THE PRODUCTION, PURIFICATION AND CHARACTERIZATION OF A MONOCLONAL ANTIBODY AGAINST OCHRATOXIN A

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

Cynthia Lorraine Porter

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

Food Science Department

August 1990

ISBN 0-315-63226-7

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ABSTRACT

Grains contaminated with the storage mycotoxin, ochratoxin A (OA), present health hazards to the consumer. The ubiquitous nature of this nephrotoxin requires detection methods that are sensitive, specific, rapid and reproducible. Enzyme-linked immunosorbant assays (ELISAs) meet all the criteria for OA detection. Since all ELISAs require antigen-specific antibodies for their operation, the purpose of this research project was to develop a monoclonal antibody against OA for the eventual production of an ELISA kit to routinely screen grain samples. A monoclonal antibody (MAb) specific for OA (OA-MAb) was produced, purified and characterized. The OA-MAb was produced through mouse immunizations and hybridoma technology. The OA-MAb was purified using ammonium sulfate precipitation and gel filtration chromatography. The OA-MAb was characterized for immunoglobulin classes and subclasses, molecular weight, sensitivity and specificity. The OA-MAb was isotyped as immunoglobulin M with an apparent molecular weight of 1.3 x 10^6. The OA-MAb displayed a lowest detection limit of 1 μg OA/ml and an antibody displacement measurement of 88.4% at 100 μg OA/ml. The OA-MAb demonstrated excellent specificity with cross-reactivity measurements of 31% for ochratoxin α, 23% for citrinin and 19% for ochratoxin B.

ACKNOWLEDGEMENTS

This project was initiated by Dr. M. Dawood and funded by Agriculture Canada under operation grant #88013 and by Agrobiotech International Inc. under the National Research Council of Canada Industrial Research Program -M; grant #9-6651-M-16. Many helpful comments and valid suggestions regarding both laboratory and thesis work were graciously provided by my advisor, Dr. J. Zawistowski. He supplied the focus and direction necessary for this project to succeed. The support and encouragement provided by my committee members; Drs. E.D. Murray and R.R. Marquardt, were most appreciated.

Essential technical contributions were made by James Clarke and Dr. A.A. Frohlich of the Animal Science Department and Anna Berczi and Lidia Gosek of the Food Science Department. I am indebted to Anna for her friendship, patience and vast expertise. Invaluable social contributions were made by my friends and fellow graduate students; Rob, Marta, Himalee, Lori and Debbie.

Last on the list, but first in my heart, I wish to thank my husband for his infinite patience and sense of humour. Both of which were in ready supply. Without your help Scott, I would never have succeeded.

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INTRODUCTION

Ochratoxins are secondary metabolites produced by moulds belonging to several species of the genera *Aspergillus* and *Penicillium* (Chu, 1974b). The most extensively studied compound of this group, ochratoxin A (OA), has been reviewed by several researchers (Applegate and Chipley, 1973; Chu, 1974b; Steyn, 1984). As a natural contaminant, OA was first isolated in the U.S.A. from mouldy corn over 20 years ago (Shotwell et al., 1969). In Canada, this mycotoxin was found mainly in wheat but also in oat, barley and rye stored under damp conditions (Scott et al., 1970; Scott et al., 1972).

Ochratoxin A is the most potent toxin of this series. It is nephrotoxic to many animal species and has been implicated in the chronic human renal disease - Balkan endemic nephropathy (Krogh et al., 1977). This mycotoxin is a widely detected contaminant of agricultural commodities, especially cereals, in Europe and North America (Morgan et al., 1986).

The most established physico-chemical methods for OA detection include thin-layer chromatography (Nesheim et al., 1973) and high performance liquid chromatography (Engstrom et al., 1977; Osborne, 1979). Although these methods are sensitive, they are also expensive, time consuming and cumbersome (Pestka, 1988). Other disadvantages include the necessity for sophisticated equipment and highly trained personnel.

Recently, several immunochemical methods for OA detection, including radioimmunoassays (RIA) (Rousseau et al., 1985) and enzyme-linked immunosorbant assays (ELISA) (Pestka et al., 1981), have been developed. These immunoassays are preferred because they provide simplicity and specificity at relatively low expense. Since immunoassays do not require sophisticated equipment, they may be used on-site by fewer trained personnel.

Most immunoassays employed for OA detection incorporate animal antisera made by conventional techniques (Pestka et al., 1981; Rousseau et al., 1985). That is, a vertebrate animal host is injected with an OA-protein conjugate until OA antibodies are detected in its serum. This antiserum is used for subsequent immunoassays.

However, there are several disadvantages to using antisera as reagents in immunoassays; the titers are low and the supply is limited (Kohler, 1981). Most importantly, the antibodies, while specific for a single determinant, are nevertheless inherently heterogeneous because they are produced by a variety of B-lymphocyte cells (Pestka, 1988). This heterogeneity makes it difficult to reproduce a specific antibody in a new animal.

To overcome these problems, Kohler and Milstein (1975) have established the method by which specific antibody-secreting cell lines can be obtained by the hybridization of immune mouse spleen cells with mouse myeloma cells, followed by growth in selective media. Their contribution to

hybridoma technology has made it possible to immortalize and propagate individual antibody-forming cells. This has allowed the generation and characterization of homogeneous monoclonal antibodies (MAbs) specific for a variety of antigens.

In an effort to enhance specificity, two researchers (Candlish et al., 1986 and Rousseau et al., 1987) have developed ELISA and RIA methods respectively, employing MAbs against OA. Although RIA presents a specific and rapid alternative to the traditional chromatographic detection methods, the health risks imposed by the use of radioactivity have limited its widespread application. This particular health risk is eliminated when ELISA detection methods are used.

The purpose of this thesis is to develop an MAb against OA (OA-MAb). The production, purification and characterization of a highly specific OA-MAb are described. This constitutes the first step towards the development of an ELISA kit to routinely screen grain samples.

REVIEW OF LITERATURE

Introduction

Foods spoiled by moulds have always presented a health risk to the consumer through consumption of potentially carcinogenic mycotoxins or other secondary fungal metabolites. Unfortunately, the broad implications of this problem were not recognized until the largest world outbreak of a mycotoxicosis occurred in the Soviet Union during World War II. Humans eating mouldy, over-wintered grain suffered severe dermal necrosis, haemorrhages, leukopenia and bone marrow destruction (Ciegler, 1975).

Yet it was only 30 years ago that the scientific community really focused attention on the mycotoxin problem. In 1961, almost one hundred thousand turkeys died following the consumption of aflatoxin-rich peanut meal (Blount, 1961). Interest in this new area grew rapidly over the succeeding decades, as reflected by the 6,048 papers on mycotoxins listed in the Chemical Abstracts for the 1967-90 period. According to Fischbach and Rodricks (1973), mycotoxins had become one of the world's most serious food contaminants.

By the late 1970s, seven mycotoxin classes (aflatoxins, ochratoxin A, patulin, zearalenone, tricothecenes, citrinin and penicillic acid) were considered significant natural contaminants of foods and feeds (Jelinek et al.,

1989). Among the toxins listed, ochratoxins attracted the attention of scientists because of their high toxicity and widespread occurrence in many food commodities (Chu, 1974b). Research on ochratoxins continued throughout the 1980s as more sensitive and innovative detection methods were introduced. The occurrence, toxicity and detection methods of ochratoxin A (OA), the most extensively studied compound of this group, are now reviewed.

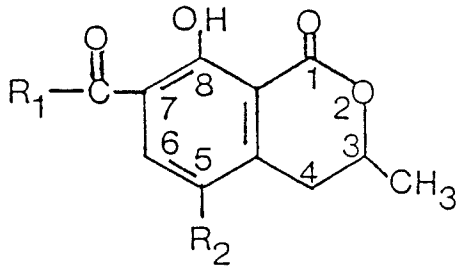
Occurrence

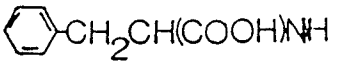
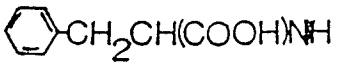
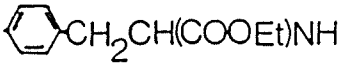
Ochratoxin-producing Fungi

Ochratoxins are the first major group of mycotoxins to be identified following the discovery of aflatoxins (Steyn, 1984). They comprise a group of structurally-related, secondary metabolites produced by several species of the genera *Aspergillus* and *Penicillium* (Rousseau et al., 1985). Their chemical structures are shown in Figure 1.

In 1965, *Aspergillus ochraceus* Wilhelm was identified as an ochratoxin producer during a study on the toxicity of moulds isolated from South African cereals and legumes (Shotwell et al., 1969). Later that same year, van der Merwe et al. (1965) isolated pure ochratoxins A (OA), B (OB) and C (OC) from corn meal inoculated with *A. ochraceus*. This fungal species was associated with the natural presence of OA in corn (Galtier et al., 1977), green coffee beans (Levi et al., 1974) and black pepper (Cristensen, 1975).

Figure 1. The chemical structures of ochratoxins.



	R_1	R_2
OA		-Cl
OB		- H
OC		-Cl
Oα	-OH	-Cl

By 1972, seven other species of *Aspergillus* were identified as OA and OB producers (Chu, 1974b).

Van Walbeek et al., (1969) identified *Penicillium viridicatum* Westling as the first *Penicillium* species to produce OA. *Penicillium viridicatum* was frequently isolated from stored corn (Cristensen, 1975), dried beans (Pohland and Mislivec, 1976), fermented and cured meats (Mintzlaff et al., 1972; Leistuer and Ayres, 1968) and hard cheese (Northolt and Soentoro, 1979). In addition, other *Penicillium* species (*P. cyclopium* and *P. palitans*) were subsequently found to be OA producers.

Both the *Aspergillus* and *Penicillium* subgenera of mould have been widely distributed in the environment (Applegate and Chipley, 1973). Likewise, reports of OA occurrence have been made by researchers from 17 countries. These countries have represented all regions of the world (Scott, 1989).

Natural Occurrence of OA

The first natural occurrence of OA was reported by Shotwell et al. (1969) during a routine U.S.A. corn survey. They found corn samples contaminated with approximately 150 μg of OA/kg. In Canada, this mycotoxin was found at levels ranging from 20 to 2,700 μg/kg in samples of wheat, oat, barley and rye stored under damp conditions (Scott et al., 1970; Scott et al., 1972).

In Denmark, OA-contaminated samples of barley and oats were observed at levels as high as 27,500 μg/kg (Krogh, 1977). Most of the cereal samples reported were collected from lots which were intended to be used as animal feed. In addition, animal feed contaminated with OA was reported in Australia, Canada, Poland, Sweden and Yugoslavia at levels approximating 1,000 μg/kg (Scott, 1989). Reports of OA in meat and meat products indicated that when the OA contamination of feeds was significant, this mycotoxin could be found in swine kidney and smoked meat products at levels ranging from 2-920 μg/kg (Scott, 1989).

The most recent reports of OA contamination include its presence in pig serum from a western Canadian slaughter house at levels greater than 10 ηg OA/ml serum (Marquardt et al., 1988) and in commercial roast coffee, purchased from a Japanese market, at levels ranging from 3-17 μg OA/kg (Tsubouchi et al., 1988). The widespread contamination of OA has been demonstrated by the literature cited. Of the ochratoxins identified, OA is the most potent toxin.

Toxicity

Ochratoxin A, the 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methyl isocoumarin amide of L-β-phenylalanine (Fig. 1), has been established as the major and most toxic metabolite of the ochratoxin family (van der Merwe et al., 1965). The toxicity of OA to a variety of animals has been well

documented over the past two decades. Incidents and possible mechanisms of OA toxicity have been reported by researchers worldwide.

Acute Toxicity of OA

The acute clinical toxicity of OA was extensively studied in chicks, rats, swine, sheep and rainbow trout (Chu, 1974b). Although the OA toxicity to one day-old chicks, through feeding trials, approximated the toxicity level of aflatoxin B_1, the lethal dose of OA differed from species to species (Chu, 1974b). The LD_{50} values for orally administered OA varied from 2.1 to 4.7 mg/kg for chicks, swine and trout (Ueno, 1985). OA was also reported as a teratogen and carcinogen to rodents (Ueno, 1985).

The main pathological changes associated with fatal OA dosing were confined primarily to kidney necrosis with extensive damage to renal tubules. Enteritis and necrosis of liver cells were also observed (Chu, 1974b). Other symptoms, including bone abnormalities, intestinal lesions and immunosuppression were noted (Chu, 1974b). According to Fukui et al. (1987), OA accumulated in the kidney, liver, blood, brown fat, gastro-intestinal and lymphatic tissues.

Chronic Toxicity of OA

Incidents of natural OA intoxication, through consumption of contaminated feed, were observed in swine and poultry. In Denmark, pigs fed mouldy cereal feeds developed severe renal lesions including degeneration

of the proximal tubules and interstitial fibrosis (Elling, 1977). Those epidemic outbreaks were always associated with extreme weather conditions. Thus a positive correlation was observed between the prevalence of porcine nephropathy and the frequency of OA contaminated feed (Krogh, 1977).

Diseases arising from OA intoxication have become a serious problem for the poultry industry. Four outbreaks, involving two million chickens, were attributed to the consumption of OA-contaminated feed (Steyn, 1984). A research group at North Carolina State University reported that OA was the most potent mycotoxin studied in chickens on the basis of LD_{50} dose and minimal growth response (Huff et al., 1975).

