This is to certify that the

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Novel Approaches for the Immunoassay of *Fusarium* Mycotoxins

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Sutikno

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James J Pestka

Dr. James J. Pestka
Major professor

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NOVEL APPROACHES FOR THE IMMUNOASSAY OF FUSARIUM MYCOTOXINS

By

Sutikno

A DISSERTATION

Submitted to
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for the degree of

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1995

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ABSTRACT

NOVEL APPROACHES FOR THE IMMUNOASSAY OF *FUSARIUM* MYCOTOXINS

By

Sutikno

The fumonisin and trichothecence mycotoxins are a group of secondary metabolites produced by toxigenic strains of *Fusarium* fungi. These mycotoxins are commonly toxic to both human and animals and found in agricultural products. The presence of these toxins in agricultural commodities are primarily dictated by environmental and biologic factors. Since controlling these factors is almost impossible, mycotoxin entry into human and animal foods is typically prevented through detection and diversion. Although mycotoxin detection is performed using conventional methods such as high performance liquid chromatography (HPLC), immunoassay techniques such as enzyme-linked immunosorbent assay (ELISA) are inexpensive, simple, rapid, specific, and sensitive. In this thesis, several new approaches for the generation and application of antibodies to *Fusarium* mycotoxins were explored. Firstly, mouse monoclonal, rabbit and sheep polyclonal antibodies against fumonisin B_1 (FB_1) were produced via a novel immunization procedure with keyhole limpet hemacyanin as a protein carrier. These antibodies were used to improve the sensitivity and

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specificity of ELISAs that were previously developed in our laboratory. ELISA sensitivity and specificity were much higher when FB_1 -sheep antisera were used in competitive direct ELISA. FB_1 detection in *Fusarium* culture, and corn products with this ELISA provided approximately two fold higher FB_1 estimates than that with HPLC. In addition, ELISAs had a strong positive relationship with HPLC, suggesting that they are suitable for fumonisins screening from human and animal foods. Secondly, attempts to generating high affinity and specificity antibodies against deoxynivalenol (DON) by immunizing mice and rabbits with a variety of DON-bovine serum albumin conjugates were made to improve sensitivity of previous DON competitive ELISA . High titer antisera were produced but they could not be used in DON competitive ELISAs. Finally, attempts were made to generate antibodies specific to the trichothecene-yeast ribosomal binding site by immunizing mice with 80S ribosomal subunits. Although all animals exhibit high antiserum titers for the ribosomes, these antisera were not effective in a variety of ELISA configurations to measure "total load" trichothecenes in foods.

To my parents and my grandparents for their moral support and prayer.

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patience, kindness, and understanding.

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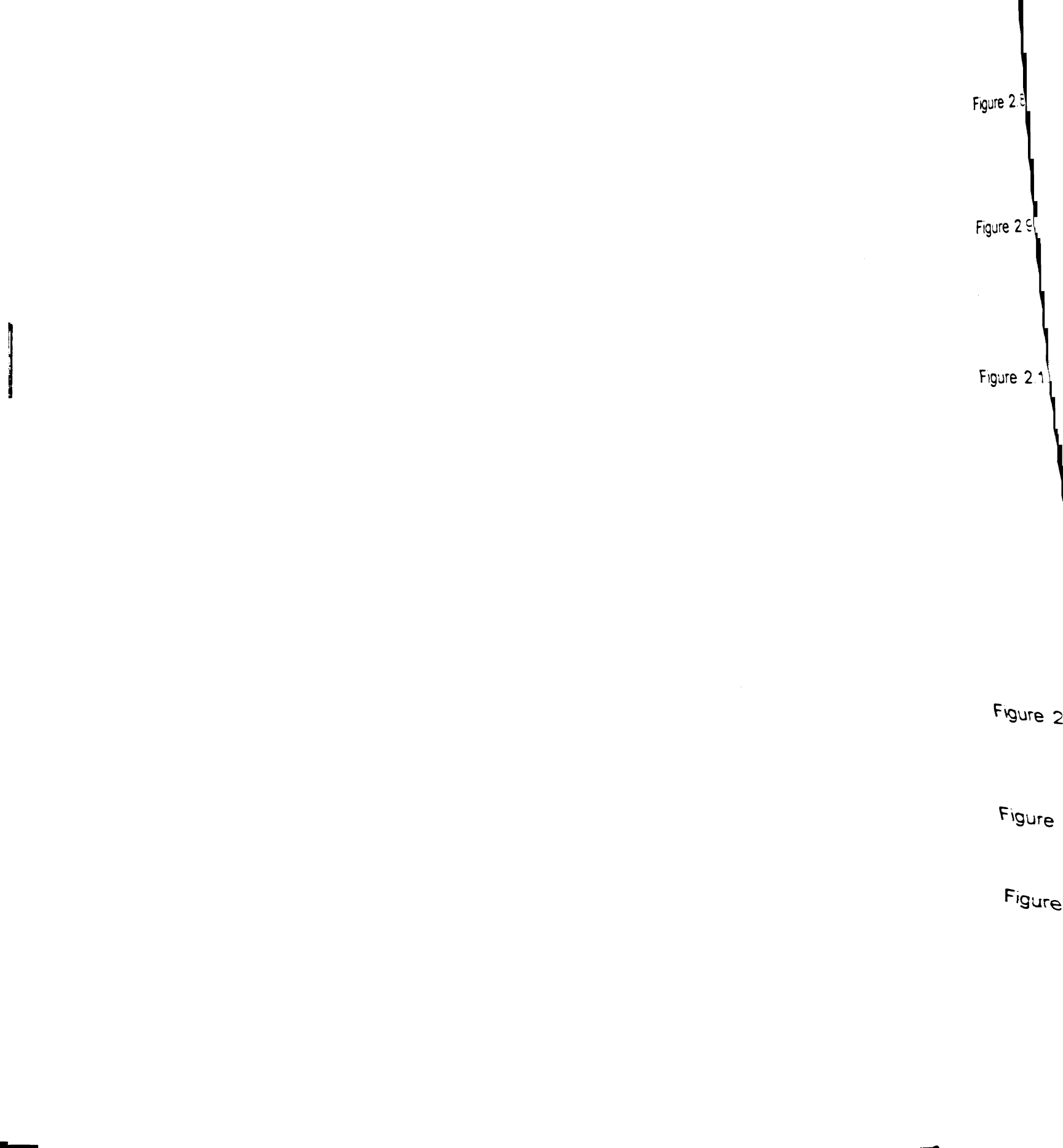
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INTRODUCTION

Mycotoxins are a group of secondary metabolites produced by toxigenic strains of fungi. One fungal genus that contains species capable of mycotoxin production is *Fusarium*. Among the *Fusarium* mycotoxins that are toxic to both human and animals and commonly found in grain and grain products are fumonisins and trichothecenes. The presence of these toxins in agricultural commodities are primarily dictated by environmental and biologic factors. Since controlling both environmental and biological factors is almost impossible, prevention of mycotoxin entries into human foods and animal feeds is commonly accomplished via detection of the toxins in agricultural commodities. Mycotoxin detection can be carried out using either conventional methods such as thin layer chromatography (TLC), gas chromatography (GC), high performance liquid chromatography (HPLC), and mass spectroscopy (MS) or immunoassay techniques such as enzyme-linked immunosorbent assay (ELISA). ELISAs are commonly preferred to conventional methods because of simplicity, rapidity, and applicability both in fields and laboratories.

ELISAs for fumonisin B_1 (FB_1) have been developed in our laboratory. However, when these ELISA systems are used to detect FB_1 in fusarium corn cultures, corn and corn products, they provide much higher FB_1 estimates than HPLC methods. One possible explanation for these observations is that FB_1

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antibodies used in these ELISAs are not completely specific to fumonisins and, therefore, may have reacted with other compounds found in *Fusarium* cultures, and contaminated corn. To overcome this problem, it is desirable to produce antibodies which have higher affinity and specificity to the toxins.

Our laboratory has also developed an ELISA for deoxynivalenol trichothecene. When this ELISA was used to detect deoxynivalenol (DON) in corn-based foods, it had a relatively high detection limit (1000 ppb). A lower detection limit of an ELISA can be generated when DON antibodies used in the ELISA have a higher affinity and specificity to DON. Therefore, production of higher affinity and specificity antibodies against DON is desirable.

Trichothecenes are a potent inhibitor of eukaryotic protein synthesis because they bind to a common site on the 60S ribosomal subunit. These toxins compete with each others for the ribosomal binding site in proportion to their toxicity. Production of antibodies specific to the binding site would enable investigators to develop an ELISA that can assess total "trichothecene load" in agricultural commodities and, thus enhance food safety.

The research in this dissertation was undertaken to address all above rationales and this dissertation was divided into four parts: Part I (Review of *Fusarium* Mycotoxin Immunoassay); Part II (Development and Application of an ELISA for Fumonisin B_1 in Fungal Cultures, Corn and Corn Products); Part III (Production of Deoxynivalenol Antibodies); and Part IV (Production of Antibodies against Trichothecene Yeast Ribosomal Binding Site). Part I reviews *Fusarium* mycotoxins especially fumonisins and trichothecenes including their chemical

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structures, toxicity, natural occurrence, and detection methods. Part II describes in detail: the production of FB_1 antibodies, the use of the antibodies for ELISA development, and the application of the ELISA for detection of FB_1 in *Fusarium* corn cultures, corn and corn product. Part III and IV explain efforts to produce antibodies against deoxynivalenol and trichothecene yeast ribosomal binding site, respectively.

PART I.

REVIEW OF *FUSARIUM*-MYCOTOXIN IMMUNOASSAY

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FUSARIUM MYCOTOXINS

Introduction

Mycotoxins are a very diverse group of toxic compounds produced as a part of secondary metabolism in a wide variety of filamentous fungi which often contaminate agricultural commodities prior to harvest and during storage (Smith and Moss, 1985). Peanut, corn, feeds, wheat and cereals are five agricultural products which most often have mycotoxin problems (Hesseltine, 1986).

Natural occurrence of mycotoxin in foods and feeds varies from commodity to commodity, year to year and region to region and are strongly dictated by environmental factors (CAST, 1989). Trichothecene mycotoxin contamination usually occurs during cold and wet season, or in extreme drought years which favor certain fungus infections and toxin production (Vesonder et al., 1978; Ueno, 1983; Shotwell et al., 1985; Tanaka et al., 1988a; CAST, 1989). Effects of environmental conditions and genetic factors on the occurrence of mycotoxins in the human food chain are illustrated in Figure 1.1.

Mycotoxin-contaminated products can produce adverse effects on both human and farm animals when the products are consumed. The effects may include: (1) acute toxicity and death following high level exposures of a mycotoxin, (2) lower growth rate, impaired immunity, greater susceptibility to bacterial or parasitic infection, (3) decreased milk and egg production,

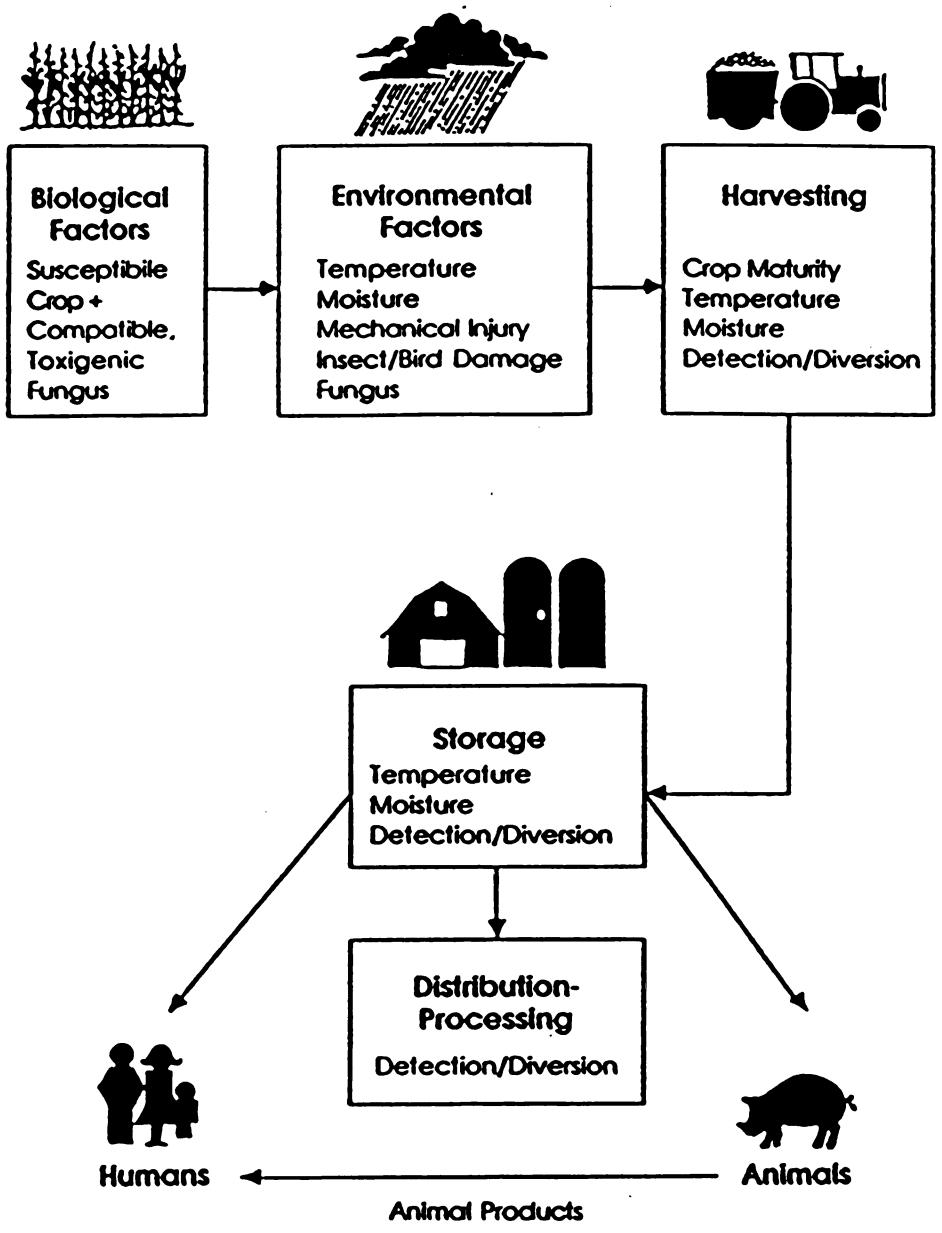

Figure 1.1. Factors affecting occurrence of mycotoxin in the human food chain (Pestka and Casale, 1990)

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(4) reduced reproductive efficiency, or (5) chronic toxicity such as cancer formation after prolonged exposure to small quantities of the mycotoxins (CAST, 1989). Consequently, mycotoxins cause economic losses to farmers and livestock producers (Hesseltine, 1986).

The economic losses (Table 1.1) are derived not only from crop and livestock losses, but also from regulatory action to protect human and animal health (CAST, 1989). In the United States, for example, the amount of aflatoxins in human foods and animal feeds has been regulated. Under the Food, Drug, and Cosmetic Act, Section 402(a)(1), the U.S. Food and Drug Administration (FDA) has established acceptable aflatoxin levels in agricultural commodities by establishing action levels that allow for the removal of violative lots from interstate commerce (CAST, 1989). The action levels of human foods are 20 part per billion (ppb) total aflatoxins, except for milk which has an action level of 0.5 ppb for aflatoxin M_1 (a metabolite of aflatoxin B_1). The action level of aflatoxins for feeds is 20 ppb, except for cottonseed meal used in feeds (300 ppb), corn used for finishing (feedlot) beef cattle (300 ppb), corn destined for finishing swine (200 ppb), and feeds used for breeding cattle, breading swine, and mature poultry (100 ppb)(CAST, 1989).

Among the most important mycotoxins are fumonisins, trichothecenes, zearalenone, aflatoxins, and ochratoxins (Hesseltine, 1986; Pohland, 1993). The first three mycotoxins are produced by *Fusarium spp.* *Fusarium* mycotoxins especially fumonisins and trichothecenes will be reviewed in this chapter.

Table 1.1.

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Food and fe handlers d and process
Governmen
Consumers (or animal)

Table 1.1. Types of economic losses and costs associated with mycotoxin contamination in foods and feeds (CAST, 1989)

Bearer	Economic losses and costs
Farmers and live stock producers	- Outright food and feed loss. - Contaminated crops provide less income and may lead to potential losses of outlet. - Reduced productivity of livestock from (1) lower quantity and quality of animal products, (2) smaller letters, (3) reduced work output, (4) loss of pregnancy, (5) reduced feed efficiency, (6) impaired resistance to disease, and (7) loss of vaccination efficacy.
Food and feed handlers, distributor and processors	- Less income from products refused, condemned, or sold at discount. - Increased storage, transport, and packing costs on such products. - Potential loss of market, trading reputation, and raw material source. - Increased costs due to litigation (may exceed cost of product), surveillance, and control.
Government	- Lower foreign exchange earnings from reduced exports. - Increased cost involved in shipment, sampling, and analyses of exported goods that are subsequently refused import entry; potential loss of overseas outlets. - Increased costs of detoxification or reconditioning abroad. - Increase costs for food or feed imports; staple food subsidies. - Increased costs of surveillance and control. - Increased costs for expenditures on human and animal health facilities and activities. - Increased costs involved in training and extension programs.
Consumers (human or animal)	- Consumption may lead to impaired health and productive capacity. - Lack of food may lead to undernutrition or higher food prices resulting from outside purchase of foods or feeds. - Possible medical and veterinary costs associated with the above conditions in previous two statements. - Possible consumer-initiated litigation costs.

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Fumonisins

Historical background. Fumonisins are a group of secondary metabolites produced by *Fusarium moniliforme* (Sheldon)(Benzuidenhout et al., 1988), and *Fusarium proliferatum* (Ross et al., 1990). These fungi are commonly found on corn, sorghum, and other grain commodities throughout the world (Marasas et al., 1984a). *Fusarium moniliforme,* which was firstly described by Sheldon (1904) as *Fusarium moniliforme* Sheldon, was implicated as a causative agent of "moldy corn poisoning" in animals in the United State (Peter, 1904). This mold has been associated with poisoning in *equine* species that is now known as equine leukoencephalomalacia (ELEM) (Ross, 1994). This disease is a neurotoxicosis that is characterized by multifocal liquefactive necrosis in the white matter of cerebral hemispheres (Marasas et al., 1988b). Although the disease symptoms have been observed since the previous century (Marasas, 1986), its causative agent [fumonisin B_1 (FB_1)] was not isolated and identified until 1988 (Benzuidenhout et al., 1988; Marasas et al., 1988b). As a consequence, interest in fumonisins has increased leading to extensive studies on their occurrence, distribution, production, toxicity, detection, and chemistry (Ross, 1994).

FB_1 is the most toxic and predominant fumonisin produced by *Fusarium moniliforme* (Gelderblom et al., 1988b), and has been confirmed as an etiologic agent of fatal animal diseases such as ELEM in horses (Kellerman et al., 1990), porcine pulmonary edema (PPE) in pigs (Harrison et al., 1990), and liver cancer in rats (Gelderblom et al., 1991). In addition, epidemiological studies indicate that fumonisins are associated with human esophageal cancer in South Africa

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(Marasas et al., 1988a). A detailed review of fumonisin toxicity and detection is given in Part II.

Chemistry. Fumonisins are diesters of 14, 15-propane-1,2,3-tricarboxylic acid and either 2-acetylamino- or 2-amino-12, 16-dimethyl-3, 5, 10, 14, 15-pentahydroxy-icosane or its C-10 deoxy analogue (Bezuidenhout et al., 1988). Chemical structures of these toxins are similar to that of *Alternaria alternata f. sp. lycopersici* (AAL) toxin and sphingosine (Figure 1.2)(Norred, 1993). In early studies, four fumonisins (FA_1, FA_2, FB_1 and FB_2) (Bezuidenhout et al., 1988) were isolated and identified by the South African researchers led by Dr. W.F.O. Marasas (Norred, 1993). Additional chemical structures for FB_3 , FB_4 (Cawood et al., 1991; Plattner et al., 1992) and FC_1 (Branham and Plattner, 1993) have now been reported. The A-series fumonisin are acetylated at the amino group whereas the B-fumonisins have a free amine (Figure 1.2). FC_1 is FB_1 which lost a methyl group at C-1 position; thus FC_1 is a diester of 13, 14-propane-1,2,3-tricarboxylic acid and 1-amino-11, 15-dimethyl-2, 4, 9, 13, 14-pentahydroxy-nonadecane (Branham and Plattner, 1993).

The biosynthetic pathways through which fungi produce fumonisins have only been partially elucidated (Norred, 1993). When *Fusarium spp.* are cultured on solid (corn) substrates, FB_1 is produced predominantly by *Fusarium moniliforme* strains, but some strains of *Fusarium proliferatum* produce FB_2 or FB_3 at higher concentrations than FB_1 (Nelson et al., 1994; Visconti and Doko, 1994). Solid substrate media are not conductive for the study of fumonisin

FUM

	R_1
FA_1	OH
FA_2	H
FB_1	OH
FB_2	H
FB_3	OH
FB_4	H

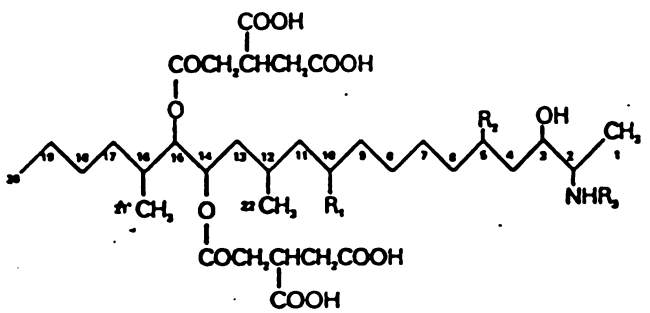
AA

SP

Figure 1.2

FUMONISINS

	R_1	R_2	R_3
FA_1	OH	OH	CH_2CO
FA_2	H	OH	CH_2CO
FB_1	OH	OH	H
FB_2	H	OH	H
FB_3	OH	H	H
FB_4	H	H	H



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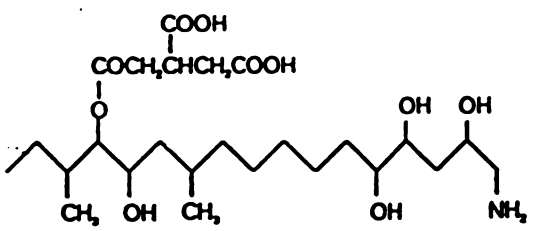

SPHINGOSINE

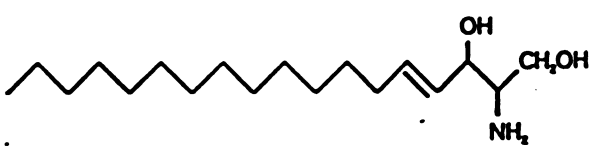

Figure 1.2. Structures of known fumonisins and of *Alternaria alternata f. sp. lycoperisici* (ALL) toxin, showing structural similarities with spingosine (Norred, 1993).

biosynthesis, and production of fumonisins in liquid media must be performed (Plattner and Shackelford, 1992; Blackwell et al., 1994). When Plattner and Shackelford (1992) fed deuterium-labeled methionine to *Fusarium moniliforme* cultures, they observed high levels of deuterium incorporation into FB_1 at the methyl groups on the C-12 and C-16 position of the fumonisin backbone. Since the structure of fumonisins is similar to that of sphingosine, these investigators speculated that biosynthesis of a fumonisin backbone is also similar to that of sphingosine. Sphingosine is synthesized through condensation of linolyl-coenzyme A and alanin, whereas the fumonisin backbone is synthesized via condensation of palmitoyl coenzyme A and serine (Plattner and Shackelford, 1992).

