin B_1 in chilli powder. *Journal of Food Science and Technology*, **36**, 163-165.

STEYN, P. S., 1971, Ochratoxin and other dihydroisocoumarins, edited by A. Ciegler, S. Kadis, and S. J. Ajl. *Microbial toxins*, Vol. 6. Academic Press Inc., New York. P. 179-205.

THIRUMALA DEVI, K., MAYO, M. A., REDDY, K. L. N., DELFOSSE, P., GOPAL REDDY., REDDY, S. V., and REDDY, D. V. R., 1999, Production and characterization of monoclonal antibodies for aflatoxin B_1. *Letters in Applied Microbiology*, **29**, 284-288.

THIRUMALA-DEVI, K., MAYO, M. A., DELFOSSE, P., GOPAL REDDY., REDDY, S. V., and REDDY, D. V. R., 2000, Production of polyclonal antibodies for ochratoxin A and its detection in chillies by ELISA. *Journal of Agricultural and Food Chemistry*, **48**, 5079-5082.

273 VELDMAN, A., BORGREVE, G. J., MULDERS, E. J., VAN DE LAGEMAAT, D.,
274 1992, Occurrence of the mycotoxins ochratoxin A, zearalenone and
275 deoxynivalenol in feed components. *Food Additives and Contaminants*, **9**, 647-
276 55.
277 VRABCHEVA, T., GAREIS, M., BRESCH, H., BODECHTEL, C., ENGEL, G.,
278 MAJERUS, P., ROSNER, H., WOLFF, J., 1998, Occurrence of ochratoxin A and
279 B in spices and herbs. *Revue Med Vet* **149**, p. 553
280 ZIMMERLI, B., and DICK, R., 1996, Ochratoxin A in table wine and grape-juice:
281 occurrence and risk assessment. *Food Additives and Contaminants*, **13**, 655-68
282

282 ***Table 1. Determination of OA in selected spice samples by of ELISA and HPLC***

283

284 | | OA contamination | |

285 | Sample type | ELISA[a] | HPLC[a] |

286

287 | Black pepper | 4.2 ± 1.2 | ND |

288

289 | Black pepper | 103.2 ± 5.1 | 98.3 |

290

291 | Coriander | 8.3 ± 0.9 | 7.8 |

292

293 | Coriander | 59.0 ± 2.4 | 53 |

294

295 | Ginger | 25.3 ± 0.8 | 23.3 |

296

297 | Ginger | 77.8 ± 4.3 | 71.3 |

298

299 | Turmeric | 32.4 ± 1.9 | 29.1 |

300

301 | Turmeric | 54.0 ± 3.6 | 47.4 |

302

303 [a] ELISA values are means of 3 estimates ± SD; HPLC values are for single estimates.

304

Table 2. Recovery of OA from artificially contaminated spice samples as determined by ELISA.

No.	Concentration of OA used for spiking (μg/kg)	Concentration of OA estimated (μg/kg)[a]	Percent recoveries of OA in spiked samples[b]
Black pepper			
1	5	4.80 ± 0.39	96.0 ± 8.07
2	10	9.60 ± 0.82	96.0 ± 8.57
3	50	49.2 ± 0.88	98.4 ± 1.78
4	100	103.9 ± 4.08	102.6 ± 3.98
Coriander			
1	5	5.90 ± 0.57	118.0 ± 9.72
2	10	10.53 ± 0.82	105.3 ± 7.75
3	50	52.47 ± 2.1	104.9 ± 4.03
4	100	108.1 ± 7.9	108.1 ± 7.38
Ginger			
1	5	4.73 ± 0.29	94.7 ± 6.22
2	10	9.30 ± 0.73	93.0 ± 7.82
3	50	49.5 ± 0.72	99.1 ± 1.45
4	100	103.9 ± 5.46	103.9 ± 5.26
Turmeric			
1	5	4.97 ± 0.05	99.3 ± 1.0
2	10	10.0 ± 0.08	100.0 ± 0.82
3	50	52.77 ± 3.11	105.5 ± 5.90
4	100	98.73 ± 1.01	98.7 ± 1.03

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

343 ***Table 3. Incidence and range of OA in selected spice samples as determined by indirect***
344 ***competitive ELISA.***

345

346		OA contamination					
347	Sample type [a]	Incidence	% contamination	No of samples with OA (μg/kg) contents in the ranges of			
348							
349							
350				10-29	30-49	50-100	110
351							
352	Black pepper	14/26	54	8	5	1	0
353							
354	Coriander	20/50	40	16	3	1	0
355							
356	Ginger	2/25	8	1	0	1	0
357							
358	Turmeric	9/25	36	3	2	3	1
359							
360							

361 [a] see text for details.

362 Figure legends

363

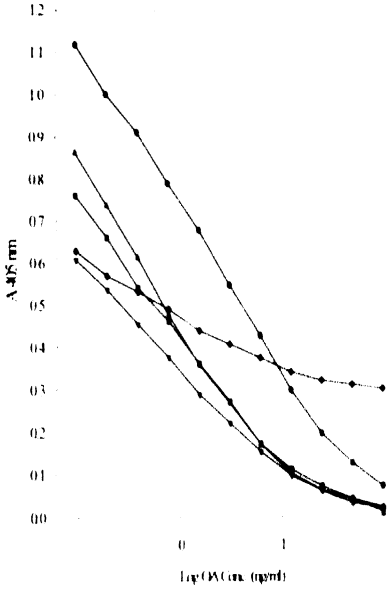
364 *Figure 1. Effect of spices extract on the standard curve of OA by indirect competitive*

365 *ELISA. OA standard curves prepared in 7% methanol (●), Black pepper (◆), Coriander*

366 *(▲), Ginger (■), and Turmeric (▼) extracts*

367

367 *Figure 1*



368

369