The most compelling aspect of OA intoxication has been its association with Balkan endemic nephropathy. This fatally chronic human disease has been characterized by contracted kidneys, tubular degeneration and interstitial fibrosis (Elling, 1977). Although of unknown etiology, the symptoms resemble porcine nephropathy. Since OA is considered the prime disease determinant of porcine nephropathy, a causal relationship between OA and Balkan nephropathy has been suggested (Krogh, 1977).

Mechanisms of Toxicity

Although many incidents of OA intoxication have been reported, the exact mechanism of toxicity has not been elucidated. Chu et al., (1972) postulated that the toxic effect of ochratoxins resulted from the interaction of their phenolic hydroxyl group with proteins and enzymes 'in vivo'. The

degree of toxicity was closely related to the acid dissociation constant (k) of each ochratoxin's phenolic hydroxyl group. For example, the toxicity and k of OB were both ten times less than the toxicity and k of OA.

Chu et al., (1972) also speculated that the chlorine atom in position 5 associated with the isocoumarin moiety of OA (Fig. 1) played an indirect role in ochratoxin toxicity. He suggested that the chlorine atom might have had a direct effect on the dissociation of the phenolic hydroxyl group in OA and OC, thus rendering them toxic. This idea was further supported by the fact that OB, the dechlorinated derivative of OA, was relatively non-toxic. However the exact mechanism by which the chlorine atom effected dissociation was not described.

It was shown that OA bound to plasma albumin 'in vivo' (rats), thus reducing its clearance from the bloodstream and consequently enhancing toxicity (Kumagai, 1988). Research by Chu (1974a) revealed that the strong binding of OA to bovine serum albumin (BSA) may have been partially due to the dissociation of its phenolic hydroxyl group. He suggested that the failure of Oα and Oβ (the hydrolysed products of OA and OB respectively) to induce toxicity in test animals may have been due to their ineffectiveness in binding proteins. He concluded that both ionic and hydrophobic forces were important in the binding of ochratoxins to BSA.

A recent study by Ueno (1985) stated that the kidneys were the sole target organ following exposure to naturally occurring levels of OA. The

proximal tubule of the nephrons was the primary target site. He suggested that OA interfered specifically with the anion transport mechanism of the proximal tubules through the release of membrane-bound alanine aminotranspeptidase.

Ueno (1985) further reported that OA was a potent inhibitor of protein synthesis in bacterial and mammalian cells. He suggested that phenylalaninyl-tRNA synthetase recognised the L-phenylalanine moiety of OA which resulted in the competitive inhibition of this enzyme's activity. Also, the immunosuppressive effects of OA may have resulted from this competition between OA and phenylalanine. Finally, immunosuppression by OA might have caused cancer in mice through the promotion of endogenous tumour cells.

Although various mechanisms for OA toxicity have been described, more research is needed in this area. The proliferating number of reports concerning OA occurrence and toxicity have forced researchers to develop sophisticated analytical techniques for mycotoxin detection and control. The evolution of these detection methods is now reviewed.

Detection Methods

The widespread occurrence and noted toxicity of OA have prompted researchers to develop rigorous detection methods for its control. Sensitive and accurate analytical procedures have evolved to accommodate survey, screening and enforcement programs. Biological and chemical techniques

have been used to analyze a variety of agricultural commodities for OA contamination.

Biological Techniques

Bioassays were once the exclusive methods employed for the determination of mycotoxicity. These general methods were used to study any mycotoxin. Many organisms, including aquatic and terrestrial animals, were the subject of biological analyses. For example, the brine shrimp larva test, described by Harwig and Scott (1971), was a popular bioassay because of its simplicity and rapidity. Unfortunately it was relatively insensitive to any mycotoxin that was only slightly water soluble.

Another method, the chick embryo test, became the most frequently used bioassay because of its sensitivity to a number of mycotoxins, including OA (Van Egmond, 1984). Chick embryo air cells were injected with extracts of a specific mycotoxin. After three to four weeks of incubation, the number of surviving chicks was calculated. Although the methodology was simple, the long incubation period required for this bioassay was a major disadvantage.

Thus bioassays were a set of simple and inexpensive methods for determining mycotoxicity. Unfortunately these methods were hindered by "their lack of specificity and sensitivity, the large variability of inherent bio-responses and the frequent occurrence of false-positive results given by food extracts" (Morgan et al., 1985). For these reasons, bioassays were useful when the screening of commodities for known or unknown mycotoxins was desired.

However, when the surveillance of commodities for known mycotoxins was necessary, a more specific, sensitive and reproducible set of assays was required.

Chemical Techniques

The limitations associated with bioassays led to the development and application of chemical techniques for OA detection and determination. The most popular of these have included primarily thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). However, recent advances in immunochemical techniques, including radioimmunoassays (RIA) and enzyme-linked immunosorbant assays (ELISA), have provided a novel alternative to the more traditional testing methods.

Regardless of the chemical technique employed, all assays require mycotoxin extraction and sample clean-up. Ochratoxin A has been extracted from a variety of agricultural commodities including, barley (Nesheim et al., 1973), flour (Osborne, 1979) and corn, peanuts and rice (Valente Soares and Rodriguez-Amaya, 1985). The various organic solvents used for the extractions have included; water-chloroform (Nesheim et al., 1973), water-acetonitrile (Hunt et al., 1978) and phosphoric acid-chloroform (Morgan et al., 1983).

Sample clean-up has been necessary to remove lipids and other interfering substances from the extract. This has been achieved by either solvent partitioning (Valente Soares and Rodriguez-Amaya, 1985) or affinity

chromatography (Rousseau et al., 1985). A combination of both methods (Lee and Chu, 1984) has also been used to clean-up samples of extracted OA.

Chromatographic methods. Before the sample was analyzed by TLC or HPLC, further purification was necessary. For example, the TLC method described by Nesheim et al., (1973) required the separation of OA and OB acids and their ethyl ester derivatives from the extract. This was followed by further purification of the derivatives on a silica gel column equilibrated with an aqueous sodium bicarbonate-methanol mobile phase. The purified samples were quantitated on TLC plates by measuring fluorescence intensity. The derivatives confirmed the presence of OA and OB. Although the lowest detection limit was 12 μg OA/kg barley, the total analysis took five hours to complete.

The reverse-phase HPLC method described by Osborne (1979) required the extensive preparation of OA samples extracted from various bakery products. A two-step extraction procedure with aqueous sodium bicarbonate-HCl buffer and chloroform was used for this purpose. The extracted samples were subjected to HPLC analysis and quantitated with a fluorescence detector.

This method displayed an overall sensitivity of 0.5 - 1.0 μg OA/kg bakery product with excellent reproducibility. However, the sample preparation was extensive and required trained personnel. Since the samples

were analyzed consecutively, rather than concurrently, much time was required for this method.

Thus, the more traditional chromatographic techniques for OA detection were hindered by expensive, tedious and time-consuming methodology. It was suggested that all of these limitations could be overcome with the further development and application of immunochemical techniques. The novel RIA and ELISA methods promised sensitivity, specificity and high sample throughput.

Immunochemical techniques. Immunochemical techniques were first developed by clinicians and used primarily for therapeutic and diagnostic purposes. Advancements in methodology, coupled with more stringent regulatory controls, led to the adoption of immunoassays by various food science disciplines. The sensitivity and specificity of immunoassays were particularly suitable for the detection of low molecular weight compounds such as; drug residues, pesticides, plant hormones and mycotoxins (Newsome, 1986).

All immunoassays have been based on the ability of an antibody to bind a specific antigen. The binding of an antibody to a specific antigen has often been referred to as a 'lock and key' interaction. This 'lock and key' interaction of antibody for mycotoxin has been quantitated by labelling the antibody with either radioisotopes (RIA) or enzymes (ELISA).

Chu et al., (1976) was the first researcher to describe the production of antibodies against OA. Antisera were produced in rabbits by multiple injections of an OA-bovine serum albumin (BSA) conjugate. The conjugation of OA to a macromolecular protein carrier (BSA) was necessary to render the low molecular weight mycotoxin immunogenic. Other protein carriers have included ethylenediamine-modified BSA (Lee and Chu, 1984), keyhole limpet hemocyanin (KLH; Morgan et al., 1983) and γ-globulin (Candlish et al., 1986).

Bovine serum albumin offers the advantages of availability, good immunogenic characteristics and known physiochemical properties (Chu, et al., 1982). Since BSA always renders its conjugating toxin immunogenic, it is more reliable than the synthetic polypeptide - polylysine (Erlanger, 1980). It is also less expensive than hemocyanin.

The actual site of conjugation between OA and BSA is primarily through the mycotoxin's highly reactive carbonly group located on the side chain of R_1 (Fig. 1; Chu, 1984). This reactive group enables OA to be directly conjugated to BSA without any prior derivatization (Chu, 1984). In addition, the phenolic hydroxyl group in the position 8 (Fig. 1) is recognized as a possible secondary coupling site for OA (Chu, 1974). The water-soluble carbodiimide (Chu et al., 1976) and mixed anhydride (Chu et al., 1982) methods are the two most commonly used techniques to conjugate a

mycotoxin to a protein carrier. Of the two, the mixed anhydride method generally couples more toxin to protein (Chu et al., 1982).

Typically, the OA polyclonal antibodies were separated from the rabbit antisera by ammonium sulfate precipitation and further purified by ion-exchange chromatography using diethylaminoethyl cellulose (Chu et al., 1976). The removal of irrelevant antibodies, through fractionation and purification, was necessary to prevent reaction of the OA antibodies with serum albumins. This procedure was used to concentrate the OA antibodies. Pestka et al., (1981) and Rousseau et al., (1986) described a similar purification procedure.

The specificity of the OA antibodies produced by Chu et al., (1976) was determined by an RIA using ^{3}H-labelled OA. The standard curve resulting from the competition of OA antibodies with ^{3}H-labelled OA displayed a lowest detection limit of 2.5 ηg OA per assay. When the OA antibodies from a similarly prepared antisera were incorporated in an ELISA method (Pestka et al., 1981), the lowest detection limit was 25 ρg OA per assay.

The major advantage associated with the ELISA method was the absence of hazardous radioisotopes. This made the ELISA a much safer detection method than the RIA. Also, the ELISA method was simple, employed stable reagents and did not require highly trained personnel nor expensive equipment (Pestka, 1988).

The first reported application of immunoassays for the analysis of OA in biological material was Morgan et al.'s (1983) ELISA to determine OA in barley. Their OA polyclonal antibodies were diluted 1 to 25000 (v/v) to generate a standard curve with a lowest detection limit of 60 ηg OA/kg barley. The high dilution of antiserum was important to eliminate non-specific effects (Morgan et al., 1985).

Other ELISA methods to analyze biological materials included the detection of OA in wheat (Lee and Chu, 1984) and in pig kidneys (Morgan et al., 1986). While other RIA methods included the detection of OA in barley (Rousseau et al., 1985) and in pig serum (Rousseau et al., 1986). All of these immunoassays used antisera raised in rabbits.

According to Chu (1984), the improvement and extent of immunoassays used for detecting mycotoxins in foods depended primarily on the availability of antibodies. Therefore a more efficient method for antibody production was needed. The advancements made in hybridoma production, initiated by Kohler and Milstein (1975), provided an alternative method for OA antibody production.

The fusion of immunized mouse spleen cells with mouse myeloma cells to produce hybridoma cells was described by Kohler et al., (1976). The resulting hybridomas were screened for antibody production. All antibody-secreting hybridomas were repeatedly cloned, using unimmunized mouse spleen cells as feeders, until a stable cell line was produced. The antibodies

resulting from the stable cell line were monoclonal and therefore of superior specificity and longevity than the polyclonal antibodies found in conventional antisera.

The first production of OA-specific monoclonal antibodies (OA-MAb) was described by Candlish et al., (1986). An OA-γ-globulin conjugate was used as the antigen for injection of female BALB/c x NZB F1 hybrid mice. Immunized mouse spleen cells were fused with mouse myeloma cells and the resulting hybridomas were cloned and propagated 'in vivo' as mouse ascites tumours to produce OA-MAb.

An ELISA was used to construct a standard curve with a lowest detection limit of 50 ηg OA/ml. The cross-reactivity of OA-MAb with its analogues was determined as an indication of antibody specificity. The OA-MAb was 150% cross-reactive with OC and 39% cross-reactive with Oα. Since OA-MAb was, by definition, 100% reactive with OA, it was more specific for OC than OA. Thus, although the OA-MAb demonstrated good sensitivity (50 ηg OA/ml) the antibody specificity was variable.

The first application of an immunoassay using OA-MAb to detect OA in biological material was Rousseau et al.'s (1987) RIA to determine OA in porcine kidneys. Although their antibody production methodology was similar to Candlish's, they developed an ELISA method to select a suitable monoclonal antibody that was more specific than the OA-MAb previously reported. Their standard curve revealed a lowest detection limit of 10 ηg

OA/ml. This limit was lower than the one described by Candlish et al., (1986).

The specificity of OA-MAb produced by Rousseau appeared much greater than the specificity demonstrated by Candlish's OA-MAb. The only OA analogue to exhibit any cross-reactivity was OB at 0.0002%. However Rousseau did not test the cross-reactivity of OA-MAb for OC.

Rousseau's RIA, which incorporated an 'in house' preparation of ^{14}C-labelled OA, was able to detect 0.2 ηg OA/g porcine kidney. This sensitivity was significantly greater than the 5 ηg OA/g porcine kidney detection limit expressed by Morgan et al., (1986) using an ELISA with polyclonal antibodies. However, the sensitivity of Rousseau's method required more sample clean-up than Morgan's procedure.