However, the above hypothesis was contested by Blackwell et al. (1994) who fed ^{13}C-labeled acetate to liquid cultures of *Fusarium moniliforme* to determine the location of labeled carbon atoms in the radiolabeled fumonisin. They observed that the methyl group (C-2) of acetate resulted in enrichment of C-20, 18, 16, 14, 12, 10, 8, 6, and 4 while the carbonyl group (C-1) of acetate labeled C-19, 17, 15, 13, 11, 9, 7, 5, and 3. When they added methionine to the cultures, FB_1 production increases up to 12-fold, with the S-methyl group from the methionine exclusively enriching positions C-21 and C22. Those resutls were similar to the results of Plattner and Shackelford (1992). Based on these findings, Blackwell et al. (1994) concluded that the backbone of fumonisins was synthesized by the fungus through condensation of acetyl coenzyme A and serine, rather than modification of palmitic acid as stated by Plattner and

Shackelford (1992). More work is still required to fully elaborate the biosynthetic pathway of the fumonisins.

Natural occurrence. *Fusarium moniliforme* , the fumonisin-producing fungus, commonly occurs in the world and it has been isolated from many countries including Australia, Brazil, Canada, Central America, China, Croatia, Egypt, German, Hong Kong, India, Indonesia, Israel, Italy, Jamaica, Japan, Nepal, New Zealand, Peru, Philippines, Poland, Portugal, Romania, South Africa, Taiwan, the United State, Turkey and Zambia (Bacon and Nelson, 1994; Doko et al., 1995). This fungus is primarily found as a corn contaminant, but can also be found on several grain commodities such as sorghum, wheat, rice, and oat and on other agricultural products such as beans, peanuts, sugar beats, and bananas (Bacon and Nelson, 1994). Natural occurrence of fumonisins has been documented in a number of countries (Table 1.2).

Conditions required for optimal production of fumonisins in the field or storage are only partially known (Bacon and Nelson, 1994). Bars et al. (1994) reported that a temperature of 20^{0}C, a 32% moisture content of corn media, and an aerated atmosphere (cotton-stoppered flasks) were the optimum condition of FB_1 production by *Fusarium moniliforme* in laboratory experiments. *Fusarium moniliforme* that was isolated from European fresh corn produced FB_1 up to 300 ppm when cultured on solid corn media at the optimum conditions for 12 days (Bars et al., 1994). Other *Fusarium moniliforme* isolates produce higher quantities (6400 ppm) of FB_1 when cultured on the same media (Nelson et al., 1991). More higher FB_1 (17,000 ppm) was produced when *Fusarium moniliforme*

MRC 826 was cultured on corn media at 20^0C for 13 weeks (Alberts, et al., 1990).

Trichothecenes

Historical background. Trichothecenes are a group of structurally similar sesquiterpenoid metabolites produced mainly by *Fusarium spp.* (Bamburg and strong, 1971; Bamburg, 1983; Ueno, 1983; Betina, 1989). The first known member of this group, trichothecin, was originally discovered as an antifungal antibiotic in 1948 (Freeman and Morrison, 1948; 1949). Since that time over eighty trichothecenes have been isolated and characterized (Betina, 1989).

Interest in trichothecenes has increased over the years since their first discovery. The former Soviet Union was the first country to conduct an extensive research on these toxins in attempts to identify and elucidate the causative agents of mycotoxicoses in the 1930's following outbreaks of alimentary toxic aleukia (ATA) and stachybotriotoxicoses (Ueno, 1980; Bamburg, 1983). Japan followed shortly thereafter because this country also suffered from a trichothecene-mediated disease known as red-mold (akakabi in Japan) disease. Interest in mycotoxins in the Western world did not emerge until the appearance of "turkey x disease", which killed thousands of turkeys in the United Kingdom in the early 1960s (CAST, 1989). The etiologic agent of the "turkey x disease" was feed contaminated by *Aspergillus flavus* which produced aflatoxins (Spensley, 1963).

Table 1.2

COUNTR
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Egypt
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Poland
Portugal
Romania
South Africa
Switzerland
USA
Zambia

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Table 1.2. Natural occurrence of fumonisins in cereals and cereal products

COUNTRY	CROP YEAR	GRAIN PRODUCTS	MEAN IN POSITIVES, PPM (POSITIVES/SAMPLES)		REFERENCE
			FB_1	FB_2	
Argentina	1991	corn	2.88 (17/17)	1.14 (17/17)	Sydenham et al., 1993
Canada	1990	cornmeal	0.05(1/2)	nd (0/2)	Sydenham et al., 1991
China:					
Linxian	1989	corn	0.87 (13/27)	0.45 (3/27)	Yoshizawa et al., 1994
Cixian	1991	moldy corn	93.13 (4/4)	-	Chu and Li, 1994
Shangqui	1989	corn	0.89 (5/20)	0.33 (1/20)	Yoshizawa et al., 1994
Shangqui	1991	moldy corn	55.00(5/5)	-	Chu and Li, 1994
Croatia	1992	corn	0.02 (11/19)	0.01 (4/19)	Doko et al., 1995
Egypt	1990	cornmeal	2.38 (2/2)	0.60 (2/2)	Sydenham et al., 1991
Italy	1989-1991	corn	0.38 (26/26)	0.14 (13/26)	Doko et al., 1995
	1991	corn feeds	1.14 (23/25)	0.30 (13/25)	Minervini et al., 1992
Peru	1990	cornmeal	0.66 (1/2)	0.14 (1/2)	Sydenham et al., 1991
Poland	1992	corn	0.02 (2/7)	0.01 (1/7)	Doko et al., 1995
Portugal	1992	corn	1.03 (9/9)	1.21 (8/9)	Doko et al., 1995
Romania	1992	corn	0.01 (3/6)	0.01 (1/6)	Doko et al., 1995
South Africa	1985	corn	1.60 (12/12)	0.50 (10/12)	Sydenham et al., 1990
	1985	moldy corn	29.3(12/12)	7.55(12/12)	-"-
	1989	corn	1.53 (5/6)	0.42 (5/6)	Rheeder et al., 1992
	1989	moldy corn	53.74(6/6)	13.68(6/6)	-"-
	1989	export corn	0.29 (28/68)	0.13 (10/68)	Rheeder et al., 1994
	1990	cornmeal	0.14 (46/52)	0.08 (11/52)	Sydenham et al., 1991
	1990	corn grits	0.13 (10/18)	0.09 (4/18)	-"-
	1990	cornflakes	nd (0/3)	nd (0/3)	-"-
Switzerland	1986-1991	corn	2.88 (7/7)	0.24 (7/7)	Stack and Eppley, 1992
USA	1989	corn	0.64 (7/7)	0.18 (6/7)	Sydenham et al., 1991
	1990	corn screenings	55.40 (6/6)	15.08 (7/7)	Stack and Eppley, 1992
	1990	corn foods	0.43 (25/36)	0.14 (18/36)	-"-
	1990	corn food[a]	0.41 (4/4)	0.15 (3/4)	Sydenham et al., 1991
	1990	cornmeal	1.05 (15/16)	0.30 (13/16)	-"-
	1990	corn grits	0.60 (10/10)	0.38 (5/10)	-"-
	1990	cornflakes	nd (0/2)	nd (0/2)	-"-
	1991	cornmeal	0.09 (2/7)	nd (0/7)	Pittet et al., 1992
	1991	corn grits	0.26 (34/55)	0.10 (13/55)	-"-
	1991	cornflakes	0.06 (1/12)	nd (0/12)	-"-
	1991	sweet corn	0.07 (1/7)	nd (0/7)	-"-
	1991	poultry feed	0.24 (6/22)	0.09 (2/22)	-"-
	1991	corn foods	0.55 (11/13)	0.13 (10/13)	Stack and Eppley, 1992
Zambia	1992	Corn	0.18 (20/20)	0.05 (15/20)	Doko et al., 1995

nd indicate not detected. - indicate not analyzed. -"- same as above

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Interest in trichothecenes arose world wide in September, 1981 after the United States indirectly accused the Soviet Union, Vietnam and Laos governments of employing these mycotoxins as chemical warfare agents (Bamburg, 1983; Watson et al., 1984). The accusation was based on several findings. Firstly, analyses of blood, urine, and internal tissues of "yellow rain" attack victims revealed extremely high levels of trichothecenes (Watson et al., 1984). Secondly, a high level (up to 20 times higher than any recorded natural outbreak) of trichothecene mycotoxins was also found in a single leaf and stem sample taken from a region of Kampuchea where "yellow rain" was reported (Seagrave, 1981). Thirdly, normal background levels of these toxins were essentially undetectable and natural occurrence of trichothecenes in Southeast Asia was absent (Bamburg, 1983).

However, a group of researchers disagreed with the above findings. They argued that "yellow rain" was merely naturally occurring bee feces where fungus grew and produced trichothecenes. In addition, consumption of trichothecene-contaminated foods might have yielded similar symptoms to the symptoms of "yellow rain" attack victims (Schiefer, 1988).

The controversy resulted in intensive research activities directing towards elucidating many aspects of trichothecene mycotoxins including their detection, natural occurrence, toxicological, biological and biochemical action (Bamburg, 1983). Based on this and other research over the last five decades, it is now recognized that trichothecene mycotoxins are etiologic agents of mycotoxicoses both in human and animals (Ueno, 1980; 1983; Bamburg, 1983; CAST, 1989).

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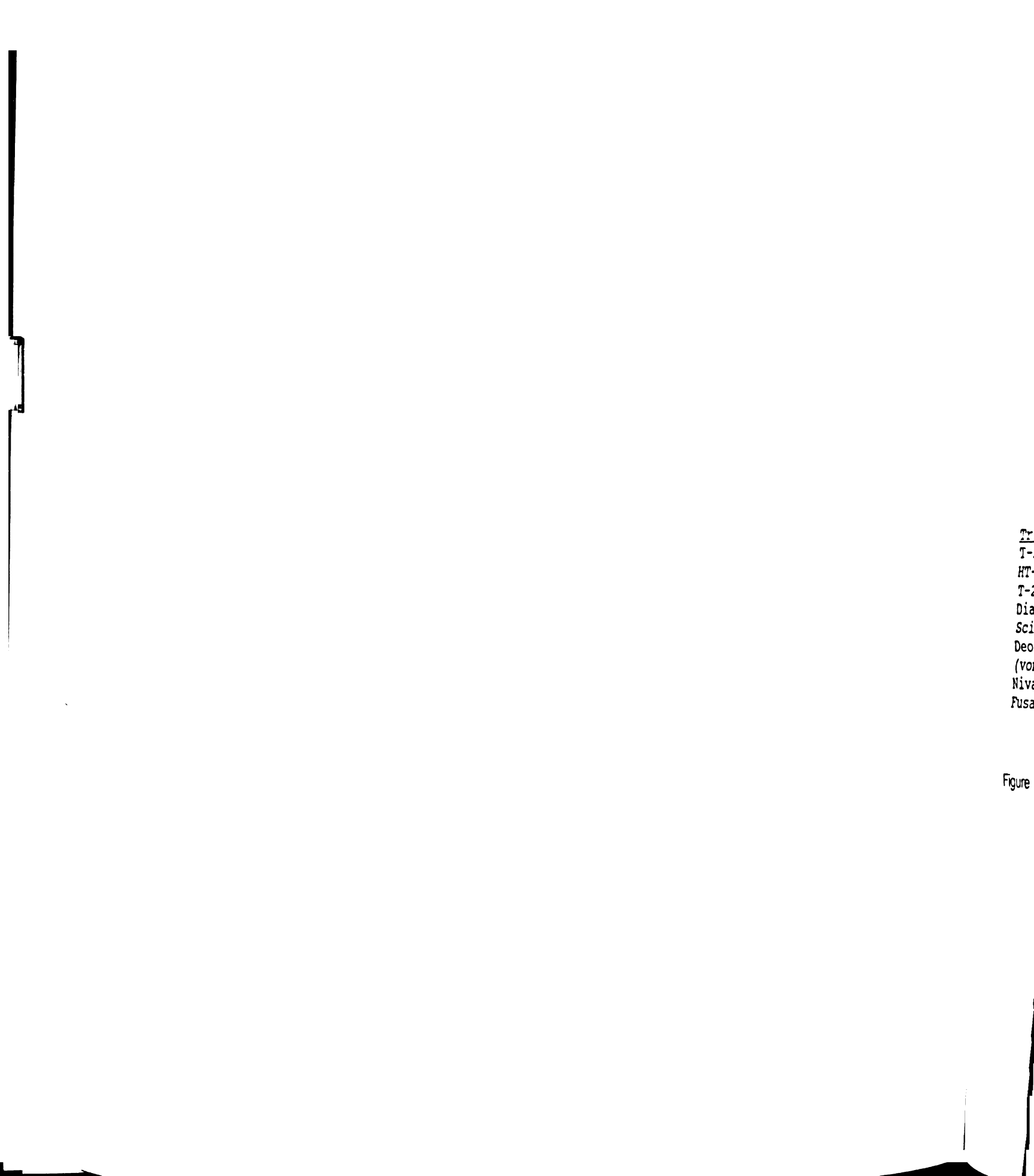
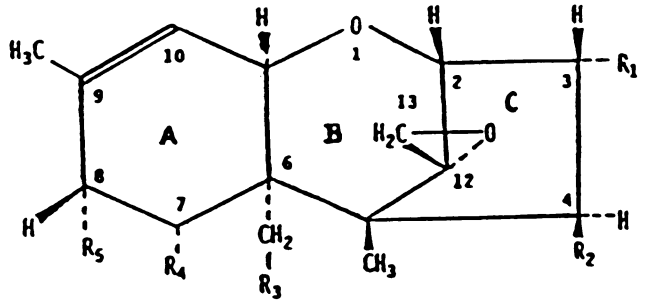
Chemistry. Trichothecenes all have tetracyclic, sesquiterpenoid structure that includes a six-member oxygen containing ring, an epoxide group in the 12, 13 position, and an olefinic bond in the 9, 10 position (Ueno, 1980; 1983; Bamburg, 1983; Betina, 1989). Structure and numbering system of some naturally identified trichothecene mycotoxins are illustrated in Figure 1.3. These toxins possess oxygen-containing substitutes at one or more C-3, 4, 7, 8, and 15 positions. The substitutes may be hydroxyl, esterified hydroxyl, keto (carbon number 8 only), or epoxide (carbons number 7, 8 only) groups (Ueno, 1983; Betina, 1989).

Based on trichothecene structural characteristics, Ueno (1980; 1983) classified trichothecenes into 4 different groups (group A, B, C and D; Figure 1.4). Group A trichothecenes have hydroxyl or acetoxy substitutes, such as T-2 toxin and trichodermin. Group B trichothecenes are 8-keto derivatives such as deoxynivalenol and nivalenol. Group C trichothecenes are 7,8 epoxide derivatives such as crotocin. Group D includes trichothecenes containing a macrocyclic ring between carbon number 4 and 5 such as verrucarin A and roridin A.(Ueno, 1980).

Trichothecenes are colorless, crystalline, optically active solids, and are generally soluble in non-polar solvents such as acetone, ethylacetate, and chloroform, but less soluble in polar solvents (e.g. water) (Bamburg and Strong, 1971; Ueno, 1980). Trichothecenes are more stable in solid condition. Their alcohol derivatives have a higher solubility in water than their esterified homologue (Betina, 1989). Under a mild alkaline solution the ester groups are readily saponified and result in less acylated derivatives or parent alcohol forms



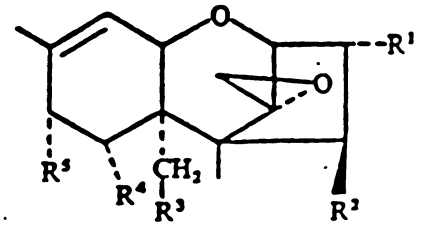
Figure



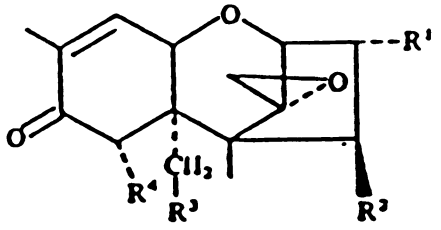
Trichothecene	R1	R2	R3	R4	R5
T-2 toxin	OH	OAC	OAC	H	$OOCCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	OAC	H	$OOCCH_2CH(CH_3)_2$
T-2 tetraol	OH	OH	OH	H	OH
Diacetoxyscirpenol	OH	OAC	OAC	H	H
Scirpenetriol	OH	OH	OH	H	H
Deoxynivalenol (vomitoxin)	OH	H	OH	OH	O
Nivalenol	OH	OH	OH	OH	O
Fusarenon-X	OH	OAC	OH	OH	O

Figure 1.3. Structure and numbering system of some naturally identified trichothecene mycotoxins (modified from Ueno, 1983).

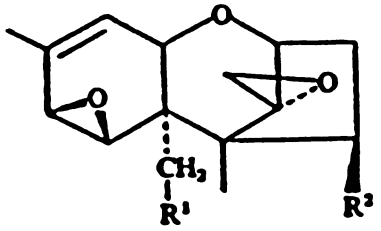
Figure 1.



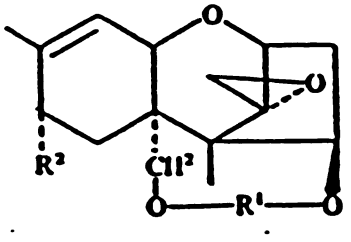
Group A



Group B



Group C



Group D

Figure 1.4. Classificasion of trichothecene mycotoxins based on their chemical structure (modified from Ueno, 1980).

(Ueno, 1980). The double bond at carbon 9, 10 can be catalytically reduced to a dihydro derivative, and the corresponding alcohol groups are then easily oxidized to ketone or aldehyde functional groups.

Natural occurrence. Only a few members of the trichothecenes, such as deoxynivalenol (DON), nivalenol (NIV),T-2 toxin (T-2), HT-2 toxin, and diacetoxyscirpenol (DAS) are detected as natural contaminants in cereal grains although over 80 members of these toxins have been characterized in the laboratory (Betina, 1989).

Production of trichothecenes in cereal grains is mainly dictated by environmental conditions (Vesonder et al., 1978; Ueno, 1983; Cote et al., 1984). Wet and cool seasons favor *Fusarium* infection and trichothecene production in the field (Tuite et al.,1974; Shotwell et al., 1977, 1983; Ueno, 1983; CAST, 1989). Tuite et al. (1974) reported that during the unusually cool and wet summer of 1972, corn produced in the United States was heavily infected with *Fusarium graminearum*, and caused outbreaks of feed refusal and emesis in swine. DON was believed to be an etiologic agent for these outbreaks. Shotwell et al. (1977, 1983) observed similar effects of weather on *Fusarium* contamination in wheat, grown in Virginia from 1975 to 1980. The weather was unusually cold and rainy during the 1975 growing season, but during the following 5 years (1976 to 1980) such weather did not occur. These investigators found zearalenone in 19 of 42 samples from wheat grains harvested in the cool and wet growing season; however, they did not find the toxin during the 1976-1980 harvest period. Later, DON was also detected in the zearalenone-positive samples (Shotwell and

Hesseltine, 1983). "Red mold disease" in Japan that is caused by *Fusarium graminearum and Fusarium nivale* (Ueno, 1983), also occurred after a long rainy season in 1963 and in 1970. Similar weather conditions resulted in "scabby wheat" (soft and shriveled wheat and often with a pink discoloration) in the United States and Canada (CAST, 1989). This "scabby wheat" was caused by *Fusarium graminearum* which produced DON (Shotwell et al., 1985; Tanaka et al., 1988a; Fernandez et al., 1994).

The natural occurrence of DON as well as NIV in cereal and cereal products has been surveyed by several investigators in several countries and the results are summarized in Table 1.3.

Human and animal mycotoxicoses. Outbreaks of human and animal diseases associated with consumption of trichothecene-contaminated foods and feeds occurs in many countries usually after a cold and wet harvest season (Bamburg, 1983). Although such outbreaks have been reported to take place since the late 19th century (Bamburg, 1983; Ueno, 1983), elucidation of trichothecenes as an etiological agent in mycotoxicoses was not reported until 1972. At that time, Hsu et al. (1972) demonstrated that 2 ppm of T-2 toxin, a trichothecene produced by *Fusarium tricinctum,* was detected in moldy corn associated with illness and death of lactating cows. Since that time other trichothecenes such as diacetoxyscirpenol, nivalenol, fusarenon-X, deoxynivalenol and HT-2 toxin have also been found naturally in feedstuffs and in cereal grains (Betina, 1989).-

Table 1.3. Natural occurrence of trichothecene deoxynivalenol (DON) and nivalenol (NIV) in cereals and cereal products

COUNTRY	CROP YEAR	GRAIN PRODUCTS	MEAN IN POSITIVES, PPM (POSITIVES/SAMPLES)		REFERENCE
			DON	NIV	
Argentina	1983	wheat	0.02(3/20)	nd (0/20)	Tanaka et al., 1988b
	1983	barley	0.24(18/20)	0.03(15/20)	-"-
	1983	corn	0.11(2/20)	nd (0/20)	-"-
Austria	1983	wheat	0.36(3/4)	0.03(3/4)	Tanaka et al., 1988b
Bulgaria	1983	wheat	0.21(1/2)	0.03(1/2)	Tanaka et al., 1988b
Canada	1980	wheat	0.30(72/72)	-	Scott et al., 1981
	1980-1984	wheat	1.26(9/10)	0.02(4/10)	Tanaka et al., 1988a
	1984	corn	0.96(1/1)	0.01(1/1)	-"-
	1982	rye	0.20(1/1)	0.01(1/1)	Tanaka et al., 1988b
China	1984	wheat	1.71(1/5)	6.64(1/5)	Ueno et al., 1986
	1985	wheat flour	0.19(7/7)	nd (0/7)	-"-
	1984	wheat	4.28(4/4)	0.16(3/4)	Tanaka et al., 1988b
England	1984	wheat	0.03(20/31)	0.10(17/31)	Tanaka et al., 1988b
Finland	1987-1978	cereal	0.13 (246/268)	-	Hietaniemi and Kumpulainen, 1991
France	1984	wheat	0.09(1/2)	0.04(2/2)	Tanaka et al., 1988b
Germany	1984	wheat	0.71(2/8)	0.27(0/8)	Tanaka et al., 1988b
	1984	barley	0.19(2/13)	0.04(1/13)	-"-
	1984	oat	0.14(4/10)	1.45(1/10)	-"-
	1984	soybean	nd (0/1)	nd (0/1)	-"-
	1984	rye	0.41(4/22)	0.01(4/22)	-"-
	1987	barley	0..40(43/44)	0.01(5/44)	Muller and Schwardorf, 1993
	1984	rye flour	0.17(1/1)	0.01(1/1)	-"-
Greece	1984	wheat	0.01(1/1)	0.01(1/1)	Tanaka et al., 1988b
Hungary	1984	wheat	0.67(2/2)	0.01(1/1)	-"-
India	1989	sorghum	nd (0/150)	-	Ramakrisna et al., 1990
	1989	corn	nd (0/102)	-	-"-
	1989	wheat	0.31(1/58)	-	-"-
	1989	whole wheat flour	4.38(11/37)	-	-"-
	1989	feed sample	nd(0/102)	-	-"-
Italy	1984	wheat	0.12(1/12)	nd (0/12)	Tanaka et al., 1988b
	1984	barley	0.19(2/5)	0.02(1/5)	-"-
	1984	corn	0.40(2/3)	nd (0/3)	-"-
	1984	oat	nd (0/5)	nd (0/5)	-"-

nd indicates not detected. -"- indicates the same as above. - indicates not analyzed.

Table 1.3. Continued

COUNTRY	CROP YEAR	GRAIN PRODUCTS	MEAN IN POSITIVES, PPM (POSITIVES/SAMPLES)		REFERENCE
			DON	NIV	
Japan	1983	wheat	0.02(4/6)	0.39(6/6)	Tanaka et al., 1985.
	1983	barley	0.25(5/5)	0.71(5/5)	-"-
Korea	1983	wheat	0.01(2/10)	0.14(9/10)	Lee et al., 1985
	1983	rye	0.01(5/5)	0.08(5/5)	Lee et al., 1985
	1984	wheat	0.02(5/9)	0.53(9/9)	Lee et al., 1986
	1983	barley	0.12(26/28)	0.55(28/28)	Lee et al., 1985
	1984	barley	0.12(31/31)	0.50(31/31)	Lee et al., 1986
	1989	barley	0.26(2/11)	0.30(3/11)	Park et al. 1991
	1989	rice	nd (0/8)	nd (0/8)	-"-
	1989	corn	0.62(1/3)	0.35(1/3)	-"-
	1989	millet	0.34 (1/6)	0.23(1/6)	-"-
	1990	barley	0.19(24/27)	1.11(27/27)	Park et al., 1992
	1993	barley	0.17(?/39)	1.01(?/39	Kim et al., 1993
	1993	corn	0.31(?/46)	-	-"-
Nepal	1984	wheat	0.06(1/10)	0.07(5/10)	Tanaka et al., 1988b
	1984	barley	nd (0/4)	0.02(1/4)	-"-
	1984	oat	nd (0/7)	0.02(4/7)	-"-
	1984	rice	nd (0/9)	0.02(2/9)	-"-
	1983	rye	nd (0/2)	nd 0/2)	-"-
	1984	corn	0.54(3/9)	0.89(6/9)	-"-
Netherlands	1984	cereals	0.22(26/29)	-	Tanaka et al., 1990
	1988-1989	feeds	0.63(19/95)	-	Veldman et al., 1992
Poland	1984	wheat	0.10(13/48)	0.05(37/48)	Tanaka et al., 1988b
	1984	barley	0.39(1/6)	0.08(3/6)	Tanaka et al., 1988b
Portugal	1984	wheat	nd (0/4)	nd (0/4)	Tanaka et al., 1988b
Scotland	1984	wheat	0.03(1/20	nd (0/2)	Tanaka et al., 1986
	1984	barley	0.04(5/8)	0.39(3/8)	-"-
S. Africa	1987	wheat	0.87(3/3)	-	Sydenham et al., 1989
Sweden	1984	wheat	nd (0/1)	nd (0/1)	Tanaka et al., 1988b
Taiwan	1984	wheat	0.56(9/12)	0.07(6/12)	Ueno et al., 1986
	1985	barley	0.08(4/4)	0.63(4/4)	-"-
	1985	wheat	0.25(3/10)	0.02(4/10)	-"-
USA	1977	corn	2.30(24/52)	-	Vesonder et al., 1978
	1981	corn	3.1(274/342)	-	Cote et al., 1984
	1982	wheat	1.78(31/33)	-	Hagler et al., 1984
	1982	wheat	1.17(45/161)	-	Shotwell et al., 1985

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Table 1.3. Continued

COUNTRY	CROP YEAR	GRAIN PRODUCTS	MEAN IN POSITIVES, PPM (POSITIVES/SAMPLES)		REFERENCE
			DON	NIV	
	1984	wheat	0.59(75/123)	-	Wood and Carter, 1989
	1984	corn	0.39(61/92)	-	-"-
	1984	dairy grain	0.10(60/101)	-	Whitlow et al., 1986
	1985	wheat	0.49(57/124)	-	Wood and Carter,1989
	1985	corn	0.47(32/106)	-	-"-
	1989	human food	4.00(46/92)	-	Abouzied et al., 1991
	1991	wheat	1.57(17/81)	-	Fernandez et al., 1994
	1990	corn	2.40(13/99)	-	Price et al., 1993
	1990	winter wheat	2.4(201/207)	-	-"-
	1990	sping wheat	0.9(120/206)	-	-"-
USSR	1984	oat	nd (0/2)	nd (0/2)	Ueno et al., 1986
	1984	spice	nd (0/3)	nd (0/3)	Tanaka et al., 1988b
	1986	wheat	0.59(6/140	-	Tutellyan et al., 1990
	1987	wheat	0.26(14/90)	-	-"-
	1988	wheat	1.13(14/62)	-	-"-
Yemen	1983	sorghum	nd (0/6)	0.09(1/6)	Tanaka et al., 1988b
	1984	wheat	0.00(1/7)	nd (0/7)	-"-
	1984	barley	0.02(2/3)	0.01(2/3)	-"-
	1984	corn	0.01(1/12)	nd (0/12)	-"-
	1984	sorghum	nd (0/5)	nd (0/5)	-"-
	1984	soybean	nd (0/2)	nd (0/2)	-"-
	1984	sesame	nd (0/7)	nd (0/7)	-"-

nd indicates not detected. -"- indicates the same as above. - indicates not analyzed.

Severe mycotoxicosis induced by trichothecene mycotoxins include alimentary toxic aleukia (ATA) in Russia (Joffe, 1962; 1965), red-mold disease in Japan (Bamburg et al., 1969), moldy corn diseases in The United States (Bamburg et al., 1969; Hsu et al., 1972), staggering grain toxicosis in Eastern Siberia (Bamburg, 1983), dendrochiotoxicosis in Russia (Bamburg, 1983), vomiting and feed refusal in the United States and other countries (Vesonder et al., 1973), bean hull poisoning in Japan (Ueno, 1980), and gastrointestinal disorders in Kasmir Valley, India (Bhat et al., 1989). Symptoms, victims, and etiologic agents of these mycotoxicosis are summerized in Table 1.4.

Table 1.4. Summary of trichothecene-induced toxicoses

Year	Toxicoses and location	Victims	Symptoms	Contaminated products	Fungus	Mycotoxin
1890	Staggering toxicosis in Siberia	Human and farm animal	Headache, vertigo, shivering, chills, nausea, vomiting, visual disturbance	Millet, barley	*Fusarium roseum*	Trichothecene (not identified)
1942-1947; 1950-1960.	Alimentary toxic aleukia (ATA) in Russia and Central Europe	Human	Vomiting, skin inflammation, diarrhea, leukopenia, multiple hemorrhage, necrotic angia, sepsis, exhaustion of bone marrow, respiratory failure, death	Overwintered millet, wheat, barley	*F. sporotrichioides, F.poae, Cladosporium epiphylum.*	Sporofusarin, poaefusarin, trichothecenes(T-2 toxin, HT-2 toxin, etc)
Since 1900	Red-mold disease (Akakabi-byo) in Japan	Human, farm animals	Vomiting , diarrhea, skin inflammation, feed refusal, infertility, jemorrage in the intestines.	Barley, wheat, millet	*F. graminearum (Fusarium roseum), Fusarium nivale.*	Trichothecenes (nivalenol, deoxynivalenol, etc.)
Since 1930	Moldy corn in the US.	Cattle and swine.	Hemorrhage, skin inflammation.	Barley, corn, rye.	*Fusarium tricinctum, Fusarium scirpi*	T-2 toxin, HT-2 toxin, diacetoxyscirpenol.
Since 1930	Vomiting and feed refusal in the US and other countries	Human and farm animals	Vomiting, feed refusal	Corn, barley, rye	*Fusarium roseum*	Deoxynivalenol, nivalenol, zearalenone (?), unknown factor.

Table 1.4. Continued

Year	Toxicoses and location	Victims	Symptoms	Contaminated products	Fungus	Mycotoxin
Since 1931	Stachybotryotoxicosis, in Russia, Hungary and other countries.	Horse, cattle, sheep, swine, and human	Skin inflammation, hemorrhage in several organs, leukocytosis, leukopenia, diarrhea, shock, depression.	Straw, hay, barley, cotton.	*Stachybotrys (S. alternans)*	Satratoxin H, etc.
Since 1937	Dendrochiotoxicosis in Russia	Horse, cattle, human	Quick death, cyanose, hemorrhage, tachycardia, leukocytosis.	Feedstuffs	*Myrothecium roridum)*	Roridin and Verrucarins.
Since 1900	Bean-hull poisoning, in Japan.	Horse	Motor irritation, circular movement, jaundice, hemorrhage, degeneration of nerve cell.	Dried bean hulls and bean plants.	*Fusarium spp.*	T-2 toxin, neosolaniol, HT-2 toxin.
1987	Gastrointestinal disorder in India	human	Abdonal pain, a feeling of fullness of abdomen.	Bread made from moldy-damaged wheat.	*Fusarium spp.*	Trichothecenes (e.g. DON, NIV, T-2 toxin)

CONVENTIONAL ANALYTICAL METHODS

The presence of trichothecenes as well as fumonisins in foods and feedstuffs is potentially hazardous to human and animal health (Ueno, 1983; CAST, 1989; Norred, 1993; Pohland,1993) and cause economic loss to farmers and livestock producers (CAST, 1989). Therefore, elimination of the mycotoxins from foods and feeds is very crucial. Although, prevention and elimination of mycotoxins from human and animal diets is very difficult, detection and diversion of contaminated raw materials from feed and food use can reduce the toxin in the diets (CAST, 1989; Pestka et al, 1995). Conventional methods for mycotoxin detection include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC) and mass spectroscopy (MS) (Pestka, 1994). Purification and detection of fumonisins will be reviewed in Part II. In the following sections purification and detection of trichothecenes are described.

Cleanup of trichothecenes prior to analytical detection.

Analyses of trichothecene mycotoxins in foods and feeds involve sample extraction and a very extensive cleanup to purify the mycotoxins before detection is made (Snyder, 1986; Chu, 1991; Gilbert, 1993). Based on trichothecene solubility in various solvents, Snyder (1986) classified trichothecene mycotoxins

into two groups. Group I toxins which have one or more acetyl groups, such as T-2 toxin, HT-toxin, diacetoxyscirpenol, are very soluble in nonpolar solvent, such as chloroform, methylene chloride, ethylacetate, diethyl ether, and acetone. Group II toxins, which have no acetyl groups (*e.g.* DON, NIV, and T-2 tetraol) are highly soluble in polar solvents, such as ethanol, methanol, water, aqueous methanol and aqueous acetonitrile because of stronger polar side groups in the group II molecules. Therefore, nonpolar solvents are suitable for extraction of group I trichothecenes, and polar ones for group II toxins (Snyder, 1986). When a mixture of group I and II compounds is extracted, this author suggested using a mixture of acetylacetate and acetonitrile as a solvent system. However, this procedure requires an extensive purification to remove the acetylacetate from the group I toxins. (Snyder, 1986).

Polar solvents such as methanol or acetonitrile in combination with water are now commonly used for extraction of mycotoxins from sample matrices because of increasing use of reverse-phase liquid chromatography column and immunoassay (Whitaker et al., 1986). In addition, those solvent systems are less toxic and less expensive than those used early. Furlong et al. (1995), for example, used a mixture of methanol and aqueous 4% KCl solution at ratio of 9:1(vol./vol.) for extraction of trichothecenes from wheat. Meanwhile, a mixture of acetonitrile and water at a ratio of 3:1 has also been used to extract DON, NIV and zearalenone from cereal grains (Tanaka et al., 1985; 1986; 1988a,b; Park et al., 1991; 1992; Muller and Schwadorf, 1993).

After extraction, trichothecenes can be separated from their crude extracts via liquid/liquid or solid/liquid partitioning techniques (Snyder, 1986; Chu, 1991). An example of a liquid/liquid partition solvent system used for T-2 toxin separation is a mixture of methanol/ethylacetate/chloroform at a ratio of 1:1:2 (vol./vol./vol.) (Hsu et al., 1972). A mixture of acetonitrile and petroleum ether at a ratio of 1:1 (vol./vol.) is also used in the separation of T-2 toxin or DAS (Mirocha et al., 1976). In solid/liquid partition systems, columns which are used for trichothecene cleanup include ferric gel (Mirocha et al., 1976), activated charcoal (Morooka et al., 1972), activated charcoal-alumina (Romer, 1984), florisil and silica gel (Tanaka et al., 1985), and charcoal:alumina:celite at a ratio of 7:5:3 (w/w/w) (Eppley et al., 1986; Trucksess et al., 1986; 1987; Fernandez et al., 1994). The availability of higher capacity and more effectively controlled size absorption packing materials led to the development of a smaller but more efficient column chromatography (Chu, 1991). Such smaller columns including SAX (Thiel et al., 1991), Amberlite IRC 50 (Shepherd and Gilbert, 1986), Sep-Pak (Mirocha et al., 1989) are now commonly used for mycotoxin purification, instead of using large columns such as silica gel or using solvent partition methods (Chu, 1991). The following step after purification is mycotoxin detection which can be performed with TLC, GC, HPLC, or MS methods (Pestka et al., 1995).

Detection of trichothecenes

TLC has always been a favorite method for analyses of mycotoxins because of its simplicity and low cost (CAST, 1989). Most of the TLC studies

usually employ silica gel as an absorbent. The thickness of the thin layer is generally 0.25 mm and various solvent systems are used as developing agents (Ueno, 1983). Retention factor (Rf) values of some trichothecene mycotoxins are summarized in Table 1.4.

Derivatization reactions are required when TLC is applied to trichothecenes because most trichothecene toxins do not have useful absorption or potential for fluorescence under ultraviolet or visible light (Ueno, 1983). Compounds used as derivatization reagents include sulfuric acid (Ueno et al., 1973; Gimeno, 1979), p-anisaldehyde (Scott et al., 1970), 4-(p-nitrobenzyl)piridine (NBP) (Takitani et al., 1979), nicotinamide-2-acetalpyridine (Sano et al., 1982), and aluminum chloride (Romer, 1986; Trucksess et al., 1987; Fernandez et al., 1994). Use of a certain derivatization reagent depends on the objectives of trichothecene analysis, the kinds of trichothecene mycotoxins to be analyzed, and the methods of toxin purification (Ueno, 1983). This author suggested to use sulfuric acid, aluminium chloride, or nicotinamide-2-acetylpiridine for analysis sensitivity; aluminium chloride, NBP, or nicotinamide-2-acetylpiridine for toxin selectivity; sulfuric acid, aluminium chloride, or NBP for procedure simplicity; aluminium chloride, or nicotinamide-2-acetylpiridine for derivative stability. For general purposes, aluminium chloride or NBP is most frequently used as a derivatization reagent (Ueno, 1983).

Color development using sulfuric acid, or p-anisaldehyde reagents is performed by spraying the reagents on a TLC plate and then heating at 100-130^{0}C for about 20 minutes (Scott et al., 1970; Ueno et al., 1973). When sulfuric

Table 1.5. Retention factor (Rf) values of some trichothecene mycotoxins on TLC plates using silica gel as absorbent (adapted from Ueno, 1983)

Solvent system	A	B	C	D	E	F	G	H	I	J	K	L
Group A												
Trichodermol	-	-	-	-	-	-	0.17	-	-	-	-	-
Trichodermin	-	-	-	-	-	-	0.51	-	-	-	-	-
Verrucarol	-	-	-	-	-	-	0.03	-	-	-	-	-
Scirpentriol	-	-	0.036	-	-	-	-	-	0.071	-	-	-
Monoacetoxyscirpenol	-	-	0.066	-	-	-	-	-	0.121	-	-	-
Diacetoxyscirpenol	-	-	0.474	-	0.52	0.50	0.14	-	0.373	0.47	0.46	0.68
7,8-Dihydroxy diacetoxyscirpenol	-	-	-	-	0.35	0.24	-	-	-	-	-	-
T-2 tetraol	-	-	0	-	-		0	-	0.21	-	-	-
Neosolaniol	-	-	0.188	-	0.38	0.29	-	-	0.152	0.15	0.25	0.32
HT-2 toxin	-	-	0.101	-	0.30	0.21	0.03	-	0.125	0.10	0.17	0.23
T-2 toxin	-	-	0.528	-	0.55	0.52	0.16	-	0.410	0.61	0.68	0.78
Group B												
Trichothecolone	-	-	-	-	-	-	0.13	-	-	-	-	-
Trichothecin	-	-	-	-	-	-	0.53	-	-	-	-	-
Nivalenol	-	-	-	0.22	0.09	0.03	-	-	-	0.02	0.01	0.04
Fusarenon-X	-	-	0.170-	0.64	0.41	0.29	-	-	0.250	0.31	0.21	0.32
Diacetylnivalenol	-	-	-	-	0.51	0.44	-	-	-	0.47	0.43	0.54
Tetraacetylnivalenol	-	-	-	-	0.62	0.63	-	-	-	-	-	-
Deoxynivalenol	-	-	0.066	0.47	0.31	0.20	-	-	0.157	-	-	-

Table 1.5. Continued

Solvent system	A	B	C	D	E	F	G	H	I	J	K	L
Group C									-	-	-	-
Verrucarin A	0.28	0.59	-	-	-	-	-	0.47	-	-	-	-
Verrucarin B	0.47	0.69	-	-	-	-	-	0.63	-	-	-	-
Verrucarin J	0.59	-	-	-	-	-	-	0.64	-	-	-	-
Roridin A	0.18	0.21	-	-	-	-	-	0.20	-	-	-	-
Roridin D	0.35	-	-	-	-	-	-	0.29				
Group D									-	-	-	-
Crotocol	-	-	-	-	-	-	0.07	-	-	-	-	-
Crotocin	-	-	-	-	0.59	0.65	0.34	-	-	-	-	-

Solvent systems:
A, B, C, and D indicate chloroform:methanol at ratio (vol:vol.) of 98:2, 97:3, 95:5, and 7:1, respectively.

E indicates benzene:acetone at ratio (vol:vol.) of 1:1. F indicates chloroform:acetone at ratio (vol:vol.) of 3:2.

G, H indicate benzene:tetrahydrofuran at ratio (vol:vol.) of 85:15. I indicates ethylacetate:toluene at ratio (vol:vol.) of 3:1.

J indicates ethylacetate:n-hexane at ratio (vol:vol.) of 3:1. K indicates chloroform:isopropanol:ethylacetate at ratio (vol:vol.:vol.) of 95:5:5.

L indicates chloroform:ethanol:ethylacetate at ratio (vol:vol.:vol.) of 90:5:5. "- " indicated not analyzed.

acid is used, group A and B trichothecenes generate a grayish black color, and a brown color, respectively. The detection limit of this system is approximately 0.25 μg per spot (Ueno et al., 1973). In addition, under 360 nm UV light the group A give a blue fluorescent color with a detection limit of 0.05 μg per spot (Ueno et al., 1973). The same detection limits is also obtained when p-anisaldehyde is used as a derivatization reagent, but group A trichothecenes give a pinkish violet color and the group B trichothecenes yield a yellowish brown color(Scott et al., 1970).

Takitani et al. (1979) reported that a detection limit of 0.02-0.2 μg per spot was possible by using NBP as a derivatization reagent. A blue-violet color for trichothecenes was observed under long wave UV light after a TLC plate was sprayed with NBP solution, heated at 150^0C for about 30 minutes, cooled at room temperature, and then dipped in tetraethylenepentamine solution. The blue-violet color was a compound which was formed through N-alkylation of N-atom on the piridine ring of the NBP reagent with the epoxy group of trichothecenes. These investigators suggested that this method was applicable to all trichothecene mycotoxins having a characteristic of 12, 13-epoxy group.

More sensitive and specific TLC methods were developed by Nelis and Sensheimer (1981). They used nicotinamide as an alkylating reagent to produce fluorescent derivatives. An epoxide-containing compound such as trichothecenes is added to the solution of nicotinamide, a ketone (e.g. acetophenone), and alcoholic KOH. A blue fluorescent color is developed by the addition of formic acid at ambient conditions. These researchers reported that this system had a

detection limit of 0.1-2.0 ng per spot. If compared to NBP method, this technique is better because it is approximately 100 folds more sensitive.

High-performance thin layer chromatography (HPTLC) has also been applied to detection of DON, fusarenon-X, and NIV in cereal grains (Trucksess et al. 1987). After purification, the toxins are spotted on a TLC plate which has been previously impregnated with aluminum chloride. The plate is then developed in two sequential solvent systems, firstly in chloroform:acetone:2-propanol at ratio of 8:1:1(vol./vol./vol.), and secondly in the same solvent mixture but at a 14:3:3 ratio. After air drying, heating at 120^{0}C for 8 minutes, and cooling at room temperature, the plate is observed under long-wave UV light. The toxins appear as blue fluorescent spots. Rf values of DON, fusarenon-X, and NIV are 0.5, 0.4, and 0.1 respectively. Average recovery of these three toxins added to cereal grains at levels of 100 and 200 ppb was 83%, and the detection limit of this system was 50 pbb (Trucksess et al., 1987). Fernandez et al. (1994) also used this technique for detection of DON in 1991 U.S. winter and spring wheat and they found that the limit of determination was 40 ppb, and average recoveries of DON-spiked wheat samples at levels of 200, 400, and 800 ppb were 83, 82 and 72%, respectively.

Trichothecenes can be analyzed either individually (Lauren and Greenhalg, 1987) or collectively (Lauren and Agnew, 1991) with HPLC methods. Lauren and Agnew (1991) developed a multitoxin screening method for *Fusarium* mycotoxins using HPLC techniques. They hydrolyzed trichothecenes (DON, NIV, scirpentriol, and T-2 tetraol) to form the toxin parent alcohol. This parent

alcohol is then detected with HPLC using a longwave UV detector. Recoveries of parent alcohol spiked into wheat and maize extracts at a level of 0.5 ppm ranged from 55 to 103%. Levels of 50 ppb or less were detectable by this method. Meanwhile, Lauren and Greenhalg (1987) observed a detection limit of 50-100 ppb for DON and NIV without hydrolysis. A higher detection limit (up to 0.02 ppb for DON and NIV) was achieved when HPLC was equipped with a fluorescent detector (Gilbert, 1991). This system involves post column heating with alkali to generate formaldehyde which is then derivatized with methyl acetoacetate and ammonium acetate in a second reaction coil (Gilbert, 1991).

Trichothecene have frequently been analyzed by GC methods (Ueno, 1983; Snyder, 1986). GC analysis require derivatization of trichothecenes to form either trimethylsilyl (TMS) or heptafluorobutyril (HFB) derivatives, prior to detection with a capillary column which is equipped with a flame ionization detector (FID) (Kamimura et al., 1981; Trucksess et al., 1987) or an electron capture detector (ECD) (Ware et al., 1984). Detection limits of GC-FID are 200 ppb and 100 ppb for group A and B trichothecenes, respectively (Kamimura et al., 1981). Detection limits of approximately 80 ppb for group A trichothecenes and 2 ppb for group B trichothecenes are achieved when ECD-GC is used (Ware et al., 1984). GC analyses are generally preferred to HPLC methods because GC has a greater sensitivity and specificity. The greater separating capacity of GC compared with that of HPLC also enables researchers to monitor a greater number of trichothecenes in the same extracts. In addition, GC when coupled

with MS analysis can simultaneously detect and confirm trichothecene mycotoxins in a food or feed sample (Gilbert, 1993).

In earlier investigations, MS detection methods were not considered to be a quantitative analytical tool for mycotoxins. However, a high resolution MS coupled with computer search and integration capabilities has led to new MS methods for both identification and quantification of mycotoxins (Plattner, 1986). MS methods are preferable for trichothecene analyses since they do not require the compounds to have a chromophore (Chu, 1991). In some cases, extensive clean-up steps are not necessary for MS and a detection limit of tandem GC/MS/MS systems is 1 ppb for T-2 toxin in urine and blood (Mirocha, et al., 1989).

In summary, analysis of trichothecene mycotoxins using a TLC method are rapid, simple and low cost. This method, however, is generally low in sensitivity and selectivity (Bamburg, 1983; Ueno, 1983). Higher sensitivity and selectivity of trichothecenes analysis can be achieved by using GC, HPLC, and MS techniques although they require laborious sample cleanups, and expensive instruments (Pestka, 1988; 1994; Chu, 1991).