Most recently, Candlish et al., (1988) developed a more sensitive OA-MAb through immunizations of female BALB/c x NZB F1 hybrid mice with OA-KLH conjugates. The improved OA-MAb demonstrated a lowest detection limit of 5 ηg/ml. This was significantly lower than the 50 ηg/ml limit of Candlish's previous OA-MAb and lower than the 10 ηg/ml limit described by Rousseau et al., (1987).

The specificity of the improved OA-MAb was determined through cross-reactivity experiments. The improved OA-MAb demonstrated cross-reactivities of 8.2% with OC and 0.6% with Oα. The specificity of the improved OA-MAb

was high when compared to the cross-reactivity measurements of 150% with OC and 39% with Oα demonstrated by Candlish's previous OA-MAb.

An indirect, competitive ELISA, incorporating the improved OA-MAb, was used to screen barley samples to a detection limit of 5 μg OA/kg. This value was lower than the 60 μg OA/kg barley limit described by Morgan et al's., (1983) polyclonal antibodies. This was the first ELISA method employing MAb to determine OA contamination of barley. As a result of this work, Ramakrishna et al., (1990) have developed an ELISA method to determine the presence of aflatoxin B_1, T-2 toxin and OA in a single extract of barley.

Although the use of monoclonal antibodies has resulted in more sensitive immunoassays, problems regarding MAb production still exist. Unfortunately the production of OA-MAb has remained relatively empirical despite published methodologies. Factors such as interspecies variation in antibody production, inaccurate hybridoma selection procedures and storage methods have influenced immunoassay sensitivity, specificity and reproducibility (Goding, 1980).

For these reasons, immunoassays have remained an interdisciplinary analysis technique. New developments resulting from clinical or pharmaceutical research have been studied by food scientists. However, additional research has been necessary to improve methodologies (Chu, 1984).

A variety of analytical methods was used to detect and determine OA contamination. These methods evolved from bioassays to chemical assays as reports of this mycotoxin's occurrence and toxicity became more pervasive. Finally, the development of very sensitive, simple and rapid immunoassays for OA detection provided results that were unobtainable by conventional chemical procedures (Vaag and Munck, 1987).

MATERIALS AND METHODS

Materials

Chemicals

Citrinin, ochratoxin A (from *A. ochraceus* NRRL 3174), ochratoxin B, ochratoxin C and ochratoxin α were provided by the Animal Science Department, University of Manitoba, Winnipeg, MB. Bovine serum albumin, triethylamine, isobutylchloroformate, pyridine, 1-ethyl-3,3-dimethylamino-propyl-carbodiimide, Fruend's complete and incomplete adjuvants, Tween 20, polyethylene glycol 4000, o-phenylenediamine, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, coumarin, 4-hydroxycoumarin, cinnamic acid, phenylalanine and mouse IgG were purchased from the Sigma Chemical Co., St. Louis, MO. Dimethyl sulfoxide and tetrahydrofuran were purchased from Fisher Scientific Co., Fair Lawn, NJ. Egg-white ovalbumin was purchased from STL Laboratories Inc., Winnipeg, MB. Mouse myeloma cells P3X63-Ag8.653 were purchased from American Type Culture Collection, Rockville, MD. Hypoxanthine, aminopterin, thymidine and RPMI-1640 tissue culture medium were purchased from Flow Laboratories Inc., McLean, VA. Fetal bovine serum was purchased from Bocknek Laboratories, Rexdale, ON. Rabbit anti-mouse IgG:horseradish peroxidase was purchased from Jackson

ImmunoResearch Laboratories Inc., Avondale, PA. Ammonium sulfate was purchased from Mallinckrodt Inc., Paris, KE. Sephacryl S-300 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Molecular weight standards for gel filtration chromatography; thyroglobulin, γ-globulin, ovalbumin, myoglobulin and cyanocobalamin and for SDS-PAGE; myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme were purchased from BIO-RAD Laboratories, Richmond, CA. All other chemicals were of analytical grade.

Plasticware

The 25 cm^2 polystyrene tissue culture flasks and the 24 and 96 well flat bottom polystyrene tissue culture plates were purchased from Corning Glass Works, Corning, NY. The 96 well Falcon Microtest III polyvinyl flexible tissue culture plates (ELISA plates) were purchased from Becton Dickinson and Co., Oxnard, CA. The 1 ml NUNC cryotubes were purchased from A/S NUNC, Kamstrup, Denmark.

Mice

All 4-8 week old female BALB/c mice were obtained from the Animal Distribution Centre, University of Manitoba, Winnipeg, MB.

Production of OA-MAb

Preparation of OA-protein Conjugates

Two ochratoxin A-protein conjugates were prepared. An ochratoxin A-bovine serum albumin (OA-BSA) conjugate was used for mouse immunizations. It was necessary to conjugate OA to the macromolecular protein carrier BSA to render the low molecular weight mycotoxin immunogenic. The OA-BSA conjugate was prepared by the modified carbodiimide method of Chu et al. (1976). Briefly, 40 mg of BSA in 4 ml of 0.1 M NaCl was mixed with 20 mg of OA in 0.5 ml of ethanol and 3 ml of 0.1 M sodium phosphate buffer (NaPB) (pH 7.0) and 150 mg of 1-ethyl-3,3-dimethylamino-propyl-carbodiimide. The solution was adjusted to pH 7.0 and stirred in the dark at room temperature (RT) for 24h. Next, the solution was dialyzed against 0.1M NaPB, pH 7.0, for 24h at RT with three changes of buffer and lyophilized to dryness.

Efficiency of OA-BSA conjugation was assessed by discontinuous, non-denaturing, polyacrylamide gel electrophoresis (PAGE), performed at 20°C, on an LKB 2001 vertical electrophoresis unit. The stacking gel consisted of 4% acrylamide and 1% N,N'-methylene-bisacrylamide (BIS) while the separating gel consisted of 11.875% acrylamide and 0.625% BIS, respectively. Both gels were polymerized by the addition of 0.13 ml of 10% N,N,N',N'-tetramethylethylene-diamine and 0.15 ml of 10% ammonium persulfate per 30 ml of gel solution. Each sample of pure OA, pure BSA, and OA-BSA

conjugate was applied to the gel in a 50% sucrose + 0.1% bromophenol blue dye solution at a protein concentration of 15 μg/well. The gels were run for 3h at a constant current of 30 mA per gel. Upon completion of the run, the gels were scanned with an ultra-violet light source (long wavelength) to determine the relative location of OA in the gel. The gels were then stained in a 0.25% coomassie brilliant blue (CBB) R-250 solution and destained in a solution of 7.5% acetic acid + 20% methanol. After staining, the relative mobility (Rf) of BSA and the OA-BSA conjugate were determined. A successful OA-BSA conjugate was one that fluoresced under ultra-violet light at the Rf value similar to pure BSA.

The ochratoxin A-ovalbumin (OA-OV) conjugate was used for all ELISA assays at a concentration of 20 μg/ml in a 0.1 M sodium carbonate buffer (pH 9.6). This second conjugate was necessary to eliminate the selection of any BSA-specific antibodies which may have been raised during the mouse immunizations. The OA-OV conjugate was prepared by the mixed anhydride method of Chu et al., (1982). Briefly, 50 μl of triethylamine in tetrahydrofuran (THF; 7.5 mg/ml) and 50 μl of isobutylchloroformate in THF (10 mg/ml) were added to a cool solution (ice-salt mixture both at -5°C) containing 1 mg of OA in 1 ml of dry THF. The solution was mixed well, kept at -5°C for 25 min and then added to 5 mg of OV in 3 ml of 33% pyridine in water at 4°C. The coupling reaction was first carried out at 4°-6°C for 30 min and then at RT overnight. The reaction mixture was then

dialyzed against 2 L of distilled water for 3 days, with the water changed daily. The amount of OA bound to OV was determined by directly measuring the absorbance of the OA-OV conjugate at the mycotoxin's absorption maximum of 333 nm. The absorbance value and the molar extinction coefficient of OA (ε = 6100; Chu et al., 1972) were then used to calculate the concentration of OA bound to protein.

Immunization Protocol

Six female BALB/c mice were immunized with the appropriate OA-BSA conjugate at four week intervals. Initially, the conjugate (75 μl; 50 μg of protein) was mixed with an equal volume of Fruend's complete adjuvant and administered to each mouse by an intramuscular injection. The second injection was also performed intramuscularly with Fruend's incomplete adjuvant. Three subsequent injections of conjugate in distilled water (without adjuvant) (150 μl; 50 μg of protein) were performed intraperitoneally. The mice were sacrificed three days after the final injection.

Hybridoma Production

Fusion. Immunized mouse spleen cells were fused with P3X63-Ag8.653 mouse myeloma cells by a modified method of Goding (1982). Approximately 10^8 spleen cells were mixed with myeloma cells at a 4:1 ratio in RPMI-1640 serum-free medium (RPMI incomplete medium) and centrifuged at 1500 x g for 10 min at RT. Unless otherwise stated, all subsequent fusion steps were

performed at 37°C. After the supernatant was removed, 1.0 ml of a 50% polyethylene glycol (PEG) solution, prewarmed to 37°C, was added dropwise to the cells for 1 min. Following a 1 min incubation, the PEG-cell solution was diluted with the rapid addition of 1 ml RPMI incomplete medium. Then, an additional 10 ml of RPMI incomplete medium was added over a five minute time interval. Following a 2 min incubation, the cells were centrifuged at 1000 x g for 10 min at RT. After the supernatant was removed, the cells were resuspended in HAT selective medium (RPMI-1640 medium + 15% fetal bovine serum (FBS) supplemented with 1.0×10^{-4} M hypoxanthine, 4.0×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine) to a concentration of 5×10^{5} cells/ml. These cells were then plated onto 96 well tissue culture plates containing a peritoneal feeder cell suspension of 5×10^{4} cells/well in complete tissue culture medium (RPMI-1640 + 10% FBS). The cells were incubated at 37°C in a 5% CO_2 atmosphere. Two weeks later, the cells were gradually weaned off aminopterin by suspension in HT medium (RPMI-1640 medium + 15% FBS supplemented with 1.0×10^{-4} M hypoxanthine and 1.6×10^{-5} M thymidine). Approximately three weeks after fusion, hybridomas were screened for antibody production by an indirect, non-competitive ELISA.

Cloning. All positive hybridomas were subcloned by the limiting dilution method of Goding (1980) using a peritoneal feeder cell suspension of 5×10^{4} cells/well in complete tissue culture medium. Cloning was

necessary to gradually select and stabilize single cells that secreted antibodies specific for OA. Typically, a 10 μl sample of cells was mixed with an equal volume of a 0.1% trypan blue dye + 0.9% NaCl staining solution and counted with the aid of a haemocytometer. Once the cell number was calculated, the remaining cells were distributed onto 96 well tissue culture plates (containing the feeder cells at 100 μl/well) at concentrations of 5, 2.5 and 1 cell/well in complete tissue culture medium. All positive clones were transferred to either 24 well tissue culture plates or 25 cm^2 tissue culture flasks as a means of propagating them for frozen storage or antibody collection, respectively. Typically, the tissue culture fluid from clones grown in culture flasks was centrifuged at 1500 x g for 30 min to remove hybridomas and cellular debris. Aliquots of tissue culture supernatant were stored at -20°C for future use.

Freezing Cells

All positive hybridomas and cell clones were stored in liquid nitrogen for future use. Typically, tissue culture fluid, containing approximately 8 x 10^8 cells, was initially centrifuged at 1500 x g for 15 min at RT. After the supernatant was removed, the cells were resuspended in 4 ml of RPMI-1640 medium containing 20% FBS. Next, 4 ml of dimethyl sulphoxide was added and the solution was quickly aliquoted into 1 ml cryotubes. The cryotubes were then labelled, secured to rods and stored in a liquid nitrogen tank.

Screening of Hybridoma Clones

A modified indirect, non-competitive ELISA method of Candlish et al., (1986) was used to screen hybridomas for OA-MAb production. Prior to the screening procedure, ELISA plates were coated with 100 μl/well (20 μg/ml) of OA-OV conjugate and incubated 1h at 37°C and then overnight at 4°C. The plates were then used immediately or stored for a maximum of one month at 4°C before use. Unless otherwise stated, all subsequent incubations were at 37°C for 1h only. The plate was washed three times with excess 0.15 M phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 (PBS-T) to remove unbound coating conjugate. Next, the plate was incubated with 210 μl/well of a 1% skim milk in PBS solution to block any unbound sites. Except for the protein determination of OA-MAb, this plate preparation procedure was used for all ELISA assays. Following washing, the prepared plate was incubated with 100 μl/well of hybridoma tissue culture supernatant. After washing, the plate was incubated with 100 μl/well of rabbit anti-mouse IgG:horseradish peroxidase conjugate at a dilution of 1:5000 (recommended by the manufacturer) in PBS-T. Then, after a final washing, the plate was incubated at RT with 100 μl/well of enzyme substrate containing 0.4 mg/ml o-phenylenediamine and 0.03% H_2O_2 in 0.15 M phosphate-citrate buffer (pH 5.5). The reaction was stopped 1h later with 4 N HCl (30 μl/well) and the A_{492} was measured with a Titertek Multiscan ELISA reader (Flow Laboratories Inc., McLean, VA). The hybridomas that produced MAb yielding at least 0.600 absorbance units upon reaction with OA

were considered positive. All positive hybridomas were subsequently screened for antibody production against BSA and OV using the indirect, non-competitive ELISA method previously described with one exception. The unprepared ELISA plates were coated with 100 μl/well of BSA or OV rather than the OA-OV conjugate. The hybridomas that produced MAb yielding a minimum value of 0.600 absorbance units upon reaction with OA and a maximum value of 0.200 absorbance units upon reaction with BSA or OV were selected for future work. Likewise, this method was used to screen immunized mouse serum for antibody production against OA, BSA, and OV. The same ELISA method was used to titrate OA-MAb, serially diluted in PBS-T, to a minimum A_{492} value of 0.100 absorbance units.