IMMUNOASSAYS FOR *FUSARIUM* MYCOTOXINS

Fusarium mycotoxins are a group of secondary metabolites produced by toxigenic strains of *Fusarium* including *Fusarium moniliforme* that produces fumonisins and *Fusarium graminearum* that produces DON (Betina, 1989; Bacon and Nelson, 1994). *Fusarium* mycotoxins especially DON and fumonisins are toxic to both human and animals and commonly contaminate cereal and cereal products (Bamburg, 1983; Tanaka et al., 1988b; Marasas et al., 1988a; Sydenham et al., 1991). Elimination of mycotoxins from human and animal foods is commonly accomplished via detection and diversion mycotoxin-contaminated raw materials from feed and finished food use (Pestka, 1994). Mycotoxin detection can be carried out using either conventional methods such as TLC, GC, HPLC and MS methods or immunoassay techniques such as ELISA. ELISAs are commonly preferred to conventional methods because of simplicity, rapidity, and applicability both in the field and laboratory (El-Nakib et al, 1981).

The basis of mycotoxin immunoassays involves competition between a free mycotoxin and a labeled mycotoxin for an antibody binding site (Pestka, 1988). The purpose of the following review is to discuss *Fusarium*-mycotoxin immunoassays with specific emphasis on antibody production, mycotoxin immunoassay format, application of mycotoxin immunoassays in foods, and commercial mycotoxin immunoassay kits for foods.

Antibody production

Antibodies or immunoglobulins are glycoproteins that are produced and secreted into the body fluids by B lymphocytes of animals in response to a foreign chemical (antigen) significantly different from the animal's own chemical (Candlish, 1991). Immunogenicity refers to the capacity of an antigen to induce an animal's immune response and usually dependent on its chemical structure and its size (Pestka et al., 1995). Production of a highly specific antibody in an animal is mainly influenced by the type of antigens, dose and route of immunizations, the number and types of accessory cells such as macrophages that initially interact with antigen and induce lymphocyte activation, and the nature of responding lymphocytes (Abbas et al., 1994). Generally, immunogen must be degradable and must have an epitope that can bind to the cell-surface antibody of a B virgin cell. It must also have at least one site that can be recognized simultaneously by a class II protein and by a T-cell receptor, thus facilitating cell-to-cell communication between helper T cells and B cells can occur (Harlow and Lane, 1988). Compounds smaller than 3000 daltons (hapten) may be able to bind to the surface antibody of B cells, but may not have suitable site for the simultaneous binding of a class II protein and a T-cell receptor.

Most *Fusarium* mycotoxins are of low molecular weight (250-500 dalton) (Betina, 1989), and thus are not immunogenic by themselves (Chu, 1986). This problem can be overcome through conjugation of the toxins to larger immunogenic molecules (carriers).

Mycotoxin conjugation. Mycotoxin conjugation is a reaction of a mycotoxin and a carrier protein to form a mycotoxin-protein carrier conjugate so that the mycotoxin becomes immunogen. Carrier molecules that are commonly used for mycotoxin conjugation are capable of imparting immunogenicity to covalently coupled mycotoxins or haptens. These include bovine serum albumin (BSA), chicken ovalbumine (OA), and keyhole limpet hemacyanin (KLH), and cholera toxin (CT) (Chu, 1986; Harlow and Lane, 1988).

BSA is very soluble and a good protein carrier (Harlow and Lane, 1998). These authors reported that BSA has 30-35 lysines, 19 tyrosines, 35 cysteines, 39 aspartic acids, and 59 glutamic acids that are available for coupling. Meanwhile, OA has 20 lysines, 10 tyrosines, 6 cysteines, 14 aspartic acids, and 33 glutamic acid residues.

KLH (MW 4.5x 10^5 to 1.3x10^7), a copper containing protein, belongs to a group of non-heme proteins called hemacyanins which are found in *arthropods* and molluscs (Senozan et al., 1981). KLH is isolated from the mollusc *Megathura creulata*. In Tris buffer, pH 7.4, this protein exists in 5 five states of aggregation which have sediment coefficients of 102S, 130S, 150S, 170S, and 186S (Senozan et al., 1981; Herckovits, 1988). It will reversibly dissociate to subunits when pH changes moderately. Both lowering and raising of pH will cause dissociation (Herckovits, 1988) and at pH 8.9 it will completely dissociate to subunits. Each subunit contains oxygen binding sites which can bind to two copper atoms per molecule oxygen in KLH (Senozan et al., 1981; Herckovits,

1988). Unlike BSA, KLH is sometimes likely precipitated during coupling due to its large size, and this can make difficult in some cases (Harlow Lane, 1988).

Cholera toxin (CT) (MW 86,000) is a protein exotoxin produced by *Vibrio cholerae* and has been shown to have strong and oral systemic adjuvant properties when co-administered with unrelated antigen (Liang et al, 1978; Lycke and Holmgren, 1986). CT consists of two types of subunits, a single 'heavy' subunit of molecular weight 28,000 which noncovalently attached to a 58,000-MW aggregate of 'light' subunits (Holmgren, 1981). CT has several advantages when used for generating antibodies (Azcona-Olivera et al., 1992a; Abouzied et al., 1993). Firstly, the procedure was rapid and yield a good quality of antibodies than standard protocols when the toxin was applied to generate FB_1 antibody. Secondly, CT might be an humane alternative to Freund's adjuvant since abscesses, ulcers, or granulomas at injection sites which usually appeared after Freund's adjuvant immunization, are not observed after CT injection. Thirdly, because low doses of CT-immunogen yields a rapid and strong antibody response, CT would be valuable when mycotoxin availability is limited.

Conjugation of a mycotoxin to a protein carrier can be achieved following chemical reaction of functional groups present on the mycotoxin with functional groups present on the carrier (Chu, 1986). Mycotoxins which already contain reactive groups such as fumonisin B_1 , B_2, and B_3 can be coupled directly to protein molecules by using glutaraldehyde as a protein linker (Figure 1.5) (Azcona-Olivera, 1992a,b; Fukuda et al., 1994; Usleber et al., 1994). However, generation of protein conjugates for mycotoxins which do not contain functional

groups such as DON (Casale et al., 1988) is much more complex because a reactive group has to be introduced by chemical synthesis to the toxins prior to the conjugation reaction (Figure 1.5).

A reactive group commonly introduced to trichothecenes is carboxylic acid which is performed by reacting the toxins with bifunctional acid anhydrides, such as succinic or glutaric anhydrides at the presence of a catalyst such as 4,N,N-dimethylaminopyridine or pyridine. Hemisuccinate and hemiglutarate of T-2 toxin (Chu et al., 1979), diacetylscirpenol (Chu et al., 1984a), 3-acetyl DON (Kemp et al., 1986; Usleber et al., 1991), and DON (Usleber et al., 1993), as well as hemisuccinate of DON triacetate (Zhang et al., 1986), DON (Casale et al., 1988; Usleber et al., 1991), and 4, 15-diacetylnivalenol (Abouzied et al., 1993) have been prepared prior to the conjugation of the toxins to protein carriers.

Conjugation of hemisuccinic or hemiglutaric trichothecenes to protein carriers is usually accomplished using a mixed-anhydride (MA) or an activated ester (AE) method. In MA method the trichothecene derivatives are generally activated to their corresponding anhydride by isobutyl chloroformate in dry tetrahydrofuran and triethylamine and simultaneously coupled with protein (Gendloff et al., 1986); where as, in the AE method, *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in dimethylformamide solution are used to activate the derivatives prior to conjugation process (Kitagawa et al., 1981).

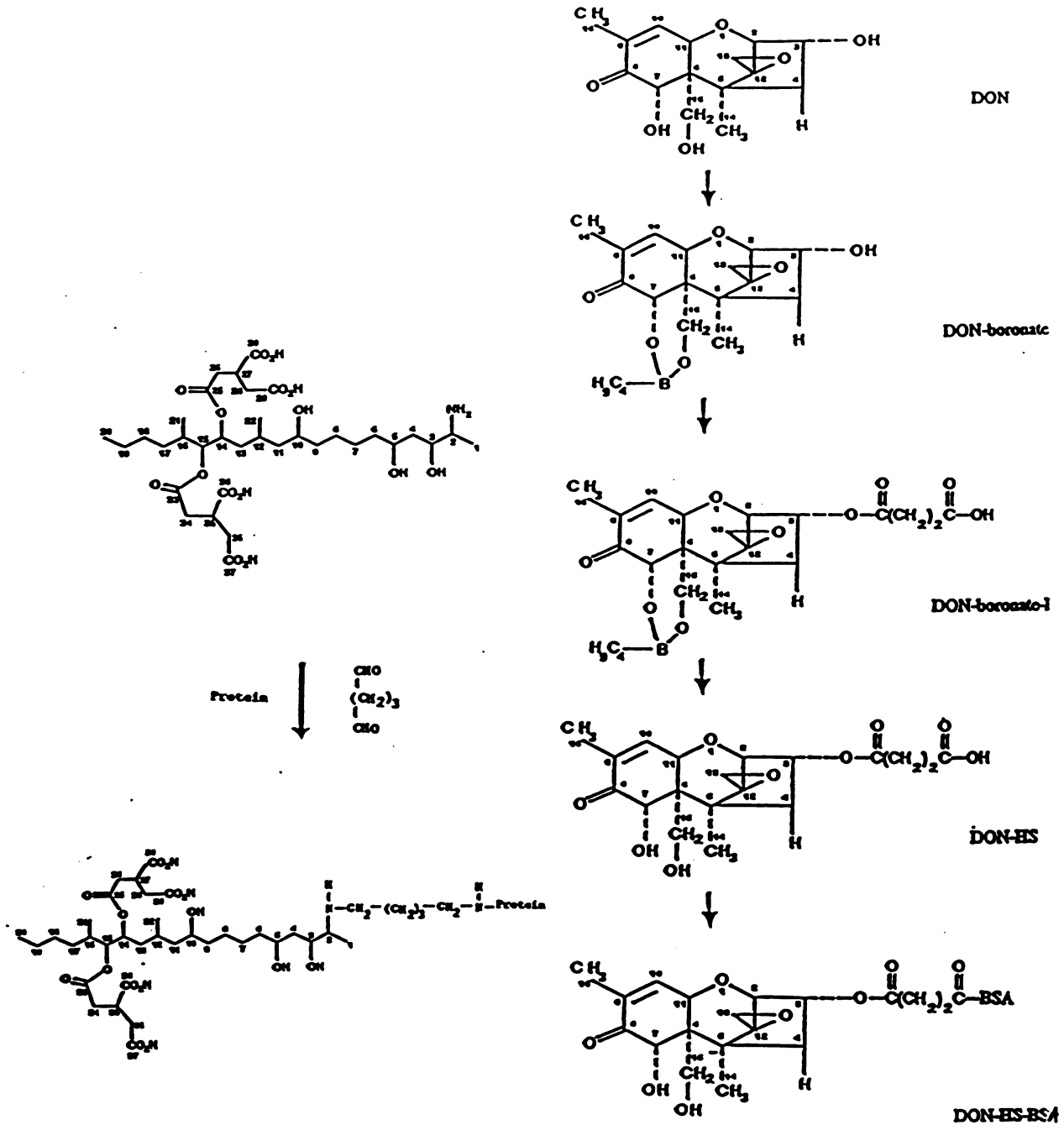


Figure 1.5. Preparation of immunogen for (left) fumonisins and (right) deoxynivalenol (DON) (Pestka et al., 1995)

Polyclonal antibodies. After successful production, characterization, and purification of a mycotoxin-protein conjugate, suitable animal species are then immunized with the purified conjugate (Chu, 1986; Harlow and Lane, 1988). These latter authors suggest the use of rabbits, goats, or sheep for polyclonal antibodies and mice for monoclonal antibodies because only mice which have tumor cell lines for the efficient fusion of plasma cells.

Multiple-site intracutaneous immunizations of rabbits with 100-1000 μg of mycotoxin-protein conjugate which has been mixed with an adjuvant are commonly performed to generate antibodies (Harlow and Lane, 1988). The conjugate is usually emulsified with an oil-base adjuvant containing killed *Mycobacterium* such as "complete" Freund's adjuvant at a ratio of 1:1 (vol./vol.) to allow slow release of the immunogen and nonspecifically induce immune response (Usleber, 1994). "Complete" Freund's adjuvant is used for primary immunization and "incomplete" (not containing killed *Mycobacterium*) one for subsequent injections which are usually given in 2-4 week intervals. High titer rabbit antisera commonly can be generated as early as 4 weeks after the initial injection (Zang et al., 1986; Wang and Chu, 1991; Abouzied et al., 1993; Usleber et al., 1993; 1994).

Rabbit antisera contain antibodies which are generated by multiple B-cell clones and vary in specificity; therefore, they are considered polyclonal (Pestka et al., 1995). A major advantage of polyclonal antibodies is that they are easy and inexpensive to produce, and often contain a subclone of high affinity antibody (Usleber et al., 1994). However, the quality of polyclonal antisera in terms of affinity,

specificity as well as physical and chemical stability varies from bleeding to bleeding and from rabbit to rabbit (Pestka et al., 1995). As a consequence, use of these antibodies for construction of commercial kits with defined performance characteristics is difficult. This difficulty can be eliminated when monoclonal antibodies are used.

Monoclonal antibodies. Production of monoclonal antibodies involves animal immunization and fusion of splenocytes with a tumor cell line (Kohler and Milstein, 1975). Intraperitoneal or subcutaneous immunizations of female BALB/c mice with 5-50 μg mycotoxin-protein conjugate per injection are generally used to induce B cells which can synthesize the mycotoxin-specific antibodies (Azcona-Olivera, 1992b; Fukuda et al., 1994). The immunizations are usually given three times at two weeks intervals. The first and second injections were with an emulsion of adjuvant and the conjugate, and the final one was with the conjugation without adjuvant. Four days later, the mice producing mycotoxin-specific antibodies are killed and their spleen cells which contain high numbers of antibody-secreting B cells are fused with myeloma cells (mouse cells which have all cellular processes necessary for the secretion of antibody but have no ability to produce antibody) using polyethylene glycol (Galfre and Milstein, 1981).

During fusion, only about 1% of the starting cells are fused and only 1 in 10^5 form viable hybrids (Harlow and Lane, 1988). In tissue cultures the cells from immunized mouse spleens can not grow but the unfused myeloma cells can adapt and grow well (Harlow and Lane, 1988). These unfused cells have to be eliminated,

so they do not block the growth of the hybrid cells. Unfused myeloma cells, in which the hypoxanthine-guanine phosphoribosyl transferase gene (HPRT$^-$) in the salvage nucleotide synthesis pathway has been mutated, are killed by the addition of hypoxanthine, aminopterin and thymidine to the culture media (Azcona-Olivera, 1992b). Aminopterin will block *de novo* nucleotide synthesis pathway and force every cell in the culture to synthesize its purine nucleotides via salvage pathway. Under these conditions, cells containing a non functional HPRT protein such as myeloma cells will die and hybrids between spleen B cells with a functional HPRT and myeloma cells with a non-functional HPRT will continue to grow by using hypoxanthine and thymidine for producing their nucleotides (Harlow and Lane, 1988).

Supernatants of the hybridoma cultures can be screened for the presence of the mycotoxin-specific antibodies by competitive indirect enzyme-linked immunosorbent assay (CI-ELISA) (Azcona-Olivera et al., 1992b). The selected cultures are then successively scaled up and cloned by limiting dilution at 0.5-1 cell/well (Goding, 1980). An immortalized subclone that consistently secretes antibody of desired affinity, specificity, and performance characteristics is then expanded to produce a large amount of monoclonal antibodies. Although monoclonal antibody generation is expensive, time consuming and requires for tissue culture facilities, these antibodies tend to exhibit a low degree of interassay variation and give highly reproducible results (Pestka et al., 1995). Therefore, they are suitable for use in the development of commercial kits.

Recombinant antibodies. Beside using hybridoma technology, antibodies can be produced through gene recombinant technology which was developed recently. The gene technology involves rearrangement or assembly of germ line V-genes, surface display of antibody, affinity selection, affinity maturation and soluble antibody production (Figure 1.6, Winter et al., 1994).

Initially antibody genes were taken from hybridomas, cloned into plasmid vectors and expressed as fragments in bacteria (Better et al., 1988) or as complete antibodies in mammalian cells (Oi et al., 1983). Later, Orlandi et al. (1989) isolated antibody genes directly form lymphocytes of immunized animals, and then propagated the genes by using universal primers and polymerase chain reaction (PCR) prior to expression the genes in bacteria. In addition, by building restriction sites into these primers, the amplified DNA can be cloned directly for expression in bacteria (Milstein, 1990). Like hybridoma technology, the later approach still relies on animal immunization to generate antigen specific lymphocyte cells. The hybridoma technology can immortalize these cells (Kohler and Milstein, 1975), while the gene technology can immortalize their genes (Milstein,1990). In future, it may become possible to generate antibody without animal immunization. Antibody genes are taken from unimmunized animal, and then amplify using universal primer and PCR (Orlandi et al., 1989). Prior to expression in bacteria, the genes can be readily manipulated by cutting and pasting of restriction fragments (Milstein, 1990) or by site directed mutagenesis (Better et al., 1983) to construct a new and desired antibodies.

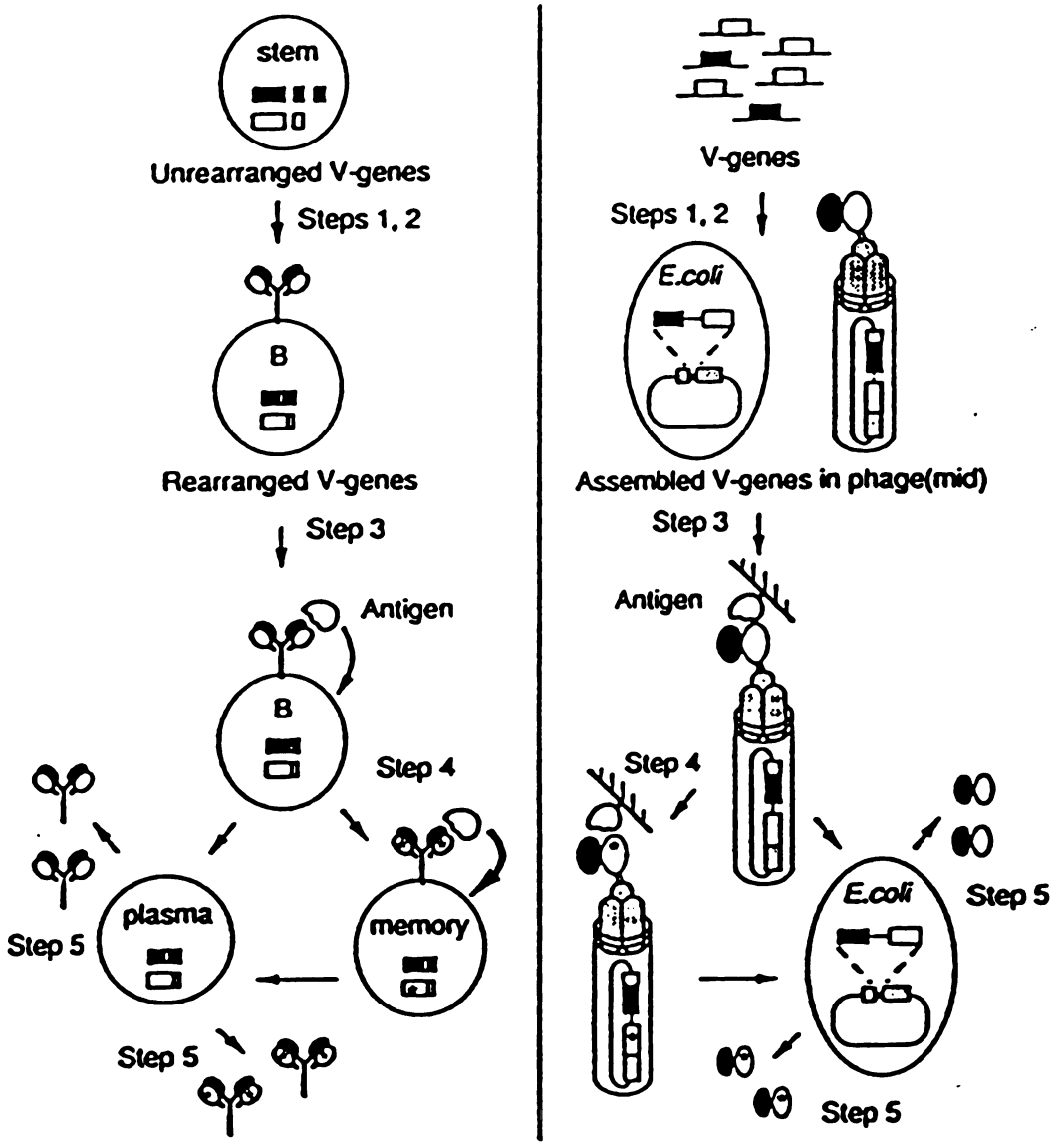


Figure 1.6. Process of antibody production *in vivo* (left) and using gene technology (right). Step 1: rearrangement or assembly of germ line V-genes; step 2: surface displaying of antibody; step 3: antigen-driven or affinity selection; step 4: affinity maturation; step 5: production of soluble antibody or antibody fragment (Adapted from Winter et al., 1994).

Mycotoxin immunoassay format

A number of immunoassay formats have been developed for mycotoxin analysis. Initially, competitive radioactive immunoassays have been developed for mycotoxins, at which a mixture of a constant amount of a radiolabeled mycotoxin and mycotoxin standards or unknown samples is incubated with specific antibodies (Pestka et al., 1995). Radioactivity of unbound radiolabeled mycotoxins in the solution is then measured after a complex of antibodies and radiolabeled toxin is removed from solution. Amount of the toxin in a sample is reversely related with unbound radiolabeled in the solution. Because of inherent problems with radioactive reagents, enzyme-linked immunosorbent assays (ELISAs) were subsequently developed on the procedures of Engvall and Perlman (1971).

Solid phase supports used for mycotoxin ELISA include microtiter plates, beads, tubes, and dipsticks (Tarcha, 1991). These solid supports are most commonly made from polystyrene because of its low cost, easy process, optical clarity and high-protein binding (Tarcha, 1991). Among solid phase supports, polystyrene microtiter plates have been most commonly used because of supporting technology, such as removable strips, multiwell pipettes, automated washers, and spectrophotometers (Pestka, 1994).

ELISA procedures commonly applied for mycotoxin detection are competitive direct ELISA (CD-ELISA) and the CI-ELISA (Figure 1.7). In the CD-ELISA, an enzyme-labeled mycotoxin and a free mycotoxin from samples are

incubated together over a solid phase-bound mycotoxin antibody (Pestka, 1988). This assay is based on the competition between free and the labeled mycotoxins. A concentration of mycotoxin in sample is inversely related to a complex of enzyme-labeled mycotoxin and the bound antibody. After the addition of an appropriate enzyme substrate, the bound antibody as well as sample mycotoxin concentrations can be calculated based on the intensity of color development which is measured spectrophotometrically. In the CI-ELISA, a solid-phase mycotoxin-protein conjugate competes with a free mycotoxin for binding to a soluble-mycotoxin specific antibody. A second antibody which has been labeled with an enzyme is then used to determine total bound antibodies and free mycotoxin concentration.