An indirect, competitive, equilibrium ELISA method was used to construct a standard curve for OA and to determine OA-MAb sensitivity of the tissue culture supernatant. Briefly, samples of pure OA were diluted in PBS-T to achieve concentrations ranging from 20 μg/ml to 0.2 ηg/ml. Each OA sample was mixed with an equal volume of tissue culture supernatant (diluted 1:50 in PBS-T) and immediately added to a prepared ELISA plate at 100 μl/well. The plate was incubated for 1h at 37°C and then overnight at 4°C. The subsequent steps of enzyme addition, enzyme substrate addition and absorbance determination were identical to the indirect, non-competitive ELISA method described above. A standard curve of absorbance values (A_{492}) for OA as a function of its $\log_{10}$ concentration, was constructed. A standard

curve was also constructed to determine the sensitivity of ammonium sulfate precipitated OA-MAb. Although the sensitivity was determined by the procedure described above, samples of pure OA were mixed with equal volumes of ammonium sulfate precipitated OA-MAb diluted 1:200 in PBS-T.

Purification Of OA-MAb

Ammonium Sulfate Precipitation of OA-MAb

The tissue culture supernatant was initially purified by ammonium sulfate [$(NH_4)_2SO_4$] precipitation at 40% (w/v) saturation. Typically, 36 g of $(NH_4)_2SO_4$ was slowly dissolved in 150 ml of tissue culture supernatant at RT and then stored overnight at 4°C to enhance precipitate formation. Next, the precipitate was collected and centrifuged at 3000 x g for 30 min at 4°C. Following centrifugation, the pellet of OA-MAb was resuspended in 5 ml of PBS buffer (pH 7.2) and dialyzed against 2 L of PBS for 48h at 4°C with three changes of buffer. The resulting ammonium sulfate precipitated OA-MAb was stored at 4°C until use.

Gel Filtration Chromatography of OA-MAb

The OA-MAb were further purified by Sephacryl S-300 gel filtration chromatography. A sample (4.5 ml; 27 mg of protein) of ammonium sulfate precipitated OA-MAb was applied to a column (2.5 x 100 cm) that had been equilibrated overnight with PBS buffer (pH 7.2). The column was then developed with the equilibrating buffer at a flow rate of 21 ml/h and 4 ml

fractions were collected. The absorbance of the eluent was continuously measured at 280 nm using the UV-1 single path monitor (Pharmacia LKB, Uppsala, Sweden). The available distribution coefficient (Kav) was determined according to the manufacturer's recommendations using blue dextran and molecular weight standards.

Concentration of OA-MAb

When required, the OA-MAb were concentrated in a 100 ml Amicon cell with a membrane (PM10) having a molecular weight cut-off of 10,000 g/mol (Amicon Canada Ltd., Oakville, ON).

Protein Determination of OA-MAb

The protein concentration of OA-MAb at each stage of the purification process was determined by the modified PIERCE-bicinchonicic (BCA) assay described by Sorensen and Brodbeck (1986). Typically, a 200 μl sample of appropriate OA-MAb was mixed with 10 μl of PIERCE-BCA working solution and incubated for 30 min at 37°C on an unprepared ELISA plate. Following incubation, the A_{592} was measured using a Titertek Multiscan ELISA reader and compared to a set of standards to determine total protein concentration.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of OA-MAb

The degree of purification attained at each stage of the process was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed at 20°C on an LKB 2001 vertical

electrophoresis unit using the discontinuous buffer system described by Laemmli (1979). The stacking gel consisted of 4% acrylamide and 0.1% N,N'-methylene-bisacrylamide (BIS) while the separating gel consisted of 10% acrylamide and 0.4% BIS respectively. Both gels contained 0.1% SDS and were polymerized by the addition of 5 μl N,N,N',N'-tetramethylethylenediamine (TEMED) and 50 μl of 10% ammonium persulfate (AP, stacking gel), or 3.3 μl TEMED and 50 μl 10% AP (separating gel) per 10 ml of gel solution. The OA-MAb samples and molecular weight standards were diluted 1:1 in a 2x treatment buffer (0.125 M Tris-HCl + 4% SDS + 20% glycerol + 10% 2-mercaptoethanol; pH 6.8) and applied to the gel at a protein concentration of 15 μg/well. The gels were run for 3.5h at a constant current of 30 mA/gel. The electrode buffer contained 0.025 M Tris + 0.192 M glycine + 0.1% SDS (pH 8.3). Following electrophoresis, the gels were stained in a 0.125% CBB R-250 and destained first in a solution of 50% methanol + 10% acetic acid for 1h and then in a solution of 5% methanol + 7% acetic acid overnight.

Enzyme-linked Immunosorbant Assay Analyses of OA-MAb

Purification process. Monoclonal antibody activity at each stage of the purification process was monitored by the same indirect, non-competitive ELISA method used to screen hybridomas (methodology previously described).

Stabilization study. Monoclonal antibody activity of the OA-MAb purified by gel filtration chromatography was monitored over a two month,

4°C storage period using the same indirect, non-competitive ELISA method used to screen hybridomas (methodology previously described). The five samples assayed were; control (antibody alone), antibody + 5% or 10% glycerol (w/v) and antibody + 5% or 10% sucrose (w/v). The samples were assayed four times during the storage period. The results of each assay were expressed as the percent absorbance of each sample relative to the sample yielding the highest absorbance value. The percent relative absorbance value of each sample was directly proportional to its monoclonal antibody activity.

Characterization of OA-MAb

Immunoglobulin Classes and Subclasses Determination of OA-MAb

The isotyping of monoclonal antibody immunoglobulin classes and subclasses was performed on antibodies from tissue culture supernatant and ammonium sulfate precipitated OA-MAb using the mouse monoclonal isotyping kit (Serotec Ltd., Oxford, UK). The samples were diluted 1:10 in PBS-T and screened against anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM antibodies. An agglutination reaction between the sample and a specific anti-mouse antibody revealed the immunoglobulin class and subclass.

Molecular Weight Determination of OA-MAb

The molecular weight of the OA-MAb was determined by gel filtration chromatography on a Sephacryl S-300 column (methodology previously described). A calibration curve was constructed according to manufacturer's

recommendations using blue dextran and the five chromatographic standards listed in the Chemicals section of Materials and Methods. The available distribution coefficient (Kav) of the purified sample was then used to calculate the molecular weight of OA-MAb.

Specificity of OA-MAb

As an indication of monoclonal antibody specificity, the cross-reactivity of ammonium sulfate precipitated OA-MAb with various test compounds was studied. The antibody specificity was determined using a modification of the indirect, competitive equilibrium ELISA method used to determine sensitivity (methodology described earlier). The test compounds were; aflatoxins B_1, B_2, G_1 and G_2, cinnamic acid, coumarin, 4-hydroxycoumarin, phenylalanine and ochratoxins A, B, C and α. Each test compound (100 μg/ml in PBS-T) was mixed with an equal volume of ammonium sulfate precipitated OA-MAb (100 μg/ml in PBS-T) and immediately added to prepared ELISA plates at 100 μl/well. The plates were incubated for 1h at 37°C and then overnight at 4°C. All other assay steps were as previously described. The extent of each compound's cross-reactivity with ammonium sulfate precipitated OA-MAb was expressed as their percent absorbance relative to pure OA.

RESULTS AND DISCUSSION

Monoclonal Antibody Production

Preparation of OA-protein Conjugates

For this study, the water-soluble carbodiimide method of Chu et al., (1976) was used to prepare the OA-BSA conjugate for mouse immunizations. The efficiency of OA-BSA conjugation was assessed by PAGE analysis using samples of OA, BSA, and OA-BSA conjugate (Materials and Methods). Following electrophoresis, the gel was examined under ultra-violet light. The protein band of the OA-BSA conjugate exhibited fluorescence at a relative mobility value similar to that of BSA. This indicated that the conjugation procedure was successful.

The mixed anhydride method of Chu et al., (1982) was employed to conjugate OA to ovalbumin (OA-OV). This conjugate was used to screen antisera and hybridomas for the secretion of antibodies against OA but not against BSA. Moreover, the OA-OV conjugate enabled the uniform and reproducible coating of mycotoxin to ELISA plates. It was essential that the conjugation of either protein not obstruct or alter the antigenicity of OA.

Mouse Immunizations

Six female BALB/c mice were immunized with appropriate OA-BSA conjugates as described in Materials and Methods. All mice produced

antibodies against both the mycotoxin and the protein carrier as determined by the indirect, non-competitive ELISA using either OA-OV or pure BSA as plate-coating antigens. However the antibody ratio of OA to BSA steadily increased following each injection. The results of a typical mouse antibody response to the OA-OV coating conjugate are shown in Table 1.

Following five injections of OA-BSA conjugate over a four month period, one mouse produced the strongest antibody response to OA. This was manifested by an absorbance value of 1.47 as assessed by an indirect, non-competitive ELISA. Subsequently, the mouse was selected for further hybridoma production. Chu et al., (1976) reported that the highest level of OA antibody production was demonstrated by a rabbit that was immunized with five injections of conjugate over a three month period.

Hybridoma Selection and OA-MAb Production

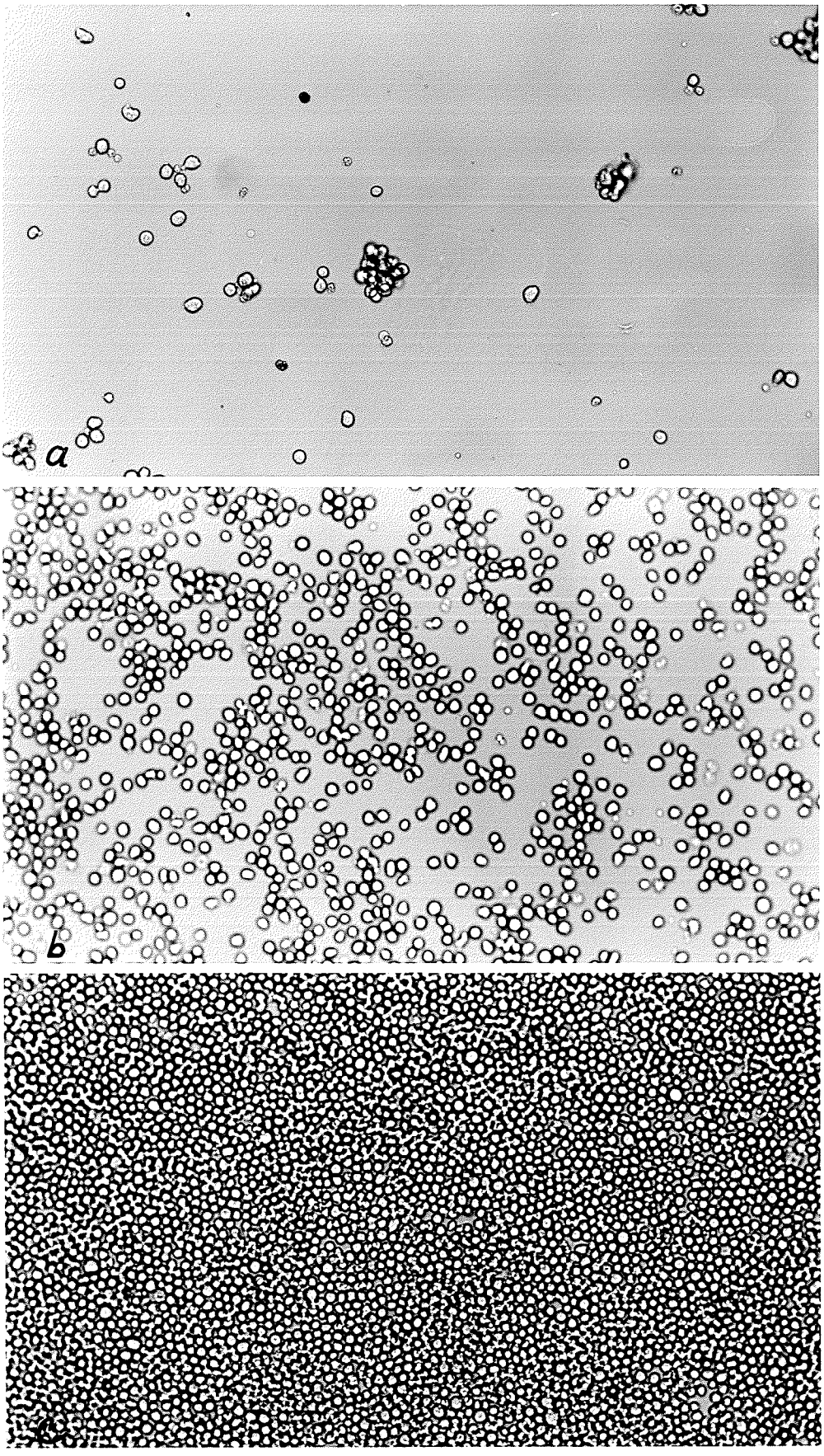
Spleen cells from the selected mouse were fused with myeloma cells. The fusion resulted in 19 hybridoma lines secreting antibodies against OA as determined by an indirect, non-competitive ELISA. The growth of several hybridoma colonies is shown in Figure 2. Most of these hybridomas were either weakly positive or unstable after cloning. In contrast, hybridoma 3D10 was stable and highly positive (typical A_{492} value was approximately 1.2). This hybridoma was cloned twice by the limiting dilution method described in Materials and Methods. The resulting hybridoma, 3D10F10^{2}A11, was propagated in tissue culture flasks to produce a ready supply of OA-MAb.

TABLE 1. Typical mouse serum absorbance values following five injections of OA-BSA conjugate.

Injections and Bleedings	Day	Absorbance (492 ηm)[a]
control (preimmune sera)	0	0
150 μg in FCA (intramuscular injection)	0	---
tailbleeding	10	0.38
150 μg in FIA (intramuscular injection)	28	---
tailbleeding	38	N.A.[b]
150 μg in H_20 (intraperitoneal injection)	56	---
tailbleeding	66	N.A.[b]
150 μg in H_20 (intraperitoneal injection)	84	---
tailbleeding	94	1.19
150 μg in H_20 (intraperitoneal injection)	112	---
tailbleeding	115	1.47
fusion	115	---

FCA; Fruend's complete adjuvant
FIA; Fruend's incomplete adjuvant
[a]The maximum absorbance value of each sample diluted 1:5 in PBS-T.
[b]Not available since insufficient sample collected for ELISA testing.

Figure 2. Hybridoma growth in tissue culture. a = early stage (5-7 days after fusion), b = mid-stage (10-14 days after fusion), c = late stage (18-21 days after fusion); magnification = 100x.


a
b
c

Assessment of OA-MAb

The OA-MAb secreted in tissue culture supernatant was assayed for dilution titer, protein concentration and immunoglobulin classes and subclasses. An indirect, non-competitive ELISA was used to titrate the OA-MAb to a dilution of 1:3200. This value was low when compared to the titer value of $1:10^5$ reported by Rousseau et al., (1987). However, their antibodies were produced 'in vivo' as ascitic fluid which, according to Kohler (1981), often gave titer values in the range of 10^2 - 10^3 fold higher than those of tissue culture supernatant.

The protein concentration of tissue culture supernatant was 4.6 mg/ml as determined by a micro-PIERCE method. This was approximately one quarter of the 20 mg total protein/ml value expressed by Rousseau et al., (1987). This low protein concentration value may have also been attributed to OA-MAb production 'in vitro' rather than 'in vivo'.

Only the total protein concentration was assessed because of the lack of a specific quantitative method to determine OA-MAb in tissue culture supernatant. However, according to Harlow and Lane (1988), tissue culture supernatant may contain approximately 0.05 mg/ml of specific antibodies. Therefore, theoretically the OA-MAb should account for about 1% of the total protein concentration.

The OA-MAb immunoglobulin classes and subclasses were determined using the commercially-available, agglutination isotyping kit described in

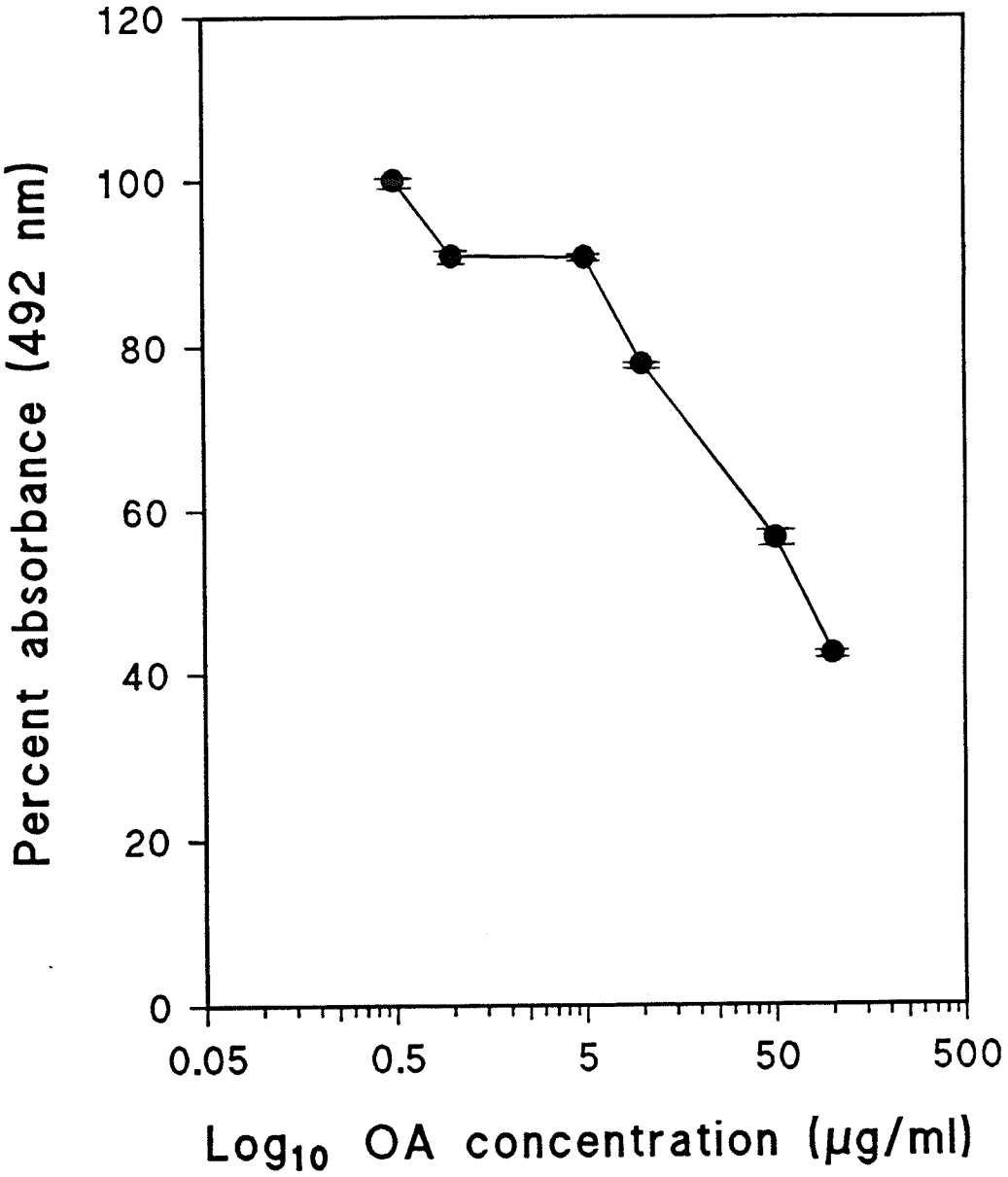
Materials and Methods. The 3D10F10^{2}A11 clone was found to secrete antibodies of the IgM class. Since the OA-MAb did not react with any other immunoglobulin antibody class or subclass, this test was a good indication of the monoclonality of OA-MAb. The OA-MAb produced by Candlish et al., (1986) were also IgM.

Sensitivity of OA-MAb

In an effort to determine antibody sensitivity, the indirect, non-competitive ELISA was used to construct the standard curve for OA, as shown in Figure 3. The lowest concentration of OA that demonstrated a maximum absorbance value significantly different from 100% represented the lowest detection limit of the ELISA and indicated the sensitivity of OA-MAb. The lowest detection limit was 1 μg OA/ml with a working range up to 100 μg OA/ml. The greatest antibody displacement was 57.4% at 100 μg OA/ml. This OA-MAb sensitivity was lower than the 75 ηg OA/ml detection limit reported by Candlish et al., (1986).

The poor performance of the OA-MAb may have been attributed to the batch of OA-OV conjugate used to coat the ELISA plates. The low molecular ratio of OA to OV may have caused fewer OA molecules to bind to the plate. This, in turn, may have reduced the number of potential OA-MAb binding sites thus lowering the overall sensitivity of the ELISA. According to Goding (1980), the screening procedure was the key to success in hybridoma production. For example, a weak coating conjugate may have selected a weak

Figure 3. ELISA standard curve for OA. Values shown are the means of three determinations $\pm$ standard deviations. The tissue culture supernatant was used as a source of OA-MAb.


120
100
80
60
40
20
0
Percent absorbance (492 nm)
0.05
0.5
5
50
500
Log10 OA concentration (µg/ml)

hybridoma. Moreover, such a conjugate may have hindered a strong antibody from detecting antigen. Since the OA-OV coating conjugate could not be replaced, a two-stage purification procedure was developed to enhance OA-MAb performance.

Monoclonal Antibody Purification

Purification of OA-MAb

The purification of IgM monoclonal antibodies has always posed special problems due to their instability and contamination with high molecular weight sera proteins such as α-macroglobulin (Clezardin et al., 1986). Several multistep procedures involving ion-exchange, gel filtration and/or affinity chromatographies have been described for the isolation of mouse IgM. Most of these purification procedures are combined with a preliminary precipitation step (Clezardin et al., 1986).

In this experiment, ammonium sulfate precipitation and gel filtration chromatography were used to concentrate and partially purify IgM monoclonal antibodies. It was essential to choose a method that did not destroy antibody activity by denaturation or aggregation. The first purification step (40% ammonium sulfate precipitation) resulted in the removal of about 85% total proteins present in the tissue culture supernatant (Table 2). This fractionation step also provided an antibody concentrating

effect. As a result, an almost two-fold increase in OA-MAb specific activity was obtained. Moreover, it yielded an almost two-fold increase in purification.

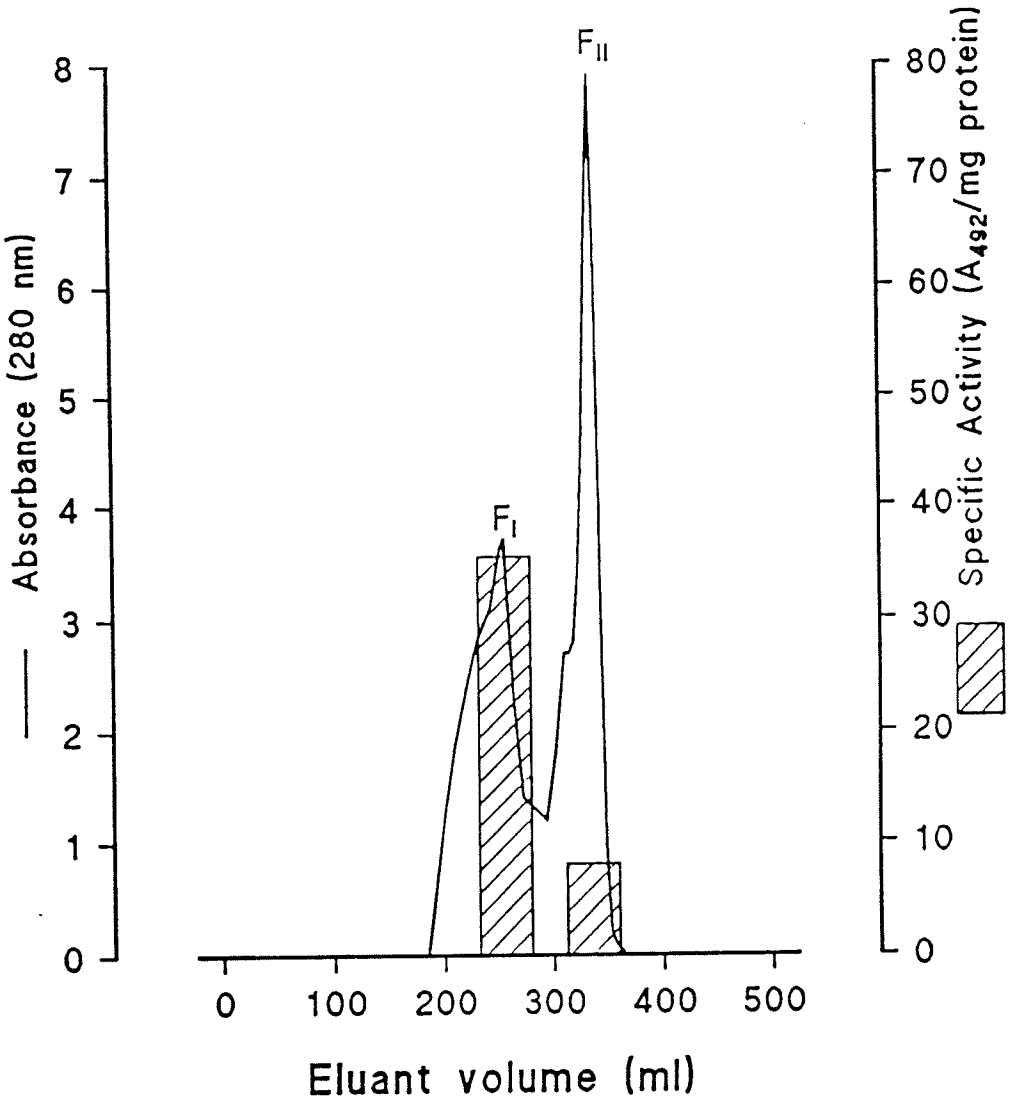
TABLE 2. Purification of OA-MAb by Sephacryl S-300 gel filtration chromatography.