Recently, Pestka (1991) developed immunoassay format, called ELISAGRAM, by combining the sensitivity and selectivity of competitive ELISA with the capability of high-performance thin-layer chromatography (HPTLC) to separate structurally related mycotoxins (Figure 1.8). ELISAGRAM involves coating nitrocellulose (NC) membrane with mycotoxin-specific monoclonal antibodies, separation of mycotoxins by HPTLC, blotted the HPTLC with the NC, incubation of NC with mycotoxin-enzyme conjugate to identify mycotoxins bound to the monoclonal antibodies, detection of bound enzyme conjugate with a precipitating substrate, and visual or densitometric assessment of inhibition bands indicative of cross-reacting mycotoxins. This format has successfully been used to detect zearalenones and aflatoxins, and the result is similar to the competitive ELISA (Pestka, 1991). A major advantage of this procedure is that detection and

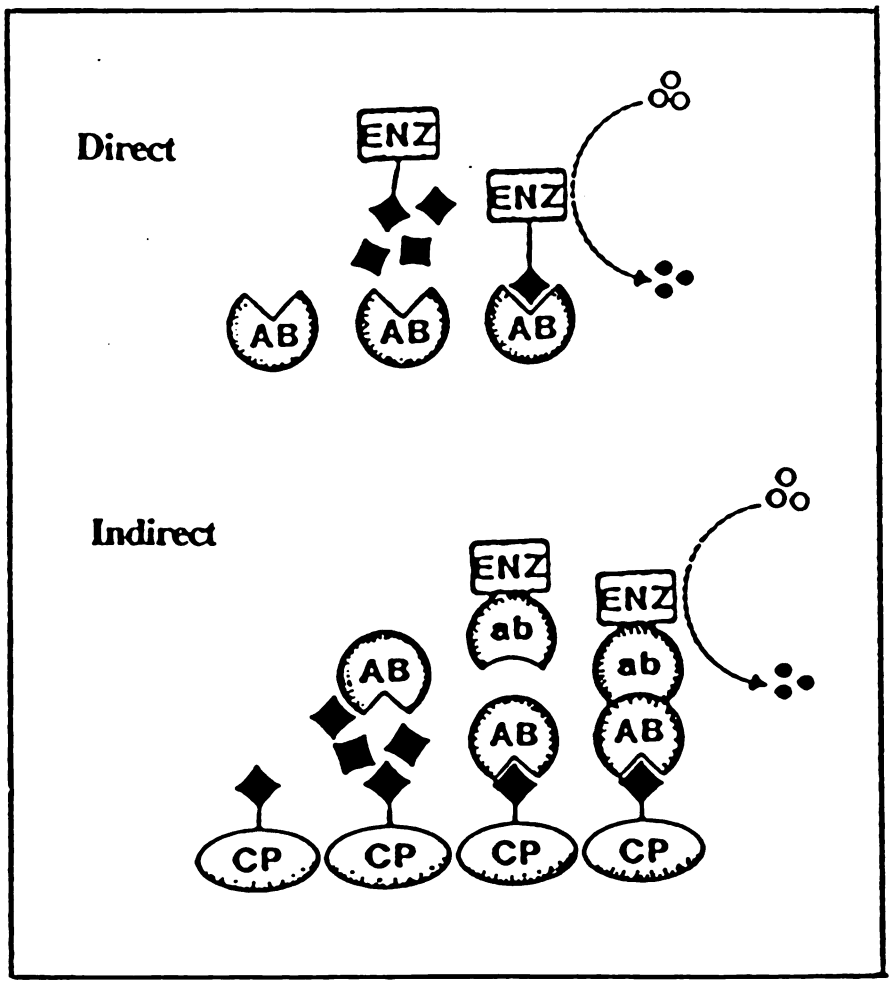


Figure 1.7. Competitive ELISA for mycotoxins (adapted from Pestka, 1988). In direct ELISA, enzyme-labeled mycotoxins (ENZ) are simultaneously incubated with free mycotoxins (□)over solid-phase bound antibodies (AB). Concentration of free mycotoxins is inversely related to bound enzyme-labeled mycotoxins and can be measured quantitatively using spectrophotometer after color development by the addition of enzyme substrate. In indirect competitive ELISA, mycotoxin specific antibodies (AB) compete with free mycotoxins (□) to solid-phase mycotoxin-carrier protein (CP) conjugate. Second antibodies (ab-ENZ) which have been labeled with enzyme are then added to determined total antibody bound. Concentration of free toxin is inversely related to bound enzyme-labeled antibodies.

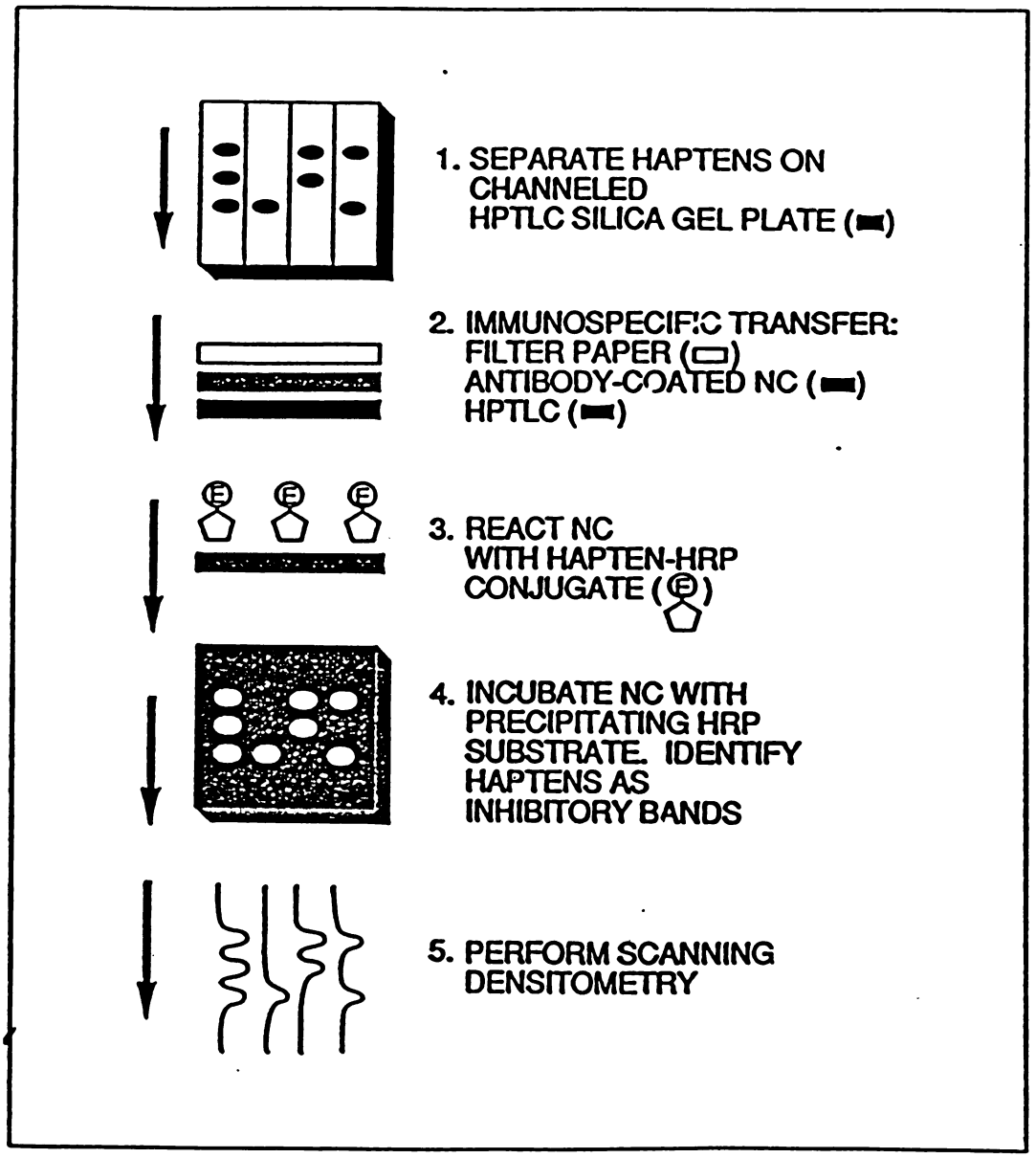


Figure 1.8. ELISAGRAM procedure for mycotoxins (Pestka, 1991).

confirmation of multiple haptens can be performed simultaneously with a single cross-reactive antibody.

More recently, a Computer-Assisted-Multianalyte Assay System (CAMAS) has been devised by Abouzied et al. (1993) for simultaneously detection of fumonisins, aflatoxins and zearalenones. In this format, monoclonal antibodies for each of the toxins are immobilized as multiple lines on nitro cellulose membrane strips and sectored into hydrophobic compartments to minimize reagent use. A modified ELISA is performed at which free mycotoxins and enzyme-labeled mycotoxins compete for binding to the nitrocellulose-bound antibodies. By addition of a precipiting substrate, line-color intensity which is inversely related to mycotoxin concentration is developed. The color intensity can be measured quantitatively using a camera, video monitor, and microcomputer equipped with a video digitizing board. An advantage of this approach is that detection of several mycotoxins can be performed simultaneously in less than 30 minutes (Abouzied et al., 1993). In addition, assay data can be recorded in the minicomputer hard disk.

Other immunochemical formats called "hit and run" assay for T-2 toxin (Warden et al., 1987) and immunochromatography for group A trichothecenes (Chu and Lee, 1989) have been also developed. In the "hit and run" assay, a T-2 toxin column is equilibrated with fluoresce isothiocyanate (FITC)-labeled Fab fragments of IgG T-2 toxin antibodies. Samples containing T-2 toxin are injected to the column so the toxin binds to FITC-Fab fragments. After washing, FITC-Fab-T-2 toxin complexes are eluted and then the toxin is determined in a

standard flow through fluorometer (Warden, et al., 1987). In the immunochromatography approach, which is a combination of HPLC and ELISA methods, group A trichothecenes are separated on C_{18} reversed-phase column. Individual fractions eluted from the column are assessed by ELISA using mycotoxin-specific antibodies. This approach can both identify and determine the concentration of individual group A trichothecene and its detection limit is 2 ng (Chu and Lee, 1989).

Application of mycotoxin immunoassays in foods

Use of immunoassay techniques for mycotoxin detection in foods and feeds is generally preferred to conventional methods, such as TLC, HPLC, GC, or MS because of lower costs, easier execution and quicker result (Pestka, 1988). Antibodies against important *Fusarium* mycotoxins such as fumonisins, trichothecenes, zearalenones have been produced and used for development of radioactive immunoassay (RIA) and ELISA methods. Both methods have been successfully used for mycotoxin screenings in a diverse array of foods and feeds. Selected examples of reported immunoassay for *Fusarium* mycotoxins are summarized in Table 1.6.

An aqueous system is required for immunoassays because antibody and enzyme conjugate activities. Originally, extraction by standard procedures, evaporation, and reconstitution in aqueous buffer have to be done prior to mycotoxin analysis in solid foods such as grain and grain products (Pestka, 1988). In some cases, a column clean up is also performed to obtain higher

Table 1.6. Selected *Fusarium* mycotoxin immunoassays in foods

Toxin	Format	Food analyzed	Detection limit (ppb)	Reference
Fumonisin B_1	ELISA	Buffer solution	50.0	Azcona-Olivera et al., 1992a,b
	ELISA	Feed	250.0	-"-
	ELISA	Buffer solution	0.6	Usleber et al., 1994
	ELISA	Corn	10.0	-"-
	ELISA	Buffer solution	10.0	Fukuda et al., 1994
	ELISA	Foods	200	Pestka et al., 1994
Trichothecenes:				
Acetyldeoxynivalenol	ELISA	Rice	1.0	Kemp et al., 1986
15-Acetyldeoxy-nivalenol	ELISA	Wheat	50.0-100.0	Usleber et al., 1993
4,15-Diacetyl-deoxynivalenol	ELISA	Buffer solution	5.0	Abouzied et al., 1993
Diacetoxyscirpenol	ELISA	Culture	16.0	Hack et al., 1989
DON	RIA	Corn, wheat	20.0	Xu et al., 1986
	ELISA	Corn	200.0	Casale et al., 1988
	ELISA	Grain-based food	1000.0	Abouzied et al., 1991
	ELISA	Buffer solution	1.0	Usleber et al., 1991
Nivalenol tetraacetate	RIA	Buffer solution	5.0	Wang and Chu, 1991
Roridin A	ELISA	Feed	5.0	Martbauer et al., 1988
T-2 toxin	RIA	Corn, wheat	0.1	Lee and Chu, 1981a
	RIA	Milk	2.5	Lee and Chu, 1981b
	ELISA	Corn	50.0	Gendloff et al., 1984
	ELISA	Corn, wheat	2.5	Pestka et al., 1981a
	ELISA	Milk	0.2	Fan et al., 1984
	ELISA	Wheat	0.5	Chiba et al., 1988
	ELISA	Cereal grains	100	Vetro et al., 1994
Zearalenone	ELISA	Corn	1.0	Warner and Pestka, 1986
	ELISA	Grain-based food	2.5	Warner and Pestka, 1987

ELISA and RIA indicate enzyme-linked immunoassay and radioimmunoassay, respectively.

sensitivity (Pestka et al., 1981a). However, since mycotoxin-horseradish peroxidase conjugate as well as solid-phase bound antibodies remain stable in 35% (vol./vol.) methanol in water (Ram et al., 1986), mycotoxin immunoassays can be directly applied to crude extracts of food samples (Usleber et al., 1994). In a liquid system, such as milk, mycotoxins can be detect directly with immunoassay approach although cleanup with a Sep-Pak or affinity column can increase the detection limit of immunoassays (Pestka et al., 1981b).

Sample matrix interference should be considered carefully when detecting mycotoxins in foods or feeds using immunoassay techniques (Laamanen and Veijalainen, 1992). These authors reported that certain substances in food sample might affect color development. When they detected T-2 toxin in millet grains with competitive ELISA assays, 75% of the grain gave higher optical density (OD) values than the negative control. However, a lower OD value than the negative control was obtained when the system applied to fermented foods, processed foods or feed stuffs. This problem could be eliminated by retesting the samples at higher dilution (Laamanen and Veijalainen, 1992). Incorporating toxin-free sample extracts during standard curve preparations can also minimize the false positive or negative reactions (Ram et al., 1986). Since reaction between antibodies and antigens occurs optimally at neutral pH, sample pH must also be considered prior to mycotoxin immunoassays (Pestka et al., 1995).

Commercial mycotoxin immunoassay kits for foods

Extensive research in ELISA has been performed since its discovery in 1971 (Engvall and Permann, 1971) and the results demonstrate that ELISA methods are feasible and reliable for mycotoxin analysis in foods and feeds (Chu, 1986). As a consequence, a number of immunoassay kits have been developed and marketed in the United States as a tool for monitoring mycotoxins in foods and feeds (Table 1.7). Generally, commercial immunoassay kits have worked well both in laboratories and in the field (Azer and Cooper, 1991; Dorner et al., 1993). Some have been evaluated and approved by several professional organizations including Association of Official Analytical Chemists (AOAC) International (Park et al., 1989 a,b). To facilitate evaluation and certification of rapid test kits used for safety screening of foods, the AOAC Research Institute has been set up recently (Pestka et al., 1995). In October, 1992, this organization signed a Memorandum of Understanding with the U.S. Department of Agriculture's Federal Grain Inspection Service (FGIS) concerning the Test Kit Performance Testing Program (Pestka, et al., 1995). Aflatoxin test kits have been evaluated using FGIS protocols and certified to claim "Performance Tested in Accordance with Standards Established by FGIS for Test Kits to Detect Aflatoxin Residues in Grain and Grain Products". More recently, two DON ELISAs have been similarly certified by FGIS.

Although commercial immunoassay kits have been certified by FGIS, they must be evaluated critically prior to adopting the systems. Criteria for adoption of

Table 1.7. Commercial Immunoassay Kits for Mycotoxins available as of June 1993 (Pestka et al., 1994)

Test Kit	Analyte(s)	Format	Detection limit (ppb)	Analysis time[a] (minutes)	Cost/assay ($)[b]	Application	Comments
Afla 5, 10, 20 cup[c]	Aflatoxins	ELISA: Cup	5.0, 10.0, 20.0	5	10.00	Corn, cottonseed	Visual, pass/fail, USDA-FGIS, AOAC First Action Approval
Aflatest[d]	Aflatoxins	Affinity column	1.0	10	7.00	Corn, peanut, feed	Quantitative with fluorometer, USDA-FGIS certified, AOAC First Action Approval, IUPAC approval
	Aflatoxin M_1	Affinity column	0.1	10	10.00	Milk	Quantitative with fluorometer
Agri-screen[e]	Aflatoxins	ELISA: MW	5.0	6-10	3.50	Corn, peanuts, cottonseed, feed	Visual or ELISA reader, pass/fail, USDA-FGIS certified, AOAC First Action Approval
	Vomitoxin	ELISA: MW	1,000.0	12-20	5.50	Corn, wheat, feed	Visual or ELISA reader, pass/fail, USDA-FGIS certified
	Fumonisin	ELISA: MW	500.0	12-20	5.50	Corn, wheat, feed	Visual or ELISA reader, pass/fail
	Ochratoxin	ELISA: MW	20.0	12-20	5.75	Corn, wheat, feed	-"-
	T-2 toxin	ELISA: MW	500.0	18-30	5.50	Corn, wheat, feed	-"-
	Zearalenone	ELISA: MW	250.0	18-30	5.50	Corn, wheat, feed	-"-
Cite Probe[f]	Aflatoxin B_1 Aflatoxin B_2	ELISA: Tray	5.0, 20.0	5	10.0	Corn, cottonseed	Visual, pass/fail, USDA-FGIS certified
Detection[g]	Aflatoxin B_1	ELISA: Tube	1.5	45	9.00	Corn, peanuts, feed	Visual or reader, pass/fail
Dosage[g]	Aflatoxin B_1	ELISA: MW	0.5	45	4.00	Corn, peanuts, feed	Quantitative, with ELISA reader
Fumonitest[d]	Fumonisin B_1 Fumonisin B_2	Affinity column	2.0	15	10.00	Corn	Quantitative with fluorometer

Table 1.7. Continued

Test Kit	Analyte(s)	Format	Detection limit (ppb)	Analysis time[a] (minutes)	Cost/assay ($)[b]	Application	Comments
EZ-Screen[h]	Aflatoxins	ELISA: MC	5.0-20.0	10	5.00-7.50	Corn, peanuts	Visual, pass/fail
	Aflatoxin M_1	ELISA: MC	0.5	10	5.00-7.50	Milk	-"-
		ELISA: MC	5.0	10	5.00-7.50	Corn	-"-
		ELISA: MC	12.5	10	5.00-7.50	Corn	-"-
		ELISA: MC	50.0	10	5.00-7.50	Corn	-"-
Ochratest[d]	Ochratoxin	Affinity column	5.0	10	10.00	Corn	Quantitative with fluorometer
One-step ELISA[c]	Aflatoxin B_1	ELISA: MW	5.0	40	1.00	Corn, peanuts, cottonseed, feed	Quantitative, with ELISA reader
	Aflatoxin M_1	ELISA: MW	0.5	40	1.00	Milk	-"-
	Zearalenone	ELISA: MW	200.0	40	1.00	Corn, wheat	-"-
Veratox[e]	Aflatoxins	ELISA: MW	5.0	15	4.00	Corn, peanuts, cottonseed, feed	Quantitative, with ELISA reader, USDA-FGIS certified
	Aflatoxin M_1	ELISA: MW	0.3	40	5.50	Corn, wheat, feed	Quantitative, with ELISA reader
	Vomitoxin	ELISA: MW	300.0	20	5.50	Corn, wheat, feed	-"-
	T-2 toxin	ELISA: MW	50.0	30	6.00	Corn, wheat, feed	-"-
	Zearalenone	ELISA: MW	250.0	30	6.00	Corn, wheat, feed	-"-
Zearaletest[d]	Zearalenone	Affinity column	500.0	10	10.00	Corn, wheat	Quantitative with fluorometer

ELISA and RIA indicates enzyme-linked immunoassay.
RIA indicates radioimmunoassay, respectively.
[a] Analysis time does not include sample extraction.
[b] Based on purchase of smallest available unit.
[c] International Diagnostics, St. Joseph, MI, 49085 (phone 616-983-0972
[d] Vicam, Somerville, MA, 02145 (800-2228-4381).

MC and MW indicate multisite card and microwell, respectively
-"- indicates same as above.
[e] Neogen Corp., Lansing, MI, 48912 (phone 800-234-5333).
[f] Idexx Labs, Westbrok, ME, 04092 (800-548-6733).
[g] Transia, Lyon, France, 69007 (33-727-30381).
[h] DiAGnostix, Inc., Burlington, NC, 27215 (800-334-1116).

a mycotoxin immunoassay include: detection limits and sensitivity ranges, requirements for rapid screening and/or quantitation, mycotoxin specificity, effectiveness of recommended extraction procedures, field stability, inter-and intra- assay reproducibility (Pestka, 1988).

PART II

DEVELOPMENT AND APPLICATION OF AN ELISA FOR FUMONISIN B_1 IN FUNGAL CULTURES, CORN AND CORN PRODUCTS

ABSTRACT

DEVELOPMENT AND APPLICATION OF AN ELISA FOR FUMONISIN B_1 IN FUNGAL CULTURES, CORN AND CORN PRODUCTS

By

Sutikno

Mouse monoclonal antibodies, rabbit and sheep polyclonal antibodies against fumonisin B_1 (FB_1) were readily generated after immunization with keyhole limpet hemacyanin (KLH) as a protein carrier. When these antibodies were used in competitive inhibition enzyme-linked immunosorbent assay (ELISA), the sheep antisera had the highest affinity and were the most specific to FB_1 as compared to the mouse monoclonal and rabbit polyclonal antibodies. Cross reactivities of these sheep antisera toward fumonisin B_1, B_2, and B_3 were 100, 24 and 30%, respectively. When competitive direct ELISA (CD-ELISA) employing these FB_1 sheep antisera was used to detect FB_1 in *Fusarium* corn culture, corn and corn products, it yielded approximately two fold higher FB_1 levels than high performance liquid chromatography (HPLC). Its perfomance was much better than a monoclonal antibody based CD-ELISA that was previously developed in our laboratory. This CD-ELISA had a strong positive relationship with HPLC, suggesting that it would be useful for screening human and animal foods from fumonisins.

INTRODUCTION

Background.

Fumonisins are toxic secondary metabolites, found worldwide that are produced by *Fusarium moniliforme* and *F. proliferatum*. These toxins are elaborated by the fungi in corn in the field and during storage (Bezuidenhout et al 1988; Gelderblom et al., 1988a). Six different fumonisins, fumonisin A_1, A_2, B_1, B_2, B_3, and B_4, have been chemically characterized(Figure 1.2) (Bezuidenhout et al., 1988; Cawood et al., 1991; Plattner et al., 1992). Fumonisin B_1 (FB_1) is the most toxic and primary fumonisin produced by *Fusarium moniliforme* (Gelderblom et al., 1988b). FB_1 produces characteristic effects in different species, including hepatic cancer in rats, brain lesions in horses (equine leukoencephalomalacia, ELEM), and pulmonary lesions in pigs (porcine pulmonary edema, PPE).

Wilson et al. (1985) observed hepatic cancer in rats that were fed with corn naturally contaminated with the *Fusarium moniliforme* for extended periods (up to 176 days). Marasas et al. (1984b) also found liver tumors in rats by feeding *Fusarium moniliforme* culture materials. Gelderblom et al. (1988b) performed experiments to investigate the toxic metabolite of *Fusarium moniliforme*. Through chromatographic procedures, these investigators identified the water-soluble fraction had potential tumor promoting capacity. Bezuidenhout et al. (1988) and Gelderblom et al. (1988b) isolated fumonisin compounds from

the water-soluble material and determined their structure. Similarly, Voss et al. (1989) developed a rat bioassay for hepatotoxic lesions that were produced by feeding *Fusarium moniliforme* cultures or naturally contaminated corn. They found that a water-extractable, rather than a chloroform-extractable one, was a causal agent of both hepatocellular carcinoma in rats and brain lesions in horses.