Volume (mL)	Protein (mg/mL)	Absorbance[a] (/100 μL)	Specific Activity[b] (Abs/mg)	Yield[c]	Purification (fold)
Tissue culture supernatant					
123	4.62	0.73[d](0.036)[e]	1.57	100	1
40% ammonium sulfate precipitation					
13.9	6.24	1.73[d](0.035)	2.77	15.26	1.76
Sephacryl S-300 gel filtration fractions					
I 15.21	0.45	1.60[d](0.012)	35.44	1.20	22.56
II 12.50	1.58	1.26[d](0.014)	8.03	3.48	5.11

[a]Absorbance measured at 492 ηm.
[b]Activity measured as Abs/mg total proteins.
[c]Yield calculated as % of total proteins.
[d]Average of triplicate determinations.
[e]Standard deviations in parentheses.

Sephacryl S-300 gel filtration chromatography of the ammonium sulfate precipitated OA-MAb yielded two peaks (Figure 4), designated as fraction I and II. The gel filtration chromatography resulted in the additional removal of 10% total proteins and consequently a 23-fold and five-fold increase in the purification of antibodies was obtained for fractions I and II, respectively.

Figure 4. Sephacryl S-300 gel filtration chromatograph of purified OA-MAb where fraction I = IgM and fraction II = other.

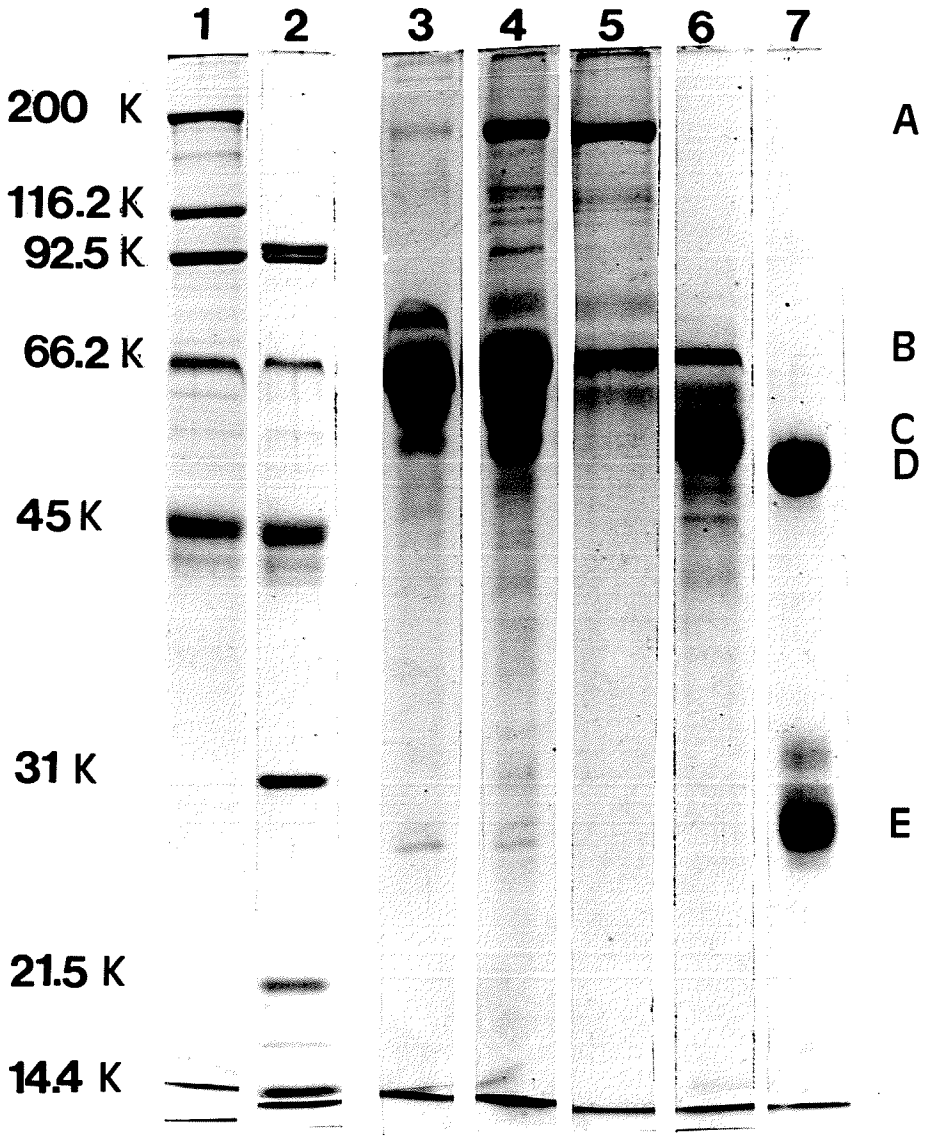

F_II
F_I
Absorbance (280 nm)
Specific Activity (A492/mg protein)
0
1
2
3
4
5
6
7
8
10
20
30
40
50
60
70
80
0
100
200
300
400
500
Eluant volume (ml)

Moreover, OA-MAb specific activities of 35.4 and 8.0 absorbance units/mg of protein were obtained for these fractions. The high specific activity of fraction I indicated that OA-MAb was primarily present in this fraction. Additional confirmation of these findings was obtained by a molecular weight (MW) determination of OA-MAb. It revealed that fraction I had an apparent MW of 1×10^6. This value was approximately the MW of IgM (Kimball, 1986). The low value of antibody activity in fraction II may have been due to the presence of low molecular weight fragments of OA-MAb caused by a partial dissociation of IgM molecules during chromatography.

The purification process was assessed by SDS-PAGE coupled with protein staining (Figure 5). Four bands or groups of bands, referred to as A, B, C, and E, were identified in the original tissue culture supernatant extract (lane 3). A comparison of OA-MAb preparations from different stages of the purification procedure indicated that the A band of low mobility was present in tissue culture supernatant (lane 3), the ammonium sulfate precipitate (lane 4) and fraction I (lane 5), but not in fraction II (lane 6).

The B band could have been the heavy chain of IgM since it had a MW value of 65 K. This value is reported to be the MW of heavy chain IgM (Roitt et al., 1989). This band was present in all four preparations. It was most visible in the fractions obtained after gel filtration chromatography. As stated earlier, the presence of the 65 K protein band in fraction II may

Figure 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of OA-MAb. Lanes: 1 = high molecular weight standards, Bio-Rad, 2 = low molecular weight standards, Bio-Rad, 3 = tissue culture supernatant containing OA-MAb, 4 = 40% ammonium sulfate precipitated OA-MAb, 5 = fraction I of OA-MAb separated by Sephacryl S-300, 6 = fraction II of OA-MAb separated by Sephacryl S-300, 7 = commercial IgG, Bio-Rad.


1
2
3
4
5
6
7
200 K
116.2 K
92.5 K
66.2 K
45 K
31 K
21.5 K
14.4 K
A
B
C
D
E

have been due to a partial fragmentation of IgM molecules during gel filtration chromatography.

The group C bands, represented as a cluster of bands, were most apparent in all preparations but fraction I. This moderate mobility group of proteins were probably removed by gel filtration chromatography. The E band could have been the light chain of either IgM or IgG since it corresponded to the MW value of 25 K reported by Roitt et al., (1989). It was present in tissue culture supernatant (lane 3) and ammonium sulfate precipitate (lane 4), but absent in fractions I and II (lanes 5 and 6 respectively). The reason for this is unknown.

Bands D and E (lane 7) represented the heavy and light chains of commercial IgG respectively. The IgG was included during the SDS-PAGE run as a marker. The electrophoretic analysis of fraction I (lane 5) revealed that most of the low molecular weight proteins were removed during gel filtration chromatography. However some high molecular weight contaminating proteins (band A, lane 5) were still present in this fraction.

Stabilization Study of OA-MAb

A two week storage of gel filtration purified OA-MAb at 4°C resulted in a 50% decrease in antibody activity as assessed by an indirect, non-competitive ELISA (results not shown). This was not surprising since immunoglobulin M was known to aggregate into large complexes unless stabilizers were added to the storage medium (Hansson and Nilsson, 1973).

It has been recommended to store solutions of purified IgM with the addition of other proteins (eg. BSA; Harlow and Lawe, 1988). Moreover, other stabilizers such as polyols (eg. glycerol; Back et al., 1979), aminoacids (Hansson and Nilsson, 1973) and sucrose (Lee and Timasheff, 1981) have been used to protect high molecular weight proteins against loss of solubility and/or denaturation during storage. Since BSA and aminoacids were relatively more expensive than polyols and sugars, sucrose and glycerol were chosen as stabilizers.

Figure 6 illustrated a time effect on OA-MAb activity during storage at 4°C in the presence of 5% and 10% glycerol (w/v). The results suggested that very little antibody activity was lost over a two month storage period when 5 or 10% glycerol was added to the medium. In contrast, the untreated antibody sample (control) retained only about 36% of its initial activity during the same time period.

The activity of OA-MAb in the presence of sucrose is shown in Figure 7. A two month storage of antibodies in the presence of either 5% or 10% sucrose (w/v) retained almost 100% of the initial antibody activity. In contrast, a 60% decrease in antibody activity was observed for the untreated OA-MAb control sample during the same time period.

The storage of ammonium sulfate precipitated OA-MAb at 4°C for two weeks in PBS buffer (pH 7.2) resulted in a 25% decrease in antibody activity (results not shown). This value was one half of that observed for the OA-MAb purified by gel filtration chromatography.

Figure 6. A time storage effect on the purified OA-MAb activity at 4°C in the presence of glycerol. Where; ● = control without glycerol, ▲ = in the presence of 5% glycerol (w/v), ■ = in the presence of 10% glycerol (w/v).

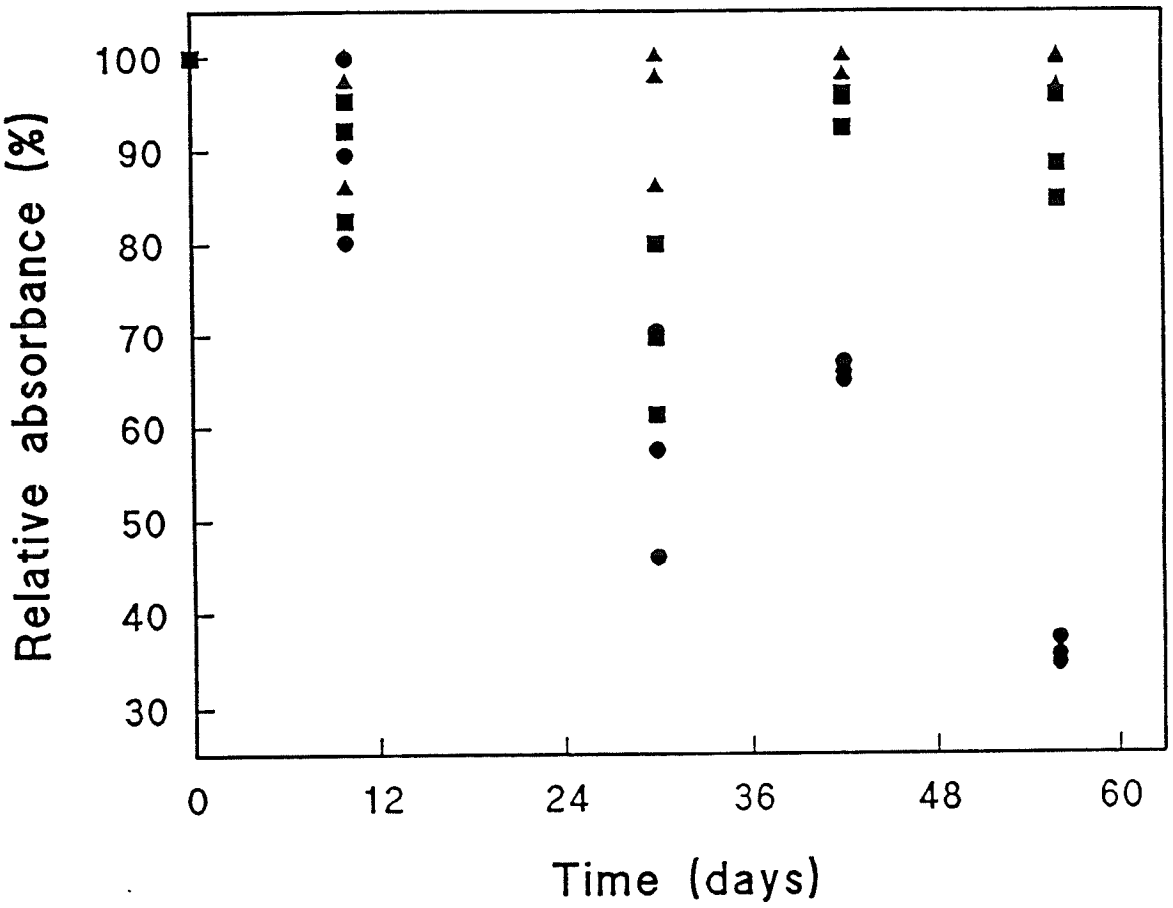
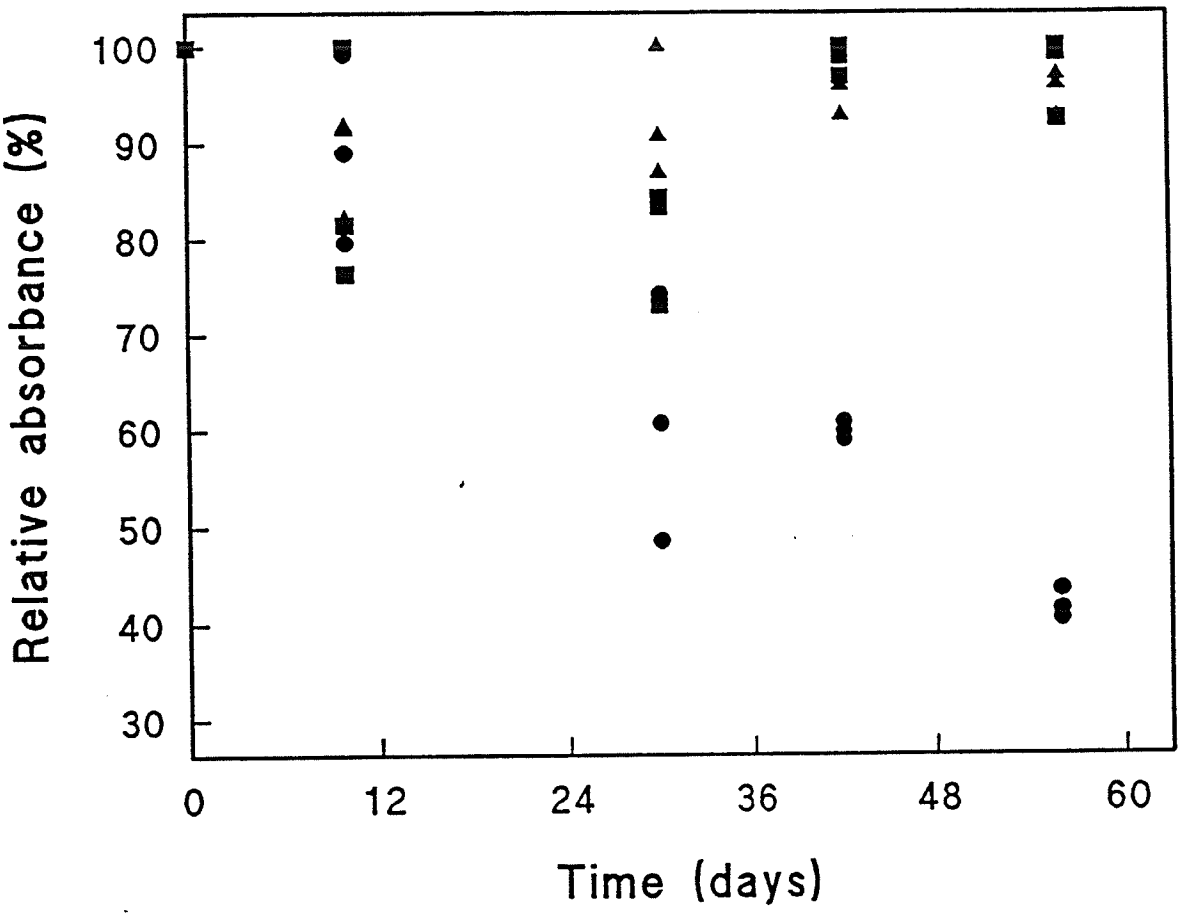

Relative absorbance (%)
100
90
80
70
60
50
40
30
0
12
24
36
48
60
Time (days)

Figure 7. A time storage effect on the purified OA-MAb activity at 4°C in the presence of sucrose. Where; ● = control without sucrose, ▲ = in the presence of 5% sucrose (w/v), ■ = in the presence of 10% sucrose (w/v).


Relative absorbance (%)
100
90
80
70
60
50
40
30
0
12
24
36
48
60
Time (days)

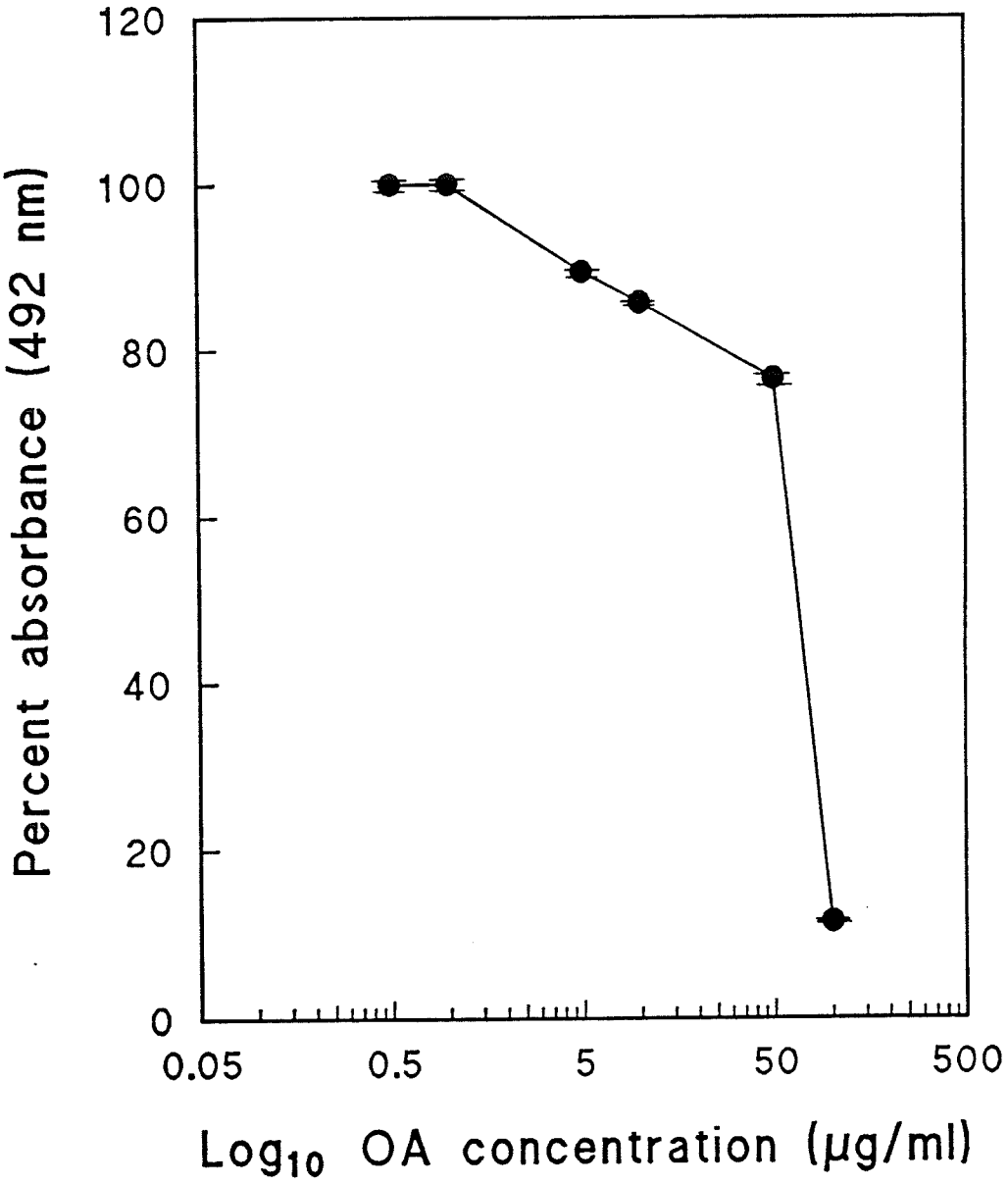
Sensitivity of Ammonium Sulfate Precipitated OA-MAb

Although the specific activity of OA-MAb was highest following gel filtration chromatography (Table 2), a two week storage at 4°C resulted in a noticeable drop in antibody absorbance measurements unless stabilizers were added to the storage medium. Because stabilizers may have effected the characteristics of antibodies, ammonium sulfate precipitated OA-MAb was used for further characterization studies. One batch of ammonium sulfate precipitated OA-MAb was used for all subsequent assays.

The OA-MAb was used to construct the standard curve for OA shown in Figure 8. This standard curve was compared to the one constructed earlier using OA-MAb from tissue culture supernatant (Fig. 3) to determine whether ammonium sulfate precipitation had improved antibody sensitivity. A four-fold dilution of the partially purified antibodies was required to construct a standard curve similar to the one produced by the original tissue culture supernatant antibodies (Fig. 3). This was advantageous since less volume of antibody was necessary for each assay.

With a lowest detection limit of 1 μg OA/ml, the antibody displacement at a concentration of 100 μg OA/ml did increase from 57.4% (Fig. 3) to 88.4% (Fig. 8) following ammonium sulfate precipitation. Although the partial purification of OA-MAb did not improve the lowest detection limit of the indirect, competitive ELISA, it did improve the sensitivity of this assay within its working detection range.

Figure 8. ELISA standard curve for OA. Values shown are the means of three determinations $\pm$ standard deviations. The ammonium sulfate precipitate was used as a source of OA-MAb.


120
100
80
60
40
20
0
Percent absorbance (492 nm)
0.05
0.5
5
50
500
Log10 OA concentration (µg/ml)

Monoclonal Antibody Characterization

Immunoglobulin Classes and Subclasses Determination of OA-MAb

An agglutination isotyping kit was used to determine the OA-MAb immunoglobulin classes and subclasses. The OA-MAb was found to be of the IgM class. This result was similar to that reported by Candlish et al., (1986).

Molecular Weight Determination of OA-MAb

The molecular weight of the partially purified OA-MAb was determined by gel filtration chromatography on a Sephacryl S-300 column. A constructed calibration curve (Materials and Methods) is presented in the Appendix. The chromatographic profile is shown in Fig. 4.

Following chromatography, the tubes corresponding to fraction I were collected and their contents pooled to determine the distribution coefficient (Kav). Calculations using the calibration curve equation [$Y = -0.2476\ X + 1.6871$, $r^2 = 0.9972$] resulted in a molecular weight determination of 1.3×10^6. This experimental value was slightly higher than the theoretical value of 9.7×10^5 quoted by Kimball (1986) for IgM.

Specificity of OA-MAb

An OA-MAb cross-reactivity study, using mycotoxins and structurally-related compounds, was conducted to determine antibody specificity. The compounds tested were aflatoxins B_1, B_2, G_1 and G_2, citrinin, coumarin, 4-

hydroxycoumarin, cinnamic acid, phenylalanine and ochratoxins A, B, C and α. For these studies an indirect, competitive ELISA was used. Although the method was similar to previous cross-reactivity experiments, the definition of cross-reactivity differed from that reported by Morgan et al., (1983) and Candlish et al., (1986).

They defined cross-reactivity as the percentage ratio of the concentration of OA giving 50% maximum absorbance to the concentration of test compound also giving 50% maximum absorbance. Thus the absorbance reading was constant and the compound concentration varied. For this method of analysis to succeed, the ELISA had to be highly sensitive with the detection limit in the range of 10 - 50 ηg OA/ml. Since the indirect, competitive ELISA used for this study demonstrated a sensitivity of approximately 1 μg OA/ml, the cross-reactivity definition had to be modified.

In this study, the cross-reactivity was defined as the percent absorbance of the test compound at 100 μg/ml compared to the absorbance of OA, also at 100 μg/ml, normalized to 100%. Thus, the concentration was constant and the absorbance reading varied. This method of analyzing cross-reactivity data was as described by Yao and Mahoney (1989). All test compounds were measured at 100 μg/ml because of the high degree of antibody displacement (88.4%) observed at this concentration from the OA standard curve (Fig. 8).

The results indicated that there was negligible cross-reactivity between OA-MAb and ochratoxin C, phenylalanine, coumarin, 4-hydroxycoumarin, cinnamic acid and the aflatoxins (Table 3). Significantly higher cross-reactivities were demonstrated for only three of the compounds tested; ochratoxin α (31%), citrinin (23%) and ochratoxin B (19%).

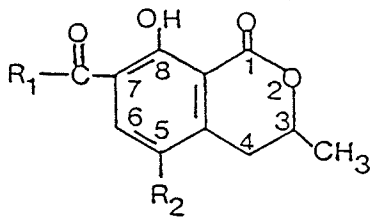
TABLE 3. Cross-reactivity of OA-MAb with various compounds.

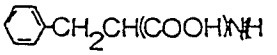
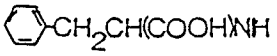
Compounds[a]	% Cross-reactivity
Aflatoxin B_1	4
Aflatoxin B_2	3
Aflatoxin G_1	3
Aflatoxin G_2	4
Citrinin	23
Ochratoxin C	4
Ochratoxin B	19
Ochratoxin α	31
Ochratoxin A[b]	100
Phenylalanine	3
Coumarin	5
4-hydroxycoumarin	5
Cinnamic acid	6

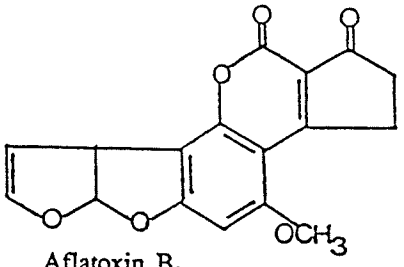
[a]PBS-T solutions (0.1 ml) containing compounds at 100 μg/ml were assayed in each well.
[b]Cross-reactivity expressed with ochratoxin A (100 μg/ml) normalized to 100%. Results represent the average of triplicate determinations.