ELEM is a neurotoxic disease that occurs only in *Equidae* and is characterized by multifocal liquefactive necrosis of cerebral white matter (Marasas et al., 1988b). ELEM has also been demonstrated in separate experiments in which horses were administered purified FB_1 either orally (Kellerman et al., 1990) or intravenously (Marasas et al., 1988b). Clinical signs begin with lethargy, head pressing and no appetite, and over a period of days progresses to convulsions and death (Ross et al., 1991). Other signs of intoxication are liver damage and altered serum sphingosine:sphinganine ratios (Wang et al., 1991). Plattner et al. (1990) identified FB_1 in extracts obtained from hepatotoxic and ELEM cases.

PPE is an unusual disease in pig, which is characterized by recumbency and death (Norred and Voss, 1994). Outbreaks of PPE have occurred in Georgia (Colvin and Harrison, 1992), and several Midwestern states (Osweiler et al., 1992; Ross et al., 1992). These outbreaks were associated with the consumption of corn screenings that were contaminated with *F. moniliforme* (Harrison et al., 1990). When purified FB_1 is injected intravenously into pigs at a dose level of 0.40 mg/kg body weight for 4 days, pigs die by day 5 and have lung edema and hydrothorax similar to pigs diagnosed with PPE. PPE does not occur when pigs are either injected with a smaller dose of FB_1 (0.17 mg/kg body weight) or with FB_2 at a dose level of 0.30

mg/kg body weight (Harrison et al., 1990). These observations strongly suggest that FB_1 is a causative agent of PPE. This finding has been confirmed by other investigators by feeding pigs with corn contaminated with FB_1 (Osweiler et al., 1992; Riley et al., 1993). Hascheck et al. (1992) reproduced PPE in pigs by injection of purified FB_1. These investigators suggested a possible mechanism for induction of PPE where by FB_1 disrupts sphingolipid biosynthesis in the liver. This leads to damaged hepatocyte membranes, which are released into the bloodstream. These membrane fragments are phagocytized by pulmonary intravascular macrophages which then release enzymes and other mediators capable of increasing capillary permeability in the lung with subsequent edema. The fact that PPE has not been found in other species, perhaps because of much lower levels of pulmonary macrophages in other species (Winkler, 1988).

Correlations between *Fusarium moniliforme* food contamination and human esophageal cancer have been reported in South Africa (Marasas et al., 1988a) and in China (Cheng et al., 1985) suggesting that fumonisins are a possible etiologic agent for this disease. Sydenham et al. (1990) surveyed corn from areas with high and low rates of human esophageal cancer in Transkei, South Africa. Samples taken from the high rate cancer area had higher levels of *Fusarium moniliforme* and fumonisins. The authors suggested that consumption of corn contaminated with *Fusarium moniliforme* may be a factor in the high rate of human esophageal cancer.

Analytical methods

Commonly used methods for detecting and quantitating fumonisins are thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and mass spectroscopy (MS). Fumonisins do not absorb ultraviolet light and do not fluoresce; therefore, derivatization reactions are required before detecting the toxins with chromatographic techniques. In order to visualize fumonisins on TLC plates, Gelderblom et al., (1988a) and Cawood et al. (1991) used 0.5% p-anisaldehyde which was able to react with amino group of fumonisins for generating reddish purple compounds when heated to 120^0C. Retention factor (Rf) values of FB_1 and FB_2 developed on normal phase silica TLC (Merck, Art. 5554) in chloroform:methanol:acetic acid (60:35:10) are 0.32 and 0.52, respectively (Ackerman, 1991). By spraying with fluorescamine, Rottinghaus et al.(1992) visualized FB_1 and FB_2 on reverse-phase C_{18} TLC plate developed in methanol:4% aqueous KCl (3:2). They observed the toxins under longwave ultraviolet light. FB_1 and FB_2 appeared as bright yellow-green fluorescent bands at Rf values of 0.5 and 0.1, respectively. The detection limit of this procedure was 0.1 ppm in corn and its recoveries from spiked corn samples averaged more than 80%. In general, TLC methods are qualitatively useful and simple but they are not accurate for quantifying fumonisins.

More accurate and lower detection limits for fumonisin quantification can be achieved using HPLC which can be performed via formation of a maleyl derivative that absorbed at 250 nm (Gelderblom et al., 1988a). The maleyl derivative procedure was initially developed by Siler and Gilchrist (1982) for

analysis *Alternaria alternata f. sp. Lucopersici* (ALL) toxin which had a chemical structure similar to fumonisins. Briefly, the toxin is dissolved in 0.1 M sodium carbonate solution pH 9.2, and then maleic anhydride crystals are slowly added over a period of 5 minutes. At least a 10:1 molar ratio of maleic anhydride crystals to toxin is used in this procedure to ensure complete derivatization. Prior to HPLC analysis, the maleylation mixtures are adjusted to pH 6-7 by dilution with the mobile phase, 0.05M KH2PO4:methanol (3:7, pH 3.5), or by addition of hydrochloric acid. This procedure is good for detecting fumonisins in corn cultures with *Fusarium moniliforme* , which generally have a high (> part per thousand, ppt) level of the toxins. It, however, does not work for detecting fumonisins in naturally contaminated corn (Norred, 1993).

HPLC sensitivity can be increased by formation of fumonisin-fluorescent derivatives with o-phthaldialdehyde (OPA)(Shephard et al., 1990). OPA derivatization should be performed between 1 and 2 minutes prior to HPLC analysis due to the OPA-derivative stability (Shephard et al., 1990). Detection limits of this procedure are approximately 50 ng/g for FB_1 and 100 ng/g for FB_2. Scott and Lawrence (1992) developed a liquid chromatographic method using 4-fluoro-7-nitrobenzofurazan (NBD-F) which yielded moderately stable derivatives and had detection limits of 1 ng/g of FB_1 and FB_2. However, derivatization with NBD-F required many steps and was time consuming.

Gas chromatography-mass spectrophotometry (GC-MS) has also been applied to detecting fumonisins in corn and feeds (Plattner et al 1990). In this method, an extract of sample is hydrolyzed in 1N potassium hydroxide at 60^0C for

1 hour followed by acidification with 0.5N HCl to form aminopentol backbones which are then analyzed with GC-MS. GC-MS is highly selective and sensitive although it requires relatively expensive equipment.

Regardless of sensitivity and selectivity, HPLC and GC-MS methods are expensive, complex, and time consuming. As an alternative to these methods, immunoassays have already been proven to be useful in screening for other mycotoxins (Pestka, 1988).

Fumonisin antibodies

Previously, both mouse polyclonal and monoclonal antibodies against fumonisin B_1, B_2 and B_3 were produced in our laboratory by using cholera toxin (CT) as carrier and adjuvant (Azcona-Olivera et al., 1992a,b). Concentrations of fumonisin B_1, B_2 and B_3 in buffer solution required for 50% binding inhibition were 260, 300, and 650 ng/ml respectively for polyclonal antibodies with CI-ELISA, and 630, 1800, and 2300 ng/ml, respectively for monoclonal antibodies with CD-ELISA. Recently, antibodies against fumonisins have been produced by several other investigators. Fukuda et al. (1994) generated murine monoclonal antibodies against FB_1 by using ovalbumin and keyhole limpet hemacyanin (KLH) as a protein carrier. When these monoclonal antibodies were used in a CI-ELISA, the amount of FB_1 required for 50% inhibition ranged from 65 to 255 ng/ml. FB_1-rabbit polyclonal antibodies have also been produced using a FB_1-KLH conjugate (Usleber et al., 1994). When these rabbit antibodies were used in CD-ELISA,

only 0.6 ng/ml of FB_1 was required to inhibit 50% of FB_1 peroxidase conjugate binding to the solid-phase-bound antibodies.

Rationale.

An HPLC procedure approved by AOAC and IUPAC after throughout collaborative studies has been the primary method used to detect FB_1. This method, however, requires laborious sample extraction, cleanup, and elusions, before finally detection. Several ELISA approaches have also been developed to date (Azcona-Olivera et al., 1992a,b; Pestka et al., 1994; Tejada-Simon, 1994; Fukuda et al., 1994; Usleber et al., 1994). The ELISAs are preferable to HPLC because of their simplicity, ease of sample preparation, and use of stable reagents (El-Nakib, et al, 1981). However, detection of FB_1 in food samples by CD-ELISA technique using monoclonal antibodies produced by Azcona-Olivera (1992b) provided much higher (30 fold) fumonisin estimates than GC-MS or HPLC method (Pestka et al., 1994). The CD-ELISA yielded even higher(up to 400 fold) fumonisin estimates in *Fusarium moniliforme* cultures (Tejada-Simon, 1994) than that for HPLC. One possible explanation for these observations is that the FB_1 antibody generated by using cholera toxin (CT) as the carrier-adjuvant is not completely specific to fumonisins and, therefore, may have reacted with other compounds found in *Fusarium moniliforme* cultures and contaminated grains. To overcome these problems, it is desirable to produce antibodies which have higher affinity and specificity to the toxins. By using KLH as a protein carrier, higher affinity and specificity rabbit polyclonal antibodies against FB_1 could be generated

(Usleber et al., 1994). In addition, by utilizing hybridoma technique, monoclonal antibodies which have higher specificity and affinity could be generated.

Based on the early findings, I hypothesized that use of KLH as a protein carrier, polyclonal approaches and hybridoma techniques could result in the development of higher affinity FB_1-specific antibodies. Specifically, the purposes of this study were to: (1) produce both polyclonal and monoclonal fumonisin antibodies using KLH as a protein carrier; (2) compare the applicability of these antibodies in ELISA; (3) apply the optimal ELISA techniques for detecting FB_1 in *Fusarium* corn cultures, corn and corn products; and (4) to compare the optimized ELISA to the existing reference HPLC method.

MATERIALS AND METHODS

Chemical and reagents

All organic solvents and inorganic chemical were of reagent grade or better. Ovalbumin (OA) (chicken egg albumin grade III; fraction VII), Tween 20, 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)(ABTS), hydrogen peroxide, horseradish peroxidase (HRP)(fraction VI), sodium borohydride, glutaraldehyde, penicillin/streptomycin solution (pen/strep) (100,000 units/ml), sodium pyruvate, polyethylene glycol (MW 1450) (PEG), hypoxanthine, aminopterin, thymidine, pristane, and dimethyl sulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA)(Albumin, bovine fraction V) was obtained from Amresco (Solon, Ohio). Dulbecco's Modified Eagle's Medium (DMEM), NCTC supplemental medium, and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Grand Island, NY). Tissue culture plasticware was purchased from Corning Laboratory Science Co. (Corning, NY). The myeloma cell line P3/NS1/1-Ag4-1 (NS 1) (ATCC TIB 18) was purchased from the American Type Culture Collection (Rockville, MD). Macrophage conditioned media (MCM) was prepared as described by Sugasawara et al. (1985). Sheep polyclonal antibodies used in this study were developed jointly by our laboratory and Neogen Corp. (Lansing, MI).

Conjugation of fumonisin B_1 to protein

Fumonisin B_1 (FB_1) was conjugated using glutaraldehyde to KLH (Pierce, Rockford, IL) for use as immunogen and to ovalbumin (fraction VII) for use as a solid-phase antigen for indirect ELISAs by the method of Avrameas and Ternynck (1969). The conjugation reaction was carried out at 4^0C in 0.01M phosphate-buffered (pH 7.4) saline (PBS). FB_1 was added to 1 mg/ml suspension of carrier protein at a protein/toxin weight ratio of 5:1 for FB_1-KLH, and of 3.4:1 for FB_1-OA. An equal volume of 2% (vol./vol.) glutaraldehyde in water was then added dropwise with constant stirring. After 1 hour, the reaction was stopped by adding sodium borohydride to a final concentration of 10 mg/ml. One hour later, the mixture was dialyzed for 72 hours (three changes) against 0.01M PBS pH 7.2. Both KLH and OA conjugates were aliquoted in fractions of 1 mg (total protein), lyophilized, and stored at -20^0C.

FB_1 was conjugated to horseradish peroxidase (FB_1-HRP) at an HRP/FB_1 weight ratio of 8:1 and used in the competitive direct ELISA by the periodate method (Nakane and Kawaoi, 1974). Two ml of HRP solution (8 mg/ml distilled water) was reacted with 400 µl of fresh 0.1M sodium periodate (0.21 g /10 ml distilled water) for 20 minutes at room temperature to activate the HRP. The solution was then dialyzed twice against 3 liters of 1mM sodium acetate buffer pH 4.4 overnight at 4^0C. Activated HRP was added into the FB_1 solution (2 mg FB_1 in 200 µl of 50% acetonitrile in water) and 20 µl of fresh 1M sodium carbonate (1.06 g/10 ml distilled water) was added dropwise to the solution. The conjugation reaction was carried out for 2 hours at room temperature and then 200 µl of

sodium borohydride (4 mg/ml distilled water) was added dropwise. After incubation for 2 hours at 4^0C, the conjugate was dialyzed twice against 6 liter 0.85% (wt./vol.) sodium chloride overnight, lyophilized in small portions, and stored at -20^0C.

Rabbit immunization

Three Female New Zealand rabbits (Charles River Laboratories, Wilmington, MA) were initially given a ten site subcutaneous (sc) injection with FB_1-KLH conjugate. This injection consisted of 1.0 ml of the conjugate (0.5 mg FB_1-KLH/ml) in a 1:1 ratio of saline and Freund's "complete" adjuvant (Difco Laboratory, Detroit, Michigan). Two weeks later, the rabbits were boosted subcutaneously with 0.5 ml (0.5 mg/ml) of the conjugate in saline and Freund's "incomplete" adjuvant (Difco Laboratory, Detroit, Michigan) in the ratio of 1:1(vol./vol.). Every other week after the second injection, rabbits were bled from lateral marginal earn vein. Two weeks after the fifth bleeding, rabbits were given the second boost as the first one and two weeks later, the animal were sacrificed and their blood was collected. Serum was obtained after overnight incubation of blood at 4^0C and centrifugation at 1,000 x g for 15 min. Rabbit immunoglobulins were purified by 33 percent saturation with ammonium sulfate (Hebert et al., 1973). Serum titer and antibody specificity were then determined by ELISA as described below.

Hybridoma production

Eight female BALB/c mice (6 to 8 weeks of age, Charles River Laboratories, Wilmington, MA) were immunized by intraperitoneal (i.p.) and subcutaneous (s.c.) routes. Mice were immunized three times with FB_1 -KLH conjugates at two-week intervals. The first immunization consisted of 200 μl (0.25 μg FB1-KLH/ μl) of conjugate in a 1:1 (vol./vol.) ratio of PBS and Freund's complete adjuvant. For the second and the third injections, mice received 5-50 μg FB_1-KLH conjugate which was mixed with an equal volume of Freund's incomplete adjuvant for ip injection or with an equal volume of saline for sc injection. Ten days after the second and the third immunizations, ether-anesthetized mice were bled from the tail vein and the blood was collected with a heparinized tube. The blood was incubated at 4^0C overnight and then centrifuged at 1000 x g for 15 minutes to obtain mouse plasma. Titer and antibody specificity were then determined by both direct and indirect ELISAs. Mice producing FB_1-specific and high titer antibody were chosen for fusion. At 4 days prior to the fusion, the mice were injected intravenously (i.v.) in the lateral tail vein with 4 μg of FB_1-KLH conjugate in saline.

Hybridoma production was performed by a modification of the procedure of Galfre and Milstein (1981). Spleen cells (1×10^8) from an immunized mouse were fused with NS-1 myeloma cells (1×10^7) using PEG. Fused cells were resuspended in 100 ml of complete DMEM medium supplemented with 1% NCTC (vol./vol.), 10 mM sodium pyruvate, 100 units/ml of pen/strep solution, 20% (vol./vol.) FBS, and 20% (vol./vol.) MCM, and then distributed into 875 wells of

96-well plates and incubated for 24 hours at 37^0C in a humid atmosphere of 5% CO_2 in air. One half of the supernatant from each well was removed and replaced with an equal volume of HAT medium (complete medium with hypoxanthine, aminopterin, and thymidine). This operation was repeated every 3 days during a 2 week period, after which time HAT medium was eliminated gradually and replaced by HT medium (the same composition of HAT but without aminopterin). Supernatants of hybridoma cultures were tested for the presence of FB_1 specific antibody by CI-ELISA. Cultures that produced a FB_1 specific antibody were successively scaled up and cloned by limiting dilution at 0.5-1 cell/well (Goding, 1980). Subclones yielding desirable antibody activity were then isolated and stored in FBS-dimethyl sulfoxide (9:1) under liquid nitrogen. Mass production of FB_1 monoclonal antibodies was done by expansion of the selected subclones. Antibodies were purified and concentrated from cell-free culture supernatants by precipitation with 50% saturated ammonium sulfate (Harlow and Lane, 1988).

For large scale production of monoclonal antibodies *in vivo*, mice were injected with 0.5 ml of pristane (2,6,10,14 tetramethylpentadecanoic acid) intraperitoneally (Potter, 1972). After 7-10 days, the mice were immunized with $5x10^5$ to $5x10^6$ hybridoma cells from an actively growing culture which had been centrifuged and resuspended in 0.5 ml 20% FBS-DMEM. After mouse-abdominal swelling (usually 7-10 days after hybridoma injection), ascitic fluid was tapped with an 18 gauge needle, and then purified by 45-50% saturation of ammonium sulfate (Harlow and Lane, 1988).

Indirect ELISA

Indirect ELISA was performed by a modification of the procedure of Azcona-Olivera et al. (1992a) and used to determine serum titers. Briefly, wells of polystyrene microtiter plates (Immunolon 4, Dynatech Laboratories, Alexandria, VA) were coated overnight (at 4^0C) with 100 µl of FB_1-OA (5 µg/ml) in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). Plates were washed six times by filling each well with 300 µl of 0.02% (v/v) Tween 20 in PBS (PBS-Tween) and aspirating the contents. Nonspecific binding was blocked by filling the wells with 300 µl of 1% (wt./vol.) bovine serum albumin in PBS 0.01 M (BSA-PBS), pH 7.2. After incubating for 30 minutes at 37^0C, the plate was washed six times with PBS-Tween. Fifty µl of serially diluted mouse serum was added to each well and incubated at 37^0C for 1 hour. Wells of serially diluted preimmune serum were used as controls. Unbound antibodies were removed by washing six times with PBS-Tween, and 100 µl of goat anti-mouse IgG peroxidase conjugate (2 µg/ml, in BSA-PBS, Cappel Laboratories, West Chester, PA) was added to each well. The plate was incubated for 30 minutes at 37^0C and washed eight times with PBS-Tween. Bound peroxidase was determined with ABTS substrate (1 ml of 35 mg ABTS/15 ml distilled water mixed with 11 ml of citrate buffer pH 4 and 8 µl hydrogen peroxide) as described previously by Pestka et al. (1982). Absorbance at 405 nm was read with a Vmax Kinetic Microplate Reader (Molecular Devices Corporation, Menlo Park, CA). Titer of each serum was arbitrarily designed as

the maximum dilution that yielded twice or greater absorbance as the same dilution nonimmune control serum.

A competitive indirect ELISA (CI-ELISA) was used to verify specificity of antibodies in sera toward FB_1 during the course of immunization and to determine those tissue culture wells which contained hybridomas secreting desirable antibody, following fusion and cloning. Briefly, microtiter plates were coated and blocked as described in the indirect ELISA procedure, and then 50 µl of standard FB_1 (0 - 5000 ng/ml) dissolved in PBS was simultaneously incubated with 50 µl of appropriate dilution of antibody or culture supernatant over the FB_1-OA solid phase for 1 hour at 37^0C. The assay was then completed as described above.

Direct ELISA

Direct ELISA was used to determine the titer of animal sera (Azcona-Olivera et al., 1992a). Plates were coated (125 µl/well) with serial dilution of preimmune and immune sera in 0.1 M sodium carbonate-bicarbonate buffer (coating buffer pH 9.6), then incubated overnight by drying at 40^0C in a conventional oven. After washing and blocking, 50 µl of FB_1 -HRP (2 µg/ml, in BSA-PBS) was added to each well. After 1 hour of incubation at 37^0C, plates were washed and bound peroxidase was determined as described above.

A competitive direct ELISA (CD-ELISA) was used for FB_1 detection in fungus cultures, corn and corn products. Briefly, microtiter plates with 96 wells were coated and blocked as described in the direct ELISA, and then 50 µl of serially (0 to 25 ng/ml) diluted FB_1 standard in 10% methanol or of appropriately

diluted samples in 10% methanol were simultaneously incubated with 50 μl of HRP-FB_1 (2 μg/ml in 1% BSA-PBS) for one hour at 37^0C. The assay was then completed as described in the indirect ELISA.

Veratox®

Veratox® (Neogen Corp., Lansing, MI) is an ELISA kit for fumonisin test based on CD-ELISA and was developed jointly between our laboratory and Neogen Corp. Veratox® was used to detect FB_1 in corn and corn products and performed according to its instructions. Briefly, 100 μl of serially diluted FB_1 standard (0-20 μg/ml) or diluted samples was put into red-marked microtiter wells and then simultaneously added with 100 μl of conjugate solution from the blue-labeled bottle. This solution was mixed by pipetting the liquid up and down 3 times using an 8 or 12 -channel pipetor. One hundred μl of the liquid mixture was transferred into coated-antibody micro titer wells and incubated for 15 minutes at room temperature. Every 30 seconds, the wells were swirled back and forth carefully to mix the solution. After 15 minutes, the solution was decanted. The wells were washed 5 times by filling/decanting with distilled water, and then added 100 μl substrate solution from the green-labeled bottle to develop color. The color development was then stopped by addition of 100 μl of stop reagent from the red-labeled bottle. Absorbance at 650 nm was read with a Vmax Kinetic Microplate Reader (Molecular Devices Corporation, Menlo Park, CA).

ELISAGRAM

ELISAGRAM was used to determine FB_1 and other compounds in food and culture samples which are also bound to FB_1-antibodies. It was performed as described by Pestka (1991) with modification. Briefly, fumonisin B_1 in samples was separated using silica gel 60 thin layer chromatography (Kieselgel 60, 0.063-0.200 mm, Merck, SA) with chloroform:methanol:acetic acid (60:30:10) as developing solvents. Nitrocellulose membranes (0.45 μm; Schlicher and Schuell, Keene, NH) were coated with FB_1 antibodies by soaking them in FB_1 antibody solution over night. The TLC plate was sprayed with PBS and then blotted with the FB_1-antibody-coated nitrocellulose (NC) membranes. The AB-coated NC membranes were then soaked in FB_1-HRP solution (2μg/ml in 1% BSA-PBS) and incubated at room temperature for 10 minutes. NC sheets were washed with PBS containing 0.2% Tween 20, and then rinsed briefly in distilled water. For color development, the NC sheets were incubated for 10-20 minutes at 25^0C with 10 ml of dioctylsodium sulfosuccinate (DONS)/3,5,3',5'-tetramethylbenzidine (TMB) substrate prepared as described by Koch et al. (1985). The reaction was stopped by washing the NC in distilled water and then incubating for 10 minutes in stopping reagent solution (100 mg DONS dissolved in 13 ml of ethanol and made up to 50 ml with distilled water). After drying between filter paper, the white spots on the NC indicating the presence of FB_1 toxin in the samples were observed.

***Fusarium* cultures**

Fusarium cultures used in this study were M-5986 *F. moniliforme*, isolated from samples that produced PPE (Ross et al., 1990); and M-5982 *F. moniliforme,* isolated from samples that produced ELEM (Ross et al., 1990). In addition, *F. graminearum* W-8, which did not produce fumonisins, but rather deoxynivalenol and zearalenone (Marasas et al., 1984a), was used as a negative control. These cultures were obtained from the *Fusarium* Research Center at Pennsylvania State University (University Park, P.A.).

Fusarium moniliforme cultures were inoculated onto petri dishes with V-8 juice agar medium (Stevens, 1974). Each liter of V-8 juice agar medium contained 200 ml of V-8 juice, 3 g of calcium carbonate, and 20 g of agar (Jackson and Bennet, 1990). These plates were incubated for 10-14 days at room temperature on an alternating light-dark schedule. To prepare spore suspensions, a loopfull of conidia from *F. moniliforme* petri dish cultures was transferred to V-8 juice agar slant-tubes which were then incubated at room temperature for 10-14 days on an alternating light-dark schedule. Spore suspensions were obtained by washing the slant tubes with sterile distilled water.

Unlike *Fusarium moniliforme* spores, conidial suspensions of *F.graminearum* W-8 were prepared by transferring the cultures into an 150-ml Erlenmeyer flask containing 40 ml of autoclaved CMC medium (15 g of carboxymethyl cellulose, 1 g of ammonium nitrate, 1 g of potassium phosphate, 0.5 g of magnesium sulfate heptahydrate, 1 g of yeast extract in 1 liter of distilled water) (Stevens, 1974). The flasks were then incubated at 25^0C with shaking at

220 rpm. Cultures were checked between 3 and 5 days for macroconidia production. Conidial suspensions were obtained by filtering CMC medium through 4 layers of sterile cheesecloth to remove mycelia.

Prior to inoculation, 250-ml Erlenmeyer flasks containing 40 g of ground corn and 11 ml of distilled water were autoclaved for 30 min. After autoclaving, an additional 11 ml of sterile distilled water containing 10^7 conidia was added (Stevens, 1974). The flask cultures were incubated in the dark at 25^0C for 14 to 28 days. Corn cultures were extracted with 5 ml/g of acetonitrile:water (1:1) by soaking for 2-3 hours with mixing every half hour (Plattner et al., 1992). Suspensions were filtered through Whatman # 1 filter paper (Whatman Ltd., Maidstone, UK) and the filtrate was tested for fumonisin B_1 by ELISA and HPLC.

Corn-sample extraction and clean up

Corn extraction was performed by a modification of the procedure of Thiel et al. (1993). Food, feed, or fresh corn samples were finely ground with Waring Blender Model 1042 (Winsted, Connecticut). A subsample (5 g) was put in 50 ml plastic-conical tubes, and 25 ml of 75% (vol./vol.) methanol in distilled water was added. The tubes were then shaken (American Rotator V, R4140) at 200 rpm for 20 minutes. The solution was centrifuged at 1000g for 10 minutes, and filtered through Whatman # 4 filter paper (Whatman Ltd., Maidstone, UK). The filtrates were diluted with 10% methanol at ratio of 1:40 or higher before analyzing for fumonisin B_1 with CD-ELISA and Veratox® (Neogen Corp., Lansing, MI, 48912).

For some experiments, filtrates were cleaned up by passage through a strong anion exchange (SAX) column (Bond-Elute SAX cartridges, 3 cc capacity containing 500 mg sorbent; Varian, Harbor City, CA 90710) before analyzing with HPLC. Briefly, pH of the filtered extract was checked and adjusted, if necessary, with 0.1 M KOH to pH 5.8-6.5 before running through the SAX column. SAX columns were attached to 16-port vacuum manifold (Alltech, State College, PA) and conditioned by washing successively, first with 5 ml methanol and then with 5 ml of 75% (vol./vol.) methanol in water at flow rate of no more than 2 ml/min. While maintaining the flow rate, 10 ml of the substrate was applied to the column. The column was then washed with 8 ml of 75% methanol (vol./vol.) in water, followed by 3 ml methanol.

Fumonisins were eluted with 10 ml of 1% (vol./vol.) acetic acid in methanol, at flow rate of no more than 1 ml/minute (at atmospheric pressure). The eluent was collected in 15 ml conical tubes. One half of that (5 ml) was evaporated in 4 ml capacity vials under stream of nitrogen at about 60^0C. The vial was then capped and stored in 4^0C until HPLC analysis. Prior to FB_1 detection with HPLC, the purified sample residue in the 4 ml vial was redissoved with 100 µl methanol.

HPLC

Measurement of FB_1 by HPLC was performed according to the previously described reference method (Shephard et al., 1990 and Sydenham et al., 1992). The liquid chromatography system used in this study consisted of Isco Model

2300 HPLC pump with an injector valve (Valco valve)(Lincoln, NE); H-S3 C_{18} #316 stainless steel packed C_{18} column (Perkin Elmer, Norwalk, Co) 0258-0178 reverse phase (3.3cm); Hewlett Packard model HP 3392A integrator (Avondale, PA); Linear Instruments Fluor fluorescence detector L.C. 304 (Reno,NE) fitted with a 3.1 µl flow cell, 500 psi (34 atm) maximum pressure and set at 334 nm (excitation) and 440 nm (emission) and slit widths of 12 nm or similar. The mobile phase was methanol:0.1M sodium dihydrogen phosphate (13.8g $NaH_2PO_4.H_2O$ in 1 liter distilled water)(66:34) that adjusted to pH 3.4 with ortho-phosphoric acid and filtered through 0.22 µm water GV membrane (Milipore Corporation, Bedford, MA). Before running the mobile phase, the C_{18} column was washed approximately for 2 hours with degassed absolute methanol at a flow rate of 1.0 ml/minute and then with 25% (vol./vol.) methanol in water for approx. 2 hours at the same flow rate. The mobile phase was pumped at 1.5 ml/minute when analyzing samples. After analyzing all samples, the column was washed with 25% (vol./vol.) methanol in water then with absolute methanol for approximately two hours each at a flow rate of 1.0 ml/minute.

OPA derivatizing reagent was prepared by dissolving 40 mg O-phthaldialdehyde in 1 ml methanol and diluted with 5 ml 0.1 M sodium borate (3.8 g $Na_2B_4O_7$ in 100 ml dH_2O) and 50 µl of 2-mercaptoethanol. The solution was stored no more than one week at room temperature in the dark.

Purified sample residues in a 4 ml vial were dissolved in 100 µl methanol. Fumonisin standards were serially diluted (0.5 to 100 µg/ml) in methanol. Twenty five µl fumonisin standard or samples were transferred to the base of small test

tubes and mixed with 225 μl OPA reagent. Less than 2 minutes after mixing, 10 μl of the mixture was injected into the HPLC system.

Statistics

Linear regression analyses were used to correlate ELISA and HPLC data. A linear correlation coefficient (r) equaling to 1 indicates there is a perfect positive relationship between the ELISA and HPLC methods, and r close to -1 indicates a strong negative correlation; whereas, a correlation coefficient close to 0 indicates no relationship between two variables (Steel and Torrie, 1980). The P value is the probability of being wrong in concluding that there is a true association between the variable. The smaller the P value, the greater the probability that the variables are correlated. Sigma Plot® (Scientific Graph System, Version 1.00 for Windows, Jandel Scientific, San Rafael, CA) was used to perform linear regression analyses.

RESULTS AND DISCUSSION

Rabbit polyclonal antibodies

Three rabbits were immunized and boosted with FB_1-KLH immunogen. Each rabbit was bled from a lateral marginal ear vein and its serum was prepared and stored. Titers of the rabbit polyclonal antibodies in a direct ELISA format were much lower (more than 3,200) as compared to that in an indirect ELISA format (more than 64,000) (Figure 2.1). Concentrations of FB_1 required for a 50% inhibition of antibody binding were much lower (approximately 100 ng/ml) in CD-ELISA than that in CI-ELISA (more than 10,000 ng/ml) (Figure 2.2). In a direct ELISA format, antibodies were bound to a solid phase and then enzyme-labeled antigen (FB_1-HRP) was added to detect and quantify the antibodies. Since FB_1 was conjugated to HRP directly without using glutaraldehyde as "a bridge", it is possible that FB_1-HRP conjugate was reacted with only FB_1 specific antibodies. On the other hand, unlike in FB_1-HRP conjugation, glutaraldehyde was used as "a bridge" in both FB_1-OA and FB_1-KLH conjugations. Therefore, glutaraldehyde-OA might have acted as an epitope for glutaraldehyde specific antibodies to produce non-specific binding as well as specific antibodies binding to FB_1. If this happened, then both specific and non-specific binding between rabbit antisera and the coating protein would be detected upon addition of the second enzyme-labeled antibodies (goat-anti-rabbit IgG-HRP). In that case, an

indirect ELISA format would yield greater color development, and subsequently higher titer than the direct ELISA. However, FB_1 required to inhibit 50% of the antibody binding may be much more higher in CI-ELISA as compared to that in CD-ELISA.

Mouse immunization and monoclonal antibodies

Four mice were injected intraperitoneally and the other four were immunized subcutaneously with FB_1-KLH conjugates. All immunized mice produced FB_1 specific antibodies as early as 4 weeks after initial exposure of FB_1-KLH immunogen. Competitive inhibition ELISAs for FB_1 using mouse antisera were determined with both CI- and CD-ELISA formats (Figures 2.3 and 2.4). Like FB_1 rabbit antibodies binding, mouse antiserum binding was inhibited by FB_1 to a greater extent in CD-ELISA than in CI-ELISA. CD-ELISA results were used to select optimal mice for fusion.

Mice were sacrificed and their spleen cells were fused with myeloma (NS1) cells. The fused cells were then cultured into 875 wells of 96-well plates. Hybridomas were detected in 821 wells, indicating a fusion efficiency [(number of wells with growing colonies/number of wells seeded) x 100%] of 94 %. This fusion efficiency was similar to that observed by Azcona-Olivera et al. (1992b).

After screening with CI-ELISA using FB_1-BSA coated plates, more than 20 wells were identified as FB_1-antibody producers. Of these, seven cell lines (A7D3, B1D3, B1G5, B2F7, B4D6, B4E3, B4G11) remained stable during scale-up prior to cloning. CI-ELISA standard curves of these cell lines are shown in

Figure 2.5. Concentrations of FB_1 required for a 50% inhibition of antibody binding ranged from 90 to 2000 ng/ml. The two best cell lines (B2F7, B4D6) were cloned by limiting dilution twice and from these cultures, four cell lines (N2C5, Q1C5, Q2C9, R1B5) were chosen for large scale antibody production in ascitic fluid. Mice were injected with actively growing hybridoma cells (N2C5, Q1C5, Q2C9, R1B5) after intraperitoneal injection with pristane. After mouse-abdominal swelling, ascitic fluids of FB_1 -monoclonal antibodies were tapped and purified. Titers and competitive inhibition using these antibodies were then analyzed with both CD- and CI-ELISA. Titers exceeded 40,000 with CI-ELISA and were between 500 to 1,000 with CD-ELISA (Figure 2.6). Concentrations of FB_1 required for a 50% inhibition of antibody binding for the clones ranged from 50 to 100 ng/ml with CI-ELISA and from 400 to 800 ng/ml with CD-ELISA (Table 2.1).

Comparison among different antibodies

FB_1 Rabbit polyclonal and N2C5 monoclonal antibodies were compared to the 2D5 monoclonal antibody which was previously generated by Azcona-Olivera (1992b) using FB_1 -cholera immunogen and to sheep antisera developed against FB_1 -KLH conjugate (Neogen Corp., Lansing, MI).

As previously noted, CD-ELISA for FB_1 using rabbit polyclonal antibodies was more sensitive than CI-ELISA (Figure 2.2). However, the opposite was true for the monoclonal antibodies (Figure 2.7). In order to ascertain which FB_1 antibodies had the highest avidity, CD-ELISA standard curves for FB_1 polyclonal antisera and CI-ELISA standard curves for FB_1 monoclonal antibodies were

generated (Figure 2.10). Concentrations of FB_1 required for 50% inhibition of antibody binding were 6, 80, 100, and 200 ng/ml for sheep, rabbit, N2C5, and 2D5, respectively (Table 2.2). Thus, the sheep antisera contained FB_1 specific antibodies with the highest affinity. These results support the hypothesis of this study [use of KLH as a protein carrier, polyclonal approaches and hybridoma techniques could result in the development of higher affinity FB_1-specific antibodies than the 2D5 antibodies which were previously produced by Azcona-Olivera et al. (1992a,b) in our laboratory].

The affinity of FB_1 polyclonal antisera was higher than that of the monoclonal antibodies (Table 2.2). This observation may be related to several possibilities. Firstly, when producing monoclonal antibodies, hybridomas secreting FB_1 antibodies with lower affinity, instead of with higher affinity, might be chosen during hybridoma screening. Secondly, hybridomas secreting higher affinity FB_1 antibodies might not have been stable and died before screening. Thirdly, the affinity of FB_1 antibodies might be affected by animal species since sheep, rabbits and mice produced FB_1 antibodies with different affinities. This current study result agreed with the findings of Usleber et al. (1994) and Fukuda et al. (1994) who also generated FB_1 antibodies using KLH as a protein carrier. The former researchers produced FB_1-rabbit polyclonal antibodies that required only 0.6 ng/ml FB_1 for 50% inhibition with CD-ELISA. Meanwhile, the latter researchers generated FB_1 monoclonal antibodies that required a higher concentration of FB_1 (65 ng/ml) for 50% inhibition with CI-ELISA.

Since the FB_1 sheep antisera contained FB_1-specific antibodies with the highest affinity, they were utilized in CD-ELISA for detection of FB_1 concentrations in fungal corn cultures, corn and corn products. CD-ELISA standard curves for FB_1 , FB_2 and FB_3 using these sheep antisera were graphically shown in Figure 2.11. Cross reactivities of the sheep antibodies towards FB_1, FB_2, and FB_3 [determined as (ng/ml of FB_1 required for 50% inhibition)/(ng/ml of fumonisin analogue required for 50% inhibition) x 100] were 100, 24 and 30%, respectively (Table 2.3).

Detection of FB_1-like compounds

Several investigators reported that ELISA methods tended to give higher FB_1 estimates than HPLC methods. Minervini et al. (1992) found ELISA methods yielded higher FB_1 estimates than HPLC when applied to Italian feed samples. Elevated estimates for FB_1 in feeds by ELISA as compared to HPLC have also been found in foods (Pestka et al., 1994) and in *Fusarium* corn cultures (Tejada-Simon, 1994). The latter investigator reported that ELISA methods yielded much higher FB_1 estimates (up to 400 times) than HPLC. She proposed that one reason for the different FB_1 estimates between the two analytical methods might have resulted from the presence of compounds structurally similar to FB_1 in sample extracts which were detectable by ELISA, but not by HPLC.

Detection of the FB_1-like compounds in corn and corn products might be performed by ELISAGRAM. FB_1 monoclonal antibodies (2D5) produced by Azcona-Olivera et al., (1992b) and FB_1 -sheep antisera developed jointly by our

laboratory and Neogen Corp. (Lansing, MI) were used in the ELISAGRAM. Before analyzing samples, a standard of FB_1 (2.5 μg/ml) was used to test whether the ELISAGRAM system worked or not for FB_1 toxin. Twenty μl of the FB_1 standard was directly spotted on silica gel thin layer chromatography, and then transferred onto FB_1-antibody-coated nitrocellulose membrane (NC). The NC was incubated with FB_1 -HRP, washed and finally incubated in dioctylsodium sulfosuccinate (DONS)/3,5,3',5'-tetramethylbenzidine (TMB) substrate for color development. However, a white dot on the NC did not appear. This indicated that FB_1 standard did not bind to a coating antibody, so the coating antibody reacted with FB_1-HRP which subsequently produced green color after incubation in HRP-substrate solution. Similar observations occurred when 10 μg FB_1 standard was directly spotted onto the NC. Thus, the ELISAGRAM that worked well for zearalenones and aflatoxins (Pestka, 1991) did not work at all for FB_1 toxin. Since FB_1 easily dissolved in water, the toxin might have run off during washing or blotting before reacting with coating FB_1 specific antibodies on the NC. As a result, a white dot on the NC was not developed although a relatively high quantity of FB_1 standard (10 μg) was spotted on the NC. Thus the ELISAGRAM would not be useful for detecting putative FB_1 -like compounds.

Detection of FB_1 in *Fusarium* cultures

FB_1 analysis was performed by HPLC and CD-ELISA using FB_1 -sheep antisera (Neogen Corp., Lansing, MI). *F. graminearum* (FW-8), *F. proliferatum* (M-5956), and *F. moniliforme* (M-5958) were grown in solid (ground corn) media.

Non FB_1-producing *F. graminearum* (FW-8) and uninoculated samples were used as negative controls. The corn cultures were harvested at 2, 3, 4, and 5 weeks, and then extracted with 50% acetonitrile, filtered, and subsequently analyzed with HPLC and CD-ELISA.

Standard curves for CD-ELISA and for HPLC analyses were generated and shown graphically in Figures 2.10 and 2.12, respectively. Based on these standard curves, FB_1 concentrations in *Fusarium* corn cultures were calculated and the results were tabulated in Table 2.4. FB_1 concentrations in the cultures ranged from 0 to 220 μg/g with HPLC and from 0 to 759 μg/g with ELISA.

In both negative controls, FB_1 was not detected by either HPLC or ELISA. However, when this ELISA was used to assay FB_1 in fumonisins producing cultures, it yielded FB_1 estimates higher (mean = 2.8-fold) than HPLC method (Table 2.4). These current study results were a vast improvement over the findings of Tejada-Simon (1994) who found that ELISA methods yielded much higher FB_1 estimates (up to 400-fold) than HPLC. The improvement was likely caused by the sheep antiserum which had higher affinity and specificity than 2D5 monoclonal antibodies used by Tejada-Simon (1994) (Table 2.2).

Detection of FB_1 in corn and corn product

Corn and corn products analyzed in this study consisted of 77 fresh corn, 14 corn food and 28 corn feed samples. Ground corn samples were extracted with 75% (vol./vol.) methanol in distilled water. After filtering through Whatman #4 filter paper (Whatman Ltd., Maidstone, UK), the raw methanolic extracts were

diluted at a 1:35 and then analyzed by CD-ELISA using FB_1- sheep antisera (Neogen Corp., Lansing, MI). Although detection of FB_1 in the food and feed extracts at a dilution of 1:35 with the CD-ELISA could be performed successfully, a problem was found when analyzing FB_1 in the fresh corn extracts. After color development, some samples had a higher color intensity (higher O.D.) than the zero FB_1 standard solution. CD-ELISA standard curves for FB_1 in extractant [10% methanol (vol./vol.) in distilled water], and raw methanolic extracts of fresh corn sample numbers 37 and 56 (at a dilution of 1:35) were graphically shown in Figure 2.8. All standard curves had relatively the same pattern, but different O.D. indicating that the raw corn extracts contained an interfering material which was able to generate non specific binding. Several possibilities might relate to this observation. First, the corn extracts might contain peroxidase which then reacted with peroxidase substrate (ABTS) resulting increase of color intensity. Second, the corn extracts might contain protein compounds which could bind to both sheep antisera and FB_1-HRP resulting higher color intensity after the addition of ABTS. Third, the pH of the corn substrate might favor to the reaction of sheep antisera with FB_1 -HRP or with peroxidase-containing compounds which subsequently increased color intensity. Fourth, the corn extracts might contain non protein compounds which could increase color intensity.

Several efforts have been performed to solve the above problem. The corn extracts was heated and then centrifuged to inactivate peroxidase and to eliminate protein compounds from the corn extracts. Addition of NaCl to the corn extracts was also performed to precipitate the interfered proteins. Measurements

of corn substrate pH and adjustment of the pH to neutral were also carried out to eliminate the effect of the sample pH. All of these efforts, however, did not solved the problem indicating that the interfered compounds were not peroxidase or proteins. The problem was eliminated after the corn extracts were diluted up to 200 times or higher with 10% methanol in distilled water. By diluting sample extracts, Laamanen and Veijalainen (1992) were also able to eliminate a similar problem when they analyzed T-2 toxin in milled grains with an ELISA method. After diluting fresh corn extracts up to 200 times, CD-ELISA standard curves for FB_1 in the extractant and in the diluted corn extracts had relatively the same O.D. and pattern (Figure 2.9).

Beside the CD-ELISA, Veratox® (Neogen Corp., Lansing, MI) and HPLC were also used to detect FB_1 in fresh corn, corn products and spiked-corn samples. After sample extraction and filtration, raw methanolic extracts were analyzed for FB_1 using both CD-ELISA and Veratox® methods. Some raw extracts were cleaned up with SAX columns and then analyzed by HPLC. A standard curve of HPLC (Figure 2.13) and standard curves of CD-ELISA and Veratox® (Figure 2.10) for analyzing FB_1 in corn and corn products were then developed. Based on these standard curves, FB_1 concentrations in corn-spiked samples, corn, and corn products were calculated.

After calculating FB_1 concentrations in corn-spiked samples containing FB_1 of 100 to 3000 ng/g, the FB_1 concentrations in the samples were divided by the added FB_1 concentrations and then multiplied by 100% to determine percent recovery of the mycotoxin (Table 2.5). Percent recoveries of FB_1 in SAX-

columned samples with HPLC ranged from 68.8 to 84.7 percent (average = 74.1) (Table 2.5) indicating some FB_1 might loss during extraction and SAX-column purification. These recovery results were relatively similar to the findings of Stack and Eppley (1992) who analyzed FB_1 in SAX-columned extracts of corn-spiked samples containing FB_1 of 500 to 2000 ng/g with HPLC, and found percent recoveries of FB_1 in the range of 63.2 and 71.5 percent. Meanwhile, by using the same method Usleber et al. (1994) reported that percent recoveries of the same toxin in corn samples spiked with 50 ng/g and 500 ng/g FB_1 were 63.2% and 65.6%, respectively.

When both Veratox® and CD-ELISA were used to analyze FB_1 in the corn-spiked samples, percent recoveries of FB_1 in the raw methanolic extracts were higher than that in SAX-cleaned extracts (Table 2.5), indicating that some FB_1 might have lost during SAX column purification. FB_1 could not be detected in SAX-clean extracts from the samples which were spiked with only 100 or 200 ng/g FB_1 (Table 2.5) because all of the spiked toxin might have lost during purification. Percent recoveries of the spiked FB_1 in the raw methanolic corn extracts determined with CD-ELISA and Veratox® ranged from 48.5 to 127.2% (Table 2.5). This variability might result from two possibilities. Firstly, the presence of FB_1 in the corn extracts might not be distributed evenly during spiking, so some samples yielded high recoveries and the other ones provided low recoveries. Secondly, the FB_1 -extraction efficiencies from corn containing different amounts of FB_1 might differ. Because of high variability, these percent

recoveries were not used to adjust the concentration of FB_1 in corn and corn products.

FB_1 concentrations in 14 food samples ranged from <0.04 to 7.11 μg/g (part per million, ppm), 0.13 to 9.59 ppm and <0.02 to 10.16 ppm when detected by HPLC, Veratox® , and CD-ELISA, respectively (Table 2.6). The ratio of Veratox® and CD-ELISA results to HPLC results ranged from 1.0 to 1.5 (Table 2.6). These ratio results were significant improvement over the findings of Pestka et al. (1994) who reported that the ELISA estimates were higher (up to 30-fold) than HPLC estimates. As in *Fusarium* corn cultures, sheep antiserum used in this current study had greater affinity and higher sensitivity than 2D5 monoclonal antibodies used by Pestka et al. (1994) (Table 2.2). These undoubted contributed to the analytical improvement. These results indicate that both Veratox® and CD-ELISA were suitable to routinely screen corn-based foods from FB_1.

The levels of FB_1 in Italian feed samples ranged from <0.04 to 4.99 ppm, 0.34 to 7.63 ppm and 0.27 to 8.43 ppm when measured by HPLC, Veratox®, and CD-ELISA, respectively (Table 2.7). Veratox® and CD-ELISA analyses yielded FB_1 estimates approximately twice higher (mean = 2.24 and 1.93 respectively) than HPLC method. Regression analyses between HPLC and Veratox® and between HPLC and CD-ELISA had linear correlation coefficients (r) of 0.961 and 0.951, respectively (Table 2.7) indicating that HPLC had a strong positive relationship with both Veratox® and CD-ELISA methods. These current study results were much better than the finding of Minervini et al. (1992) who reported

that the correlation coefficient between HPLC and ELISA was only 0.240, indicating no relationship at all between ELISA and HPLC methods. Like the above mentioned assays of *Fusarium* cultures and foods, the sheep antiserum was critical to ELISA improvement.