The structures of all compounds used for the cross-reactivity experiments are shown in Figure 9. The compounds were categorized into three general groups. Group one was all compounds that did not share any

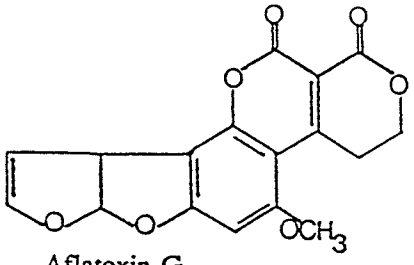
Figure 9. The chemical structures of compounds used for cross-reactivity experiments.



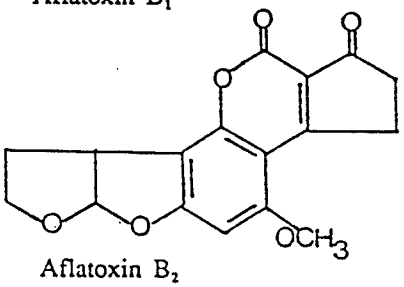
	R_1	R_2
OA		−Cl
OB		−H
OC		−Cl
Oα	−OH	−Cl



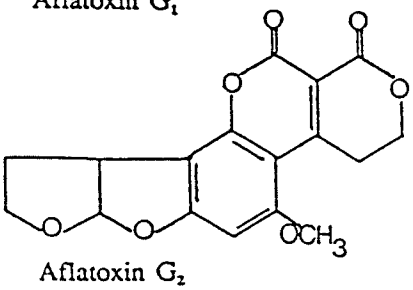
Aflatoxin B_1



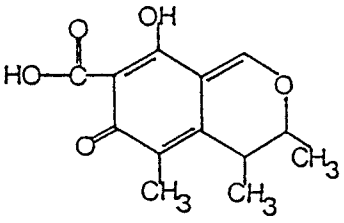
Aflatoxin G_1



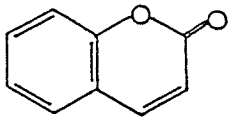
Aflatoxin B_2



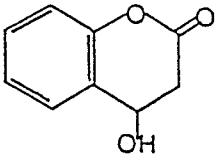
Aflatoxin G_2



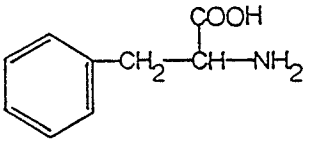
Citrinin



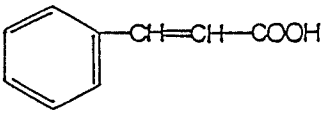
Coumarin



4-hydroxycoumarin



Phenylalanine



Cinnamic Acid

structural similarities with OA (all the aflatoxins). Group two consisted of all compounds that contained a phenylalanine structure (ochratoxins A, B and C, cinnamic acid and phenylalanine). Group three was all compounds that contained an isocoumarin ring (ochratoxins A, B, C and α, citrinin, coumarin and 4-hydroxycoumarin).

Ochratoxin C (OC) was the one compound which exhibited the greatest structural resemblance to OA. The ethyl group present on the phenylalanine moiety of OC was the only structural difference between these two ochratoxins. The structure of ochratoxin B (OB) was similar to OA except for the absence of a chlorine group at position 5 of the isocoumarin moiety. Ochratoxin α, the hydrolyzed product of OA, represented the isocoumarin moiety of OA.

According to Chu et al., (1976) several features of the OA molecule, including the amide bond, chlorinated dehydroisocoumarin residues and the conformation of intact OA, were important for antibody binding. The cross-reactivity experiments performed by Morgan et al., (1983) and Candlish et al., (1988) demonstrated a low antibody binding to Oα and a lack of binding to phenylalanine and various coumarins. They suggested that the complete OA molecule was necessary for antibody binding.

The relatively high cross-reactivity of OA-MAb for Oα (31%) suggested that a particular site on the OA molecule may have predominated in attracting and binding antibody. Thus, the cross-reactivity experiments were

also performed to determine the actual site on OA that was recognized by OA-MAb. Since all three cross-reactive compounds contained isocoumarin rings, the OA-MAb may have recognized this area of OA rather than the phenylalanine moiety or the complete OA structure. The non-reactivity of the phenylalanine test sample supported this hypothesis.

However, since the coumarin and 4-hydroxycoumarin test samples were also non-reactive, the OA-MAb may have recognized groups on the isocoumarin ring that were specific to the cross-reactors only. The carbonyl and hydroxyl groups present at positions seven and eight, respectively (Fig. 9) of the isocoumarin ring may have formed the OA binding site. These groups were present on the cross-reactors but absent on the coumarins.

The non-reactivity of OC may have been due to steric hinderance effects associated with the ethyl group attached to the carbonyl group of the phenylalanine moiety. The ethyl group may have masked or obstructed part of the binding site and thereby prevented OA-MAb recognition and attachment. The marginal cross-reactivity of cinnamic acid could not be explained.

The data collected as a result of cross-reactivity analyses were used to determine the theoretical and practical specificity of OA-MAb. Theoretically, the OA-MAb specificity was acceptable when compared to other researchers' findings. The OA-MAb produced by Candlish et al., (1986) was 150% cross-reactive with OC while our OA-MAb was only 4% cross-reactive and 39%

cross-reactive with Oα (our OA-MAb was 31% cross-reactive). Recently, Candlish et al., (1988) produced an IgG-classed OA-MAb which was only 8.2% cross-reactive with OC and 0.6% cross-reactive with Oα. However the cross-reactivity with OB was not determined.

Of practical importance, the OA-MAb was quite specific since the most cross-reactive compound detected (Oα) was a synthetic metabolite resulting from the hydrolysis of OA and therefore not usually present in the natural environment. The low cross-reactivity with citrinin was considered advantageous for screening grains since this nephrotoxin has frequently been found as a co-contaminant with OA (Jelinek et al., 1989). Likewise, the low cross-reactivity of OA-MAb for OB enabled its incorporation into a grain-screening assay.

CONCLUSIONS AND RECOMMENDATIONS

The purpose of this thesis was to develop a monoclonal antibody against ochratoxin A (OA-MAb). Both the production and purification of OA-MAb were described. Since the stability of isolated antibody was low unless 5% glycerol or 5-10% sucrose (w/v) was added to the storage medium, ammonium sulfate precipitated OA-MAb was used for all characterization studies. Characterization consisted of immunoglobulin classes and subclasses typing, molecular weight determination, antibody sensitivity and cross-reactivity experiments.

The OA-MAb belonged to immunoglobulin class M. This was determined using a commercially-available, agglutination isotyping kit. Since the OA-MAb did not react with any other immunoglobulin class or subclass, this test was a good indication of antibody monoclonality. The molecular weight of OA-MAb, as determined by gel filtration chromatography, appeared to be 1.3×10^6. This experimental value was slightly higher than the theoretical value of 9.7×10^{5} for IgM quoted by Kimball (1986).

The antibody sensitivity and specificity were both determined using indirect, competitive ELISA methods. The OA-MAb displayed a lowest detection limit of 1 μg OA/ml. However the antibody displacement measurement of 88.4%, at a concentration of 100 μg OA/ml, was acceptable for antibody cross-reactivity studies. The degree of antibody cross-reactivity

with other mycotoxins or structurally-related compounds was determined. The OA-MAb displayed excellent specificity since the most cross-reactive compound detected (Oα, 31%) was a synthetic metabolite resulting from the hydrolysis of OA and therefore not present in the natural environment. The low cross-reactivity with citrinin (23%) and OB (19%), compounds frequently found as co-contaminants with OA, was considered advantageous.

The development of an OA-MAb constitutes the first step towards the production of a routine grain screening ELISA kit. Further work in this area will involve refinements and possible modifications to the ELISA method itself. This will include the selection of an appropriate solid support medium and the reproducible production of a sensitive OA-protein plate-coating conjugate.

The resulting ELISA kit must be subjected to rigorous standardization experiments to determine its OA detection abilities in a variety of grains. The preparation of grains for sampling purposes is another aspect of this project that will require additional research. A simple and reproducible OA extraction and cleanup process must be selected which will accommodate different types of grains. The possibility of using an OA-MAb-immunoaffinity column for sample cleanup should be explored.

One of the anticipated problems during the development of an ELISA kit using OA-MAb could be its instability. This monoclonal antibody is of the IgM class and is therefore less stable than an antibody of the IgG class.

It is recommended that a class-switch experiment be performed in future studies. In such an experiment, clones producing IgM antibodies would be induced to produce IgG antibodies through a somatic mutation according to the method of Spira et al., (1985) or Rosen and Klein (1983).

SUMMARY

1. A monoclonal antibody (MAb) against OA (OA-MAb) was produced through mouse immunizations and hybridoma technology.
2. A two-step procedure, involving ammonium sulfate precipitation and gel filtration chromatography, was used to increase the purity of OA-MAb.
3. The chromatographically-purified OA-MAb retained its antibody activity when stored at 4°C for two months in the presence of 5% added glycerol or 5-10% added sucrose (w/v).
4. The ammonium sulfate precipitated OA-MAb was of the immunoglobulin M class with a molecular weight that appeared to be 1.3×10^6.
5. The OA-MAb displayed a lowest detection limit of 1 μg OA/ml with an antibody displacement measurement of 88.4% at 100 μg OA/ml.
6. The OA-MAb demonstrated excellent specificity with cross-reactivity measurements of 31% for ochratoxin α, 23% for citrinin and 19% for ochratoxin B.

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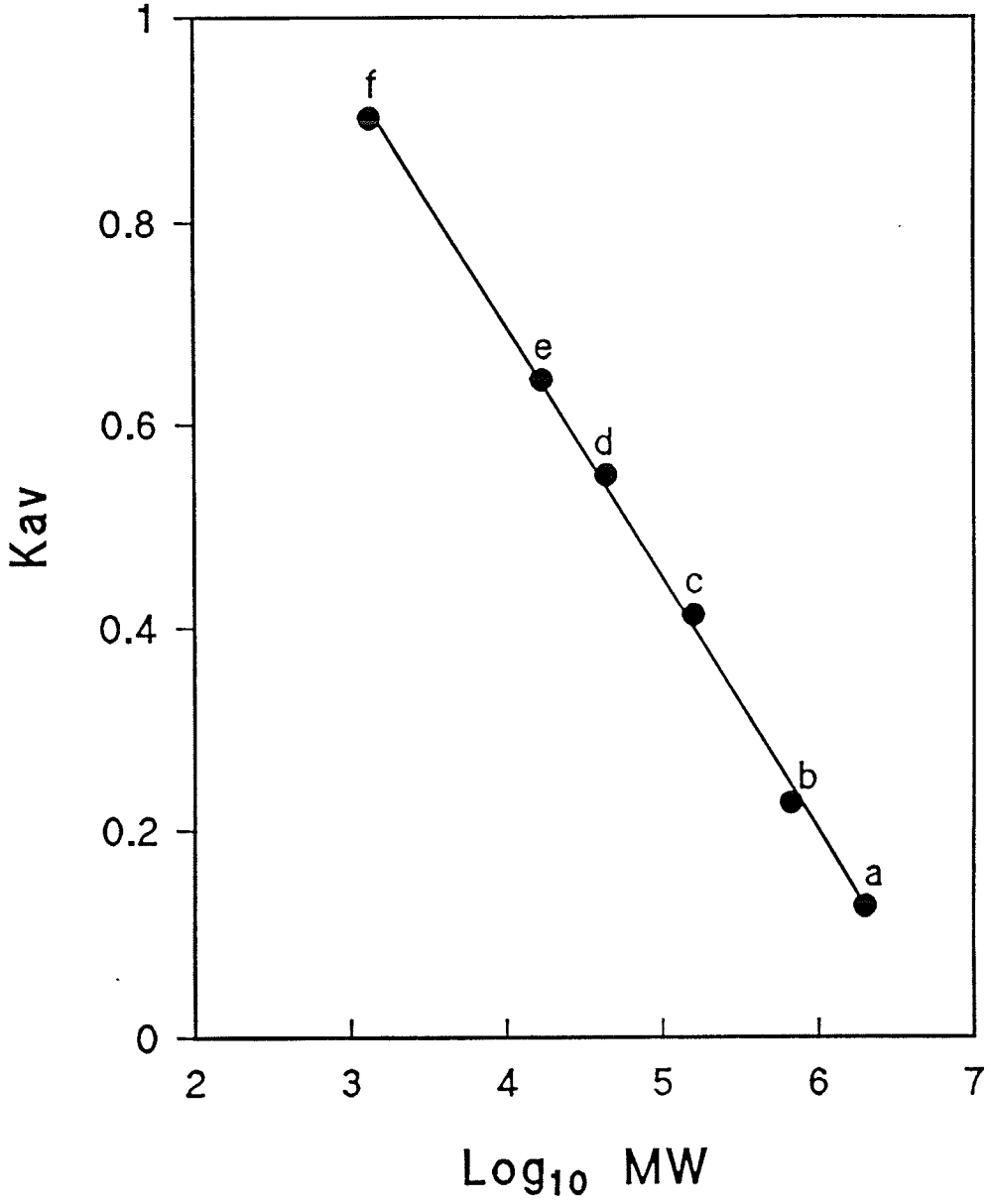
Appendix. Relationship between Kav and $\log_{10}$ MW for chromatographic standards; a = blue dextran (void volume; 2.0×10^6), b = thyroglobulin (bovine; 6.7×10^5), c = γ-globulin (bovine; 1.6×10^5), d = ovalbumin (chicken; 4.4×10^4), e = myoglobulin (horse; 1.7×10^4), f = cyanocobalamin (1.4×10^3). $Y = -0.2476X + 1.6871$, $r^2 = 0.9972$.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