Seventy seven of fresh corn samples were first analyzed by Veratox® to screen for samples contaminated with FB_1. All positive samples and approximately half of the negative samples were reanalyzed by Veratox®, CD-ELISA and HPLC. Analysis of positive fresh corn samples, Veratox® and CD-ELISA yielded higher FB_1 estimates (mean = 2.85 and 1.54, respectively) (Table 2.8) than HPLC. However, the three methods yielded the same results when applied to negative fresh corn samples (Table 2.8). Linear correlation coefficients (r) between HPLC and Veratox® and between HPLC and CD-ELISA were 0.933 and 0.836, respectively (Table 2.8) indicating that HPLC correlated well with both Veratox® and CD-ELISA methods. Thus, both Veratox® and CD-ELISA can be useful for routinely screening fresh corn from FB_1.

CD-ELISA developed in this current study was much better than the previous CD-ELISA that was developed by Azcona-Olivera et al. (1992a,b) and used by Minervini et al. (1992), Pestka et al. (1994) and Tejada-Simon (1994) for analyses of FB_1 in feeds, foods, and fungal cultures, respectively. However, when this "new" CD-ELISA was used to detect FB_1 in corn cultures, corn foods, corn feeds and fresh corn, it still provided higher FB_1 levels (means = 2.8, 1.3, 1.9, and 1.5, respectively) as compared to those obtained by HPLC (Tables 2.4, 2.6, 2.7, 2.8). This disagreement might relate to several possibilities. Firstly, HPLC

detected only FB_1 ; whereas, CD-ELISA detected not only FB_1 but also FB_2 and FB_3 since FB_1 sheep antisera used in the CD-ELISA cross-reacted with FB_2 and FB_3 (Table 2.3). Secondly, the sample extracts might contain FB_1 -similar compounds which were detectable by CD-ELISA but not by HPLC. The FB_1 -sheep antisera might not be completely specific for FB_1, so they can possibly detect other related compounds. Thirdly, because it contains an amine ($-NH_2$) functional group, FB_1 might react with protein compounds prior to or during extraction to form FB_1 -protein conjugates that cross react with FB_1 -sheep antisera, and thus increase the response of the CD-ELISA without affecting HPLC detection.

CONCLUSION

Mouse monoclonal antibodies, rabbit and sheep polyclonal antibodies against FB_1 were readily generated after immunization with keyhole limpet hemacyanin (KLH) as a protein carrier. The sheep antisera had the highest affinity and were most specific to FB_1 as compared to the mouse monoclonal and rabbit polyclonal antibodies when they used in competitive inhibition ELISA. Cross reactivities of these sheep antisera toward fumonisin B_1 , B_2 , and B_3 were 100, 24 and 30%, respectively.

CD-ELISA using these FB_1 sheep antisera provided approximately two fold higher FB_1 estimates than HPLC when used for detection of FB_1 in *Fusarium* corn cultures, corn and corn products. This was much better than CD-ELISA previously developed in our laboratory In addition, this CD-ELISA as well as Veratox® correlated well with HPLC methods, suggesting that it is suitable for routinely screening *Fusarium* cultures, corn and corn products from FB_1 toxin.

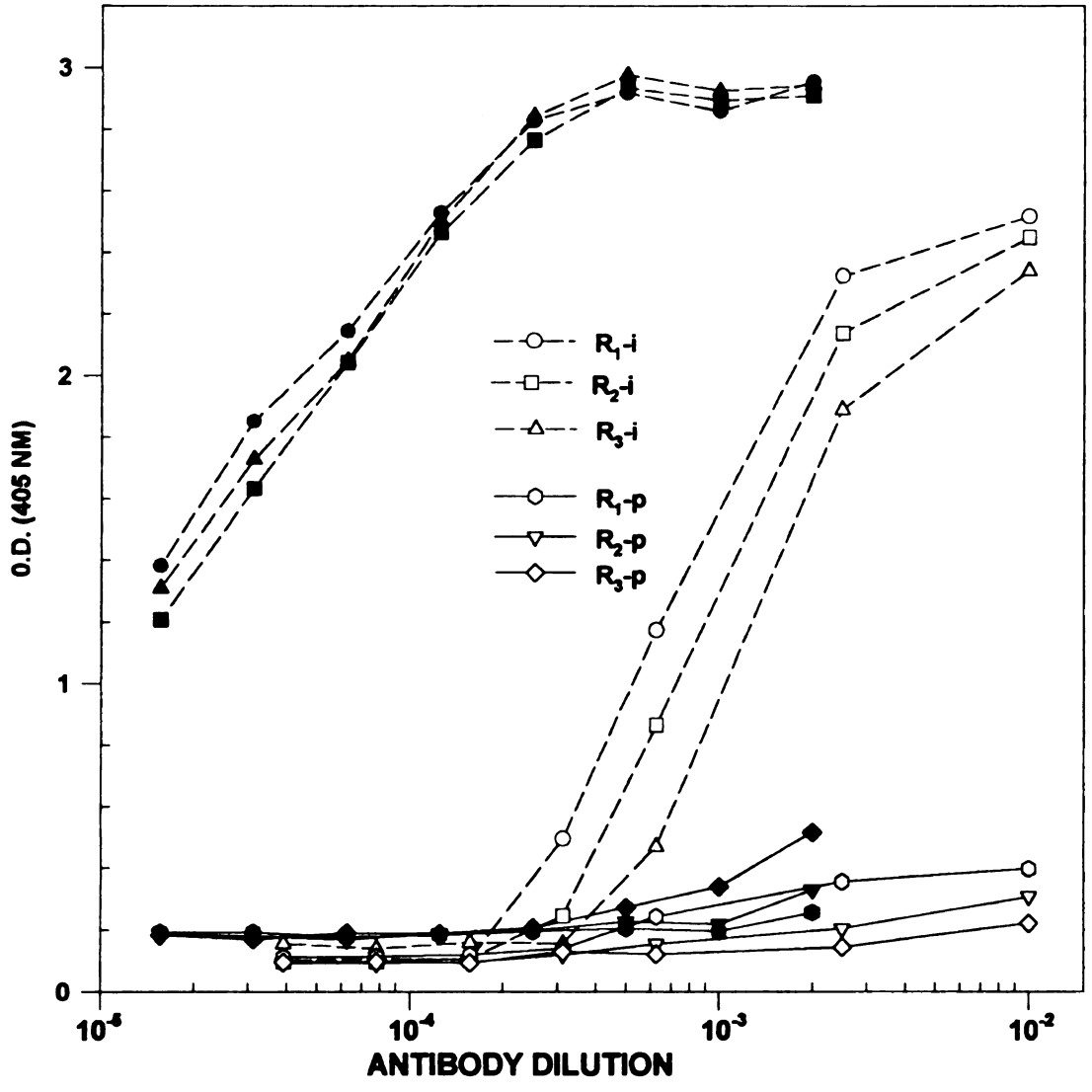


Figure 2.1. ELISA titration of rabbit polyclonal FB_1 antibodies. Sera were obtained two weeks after the second injection with FB_1-KLH immunogen. Each data point represents the average value of duplicate measurements. Filled symbols indicate indirect ELISA. Open symbols are direct ELISA. R_1, R_2 and R_3 refer to rabbit number. The letter i indicates immunized and p indicates preimmun.

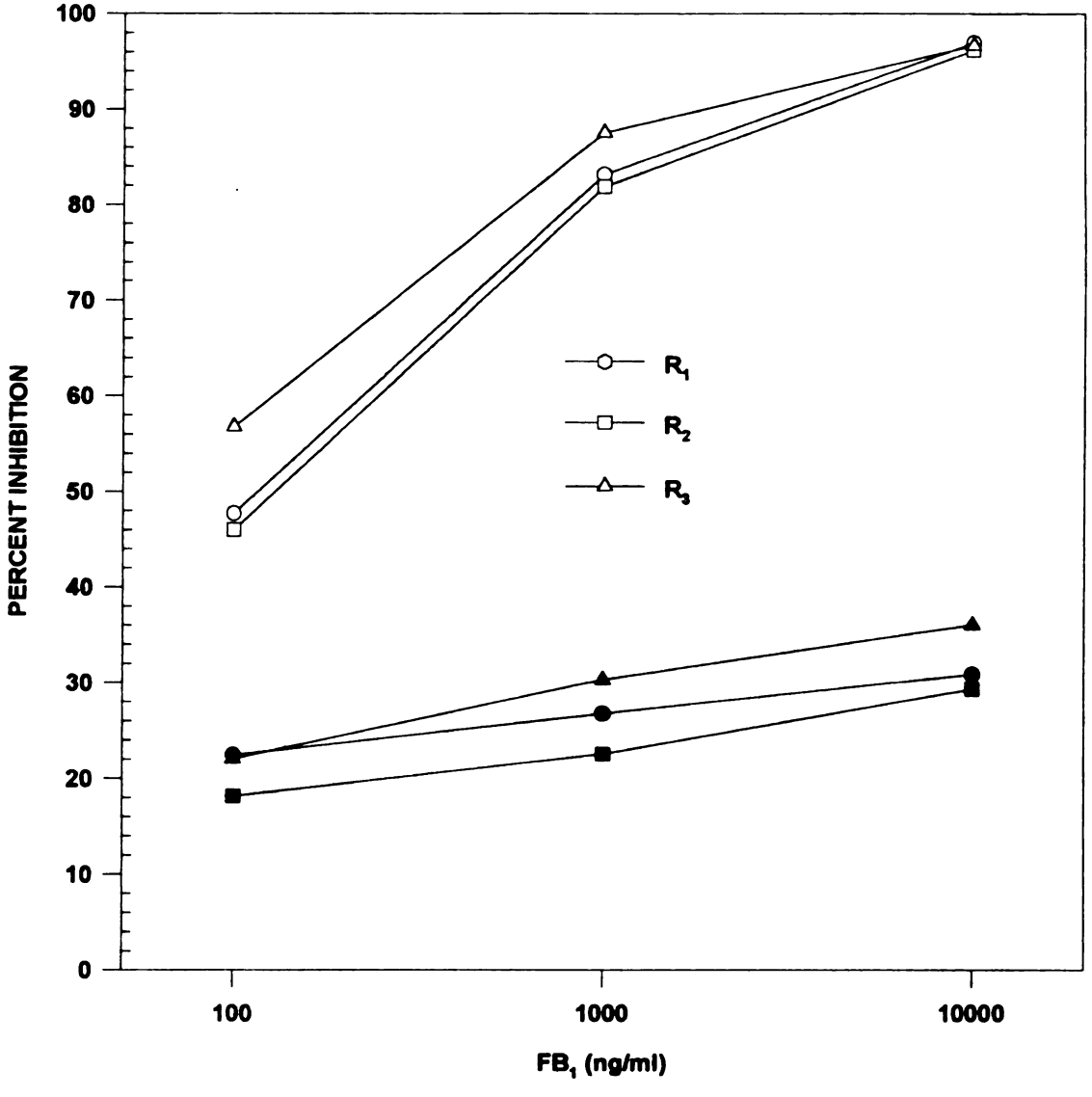


Figure 2.2. Competitive inhibition ELISA for FB_1 using different rabbit polyclonal antibodies. Sera were obtained two weeks after the second injection with FB_1-KLH immunogen. Each data point represents the average value of duplicate measurements. Filled symbols indicate CI-ELISA. Open symbols are CD-ELISA. R_1, R_2 and R_3 refer to rabbit number.

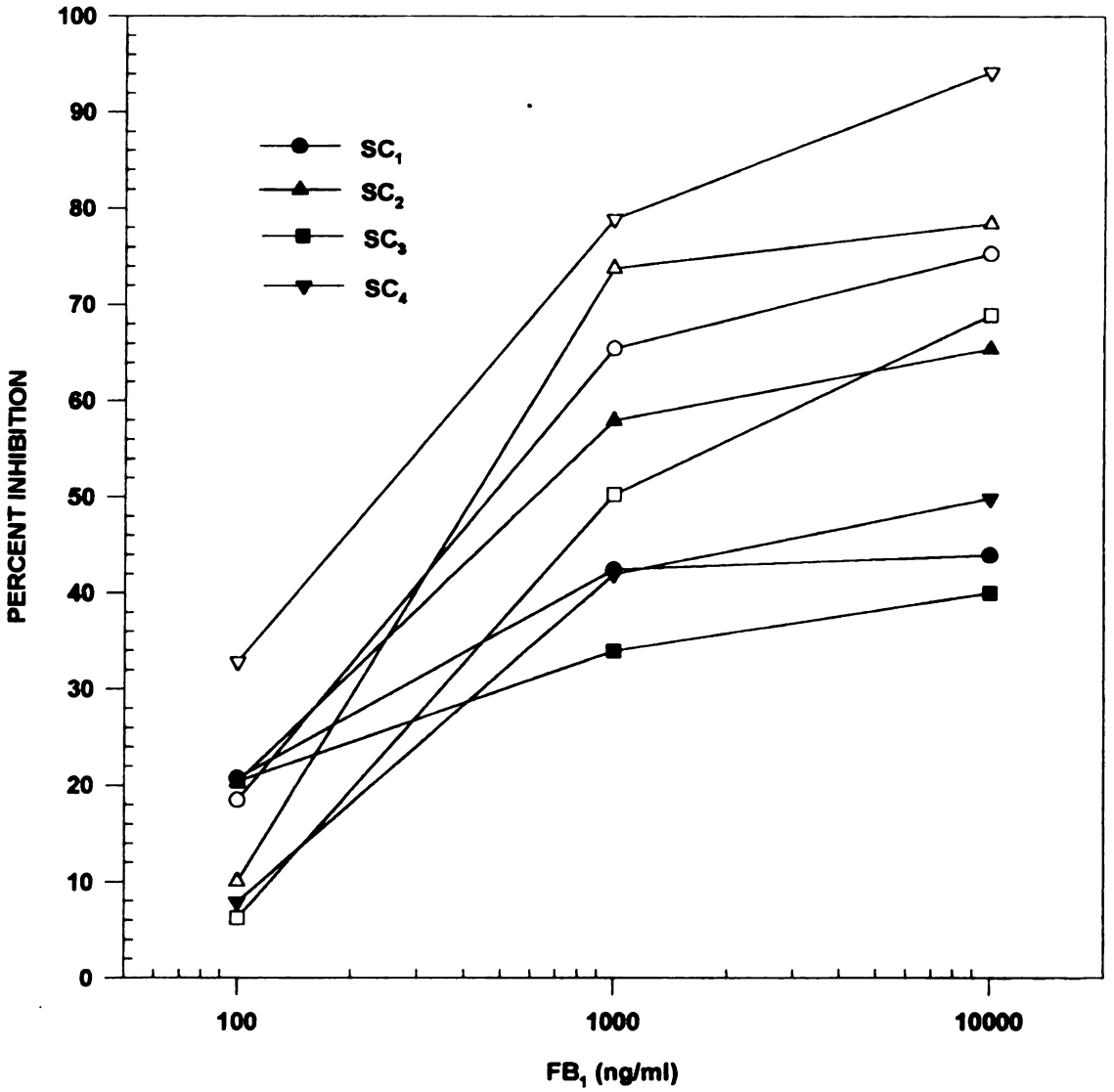


Figure 2.3. Competitive inhibition ELISA for FB_1 using mouse sera. Sera were obtained ten days after the third subcutaneous (SC) injection with FB_1-KLH immunogen. Each data point represents the average value of duplicate measurements. Filled symbols indicate CI-ELISA. Open symbols are CD-ELISA. Subscript 1-4 refers to mouse number.

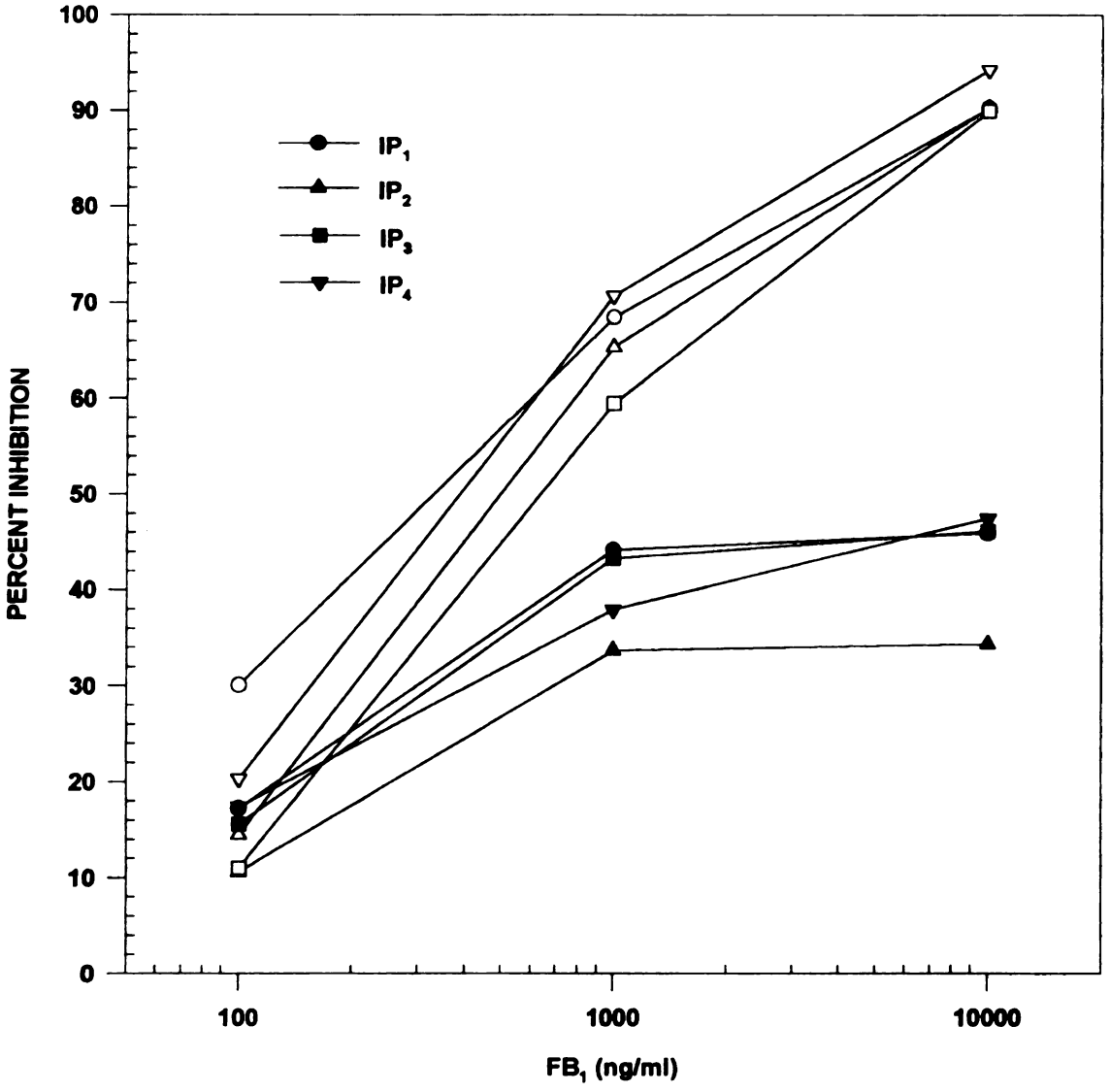


Figure 2.4. Competitive inhibition ELISA for FB_1 using mouse sera. Sera were obtained ten days after the third intraperitoneal (IP) injection with FB_1-KLH immunogen. Each data point represents the average value of duplicate measurements. Filled symbols indicate CI-ELISA. Open symbols are CD-ELISA. Subscript 1-4 refers to mouse number.

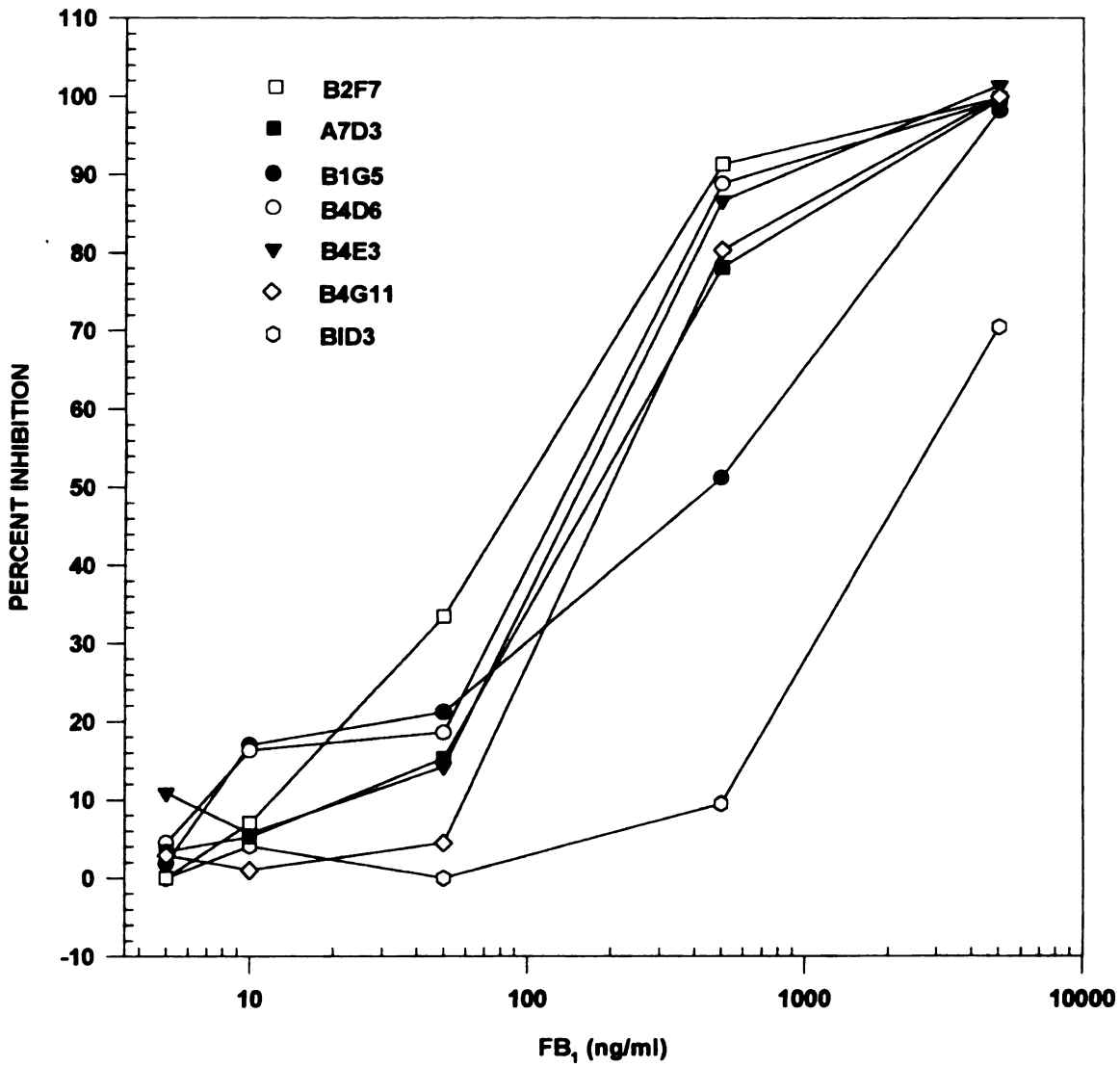


Figure 2.5. Competitive indirect ELISA curves for FB_1 using hybridoma supernatants. Supernatants were obtained from hybridoma cells producing FB_1 antibodies before cloning. Each data point represents the mean value of duplicate measurements.

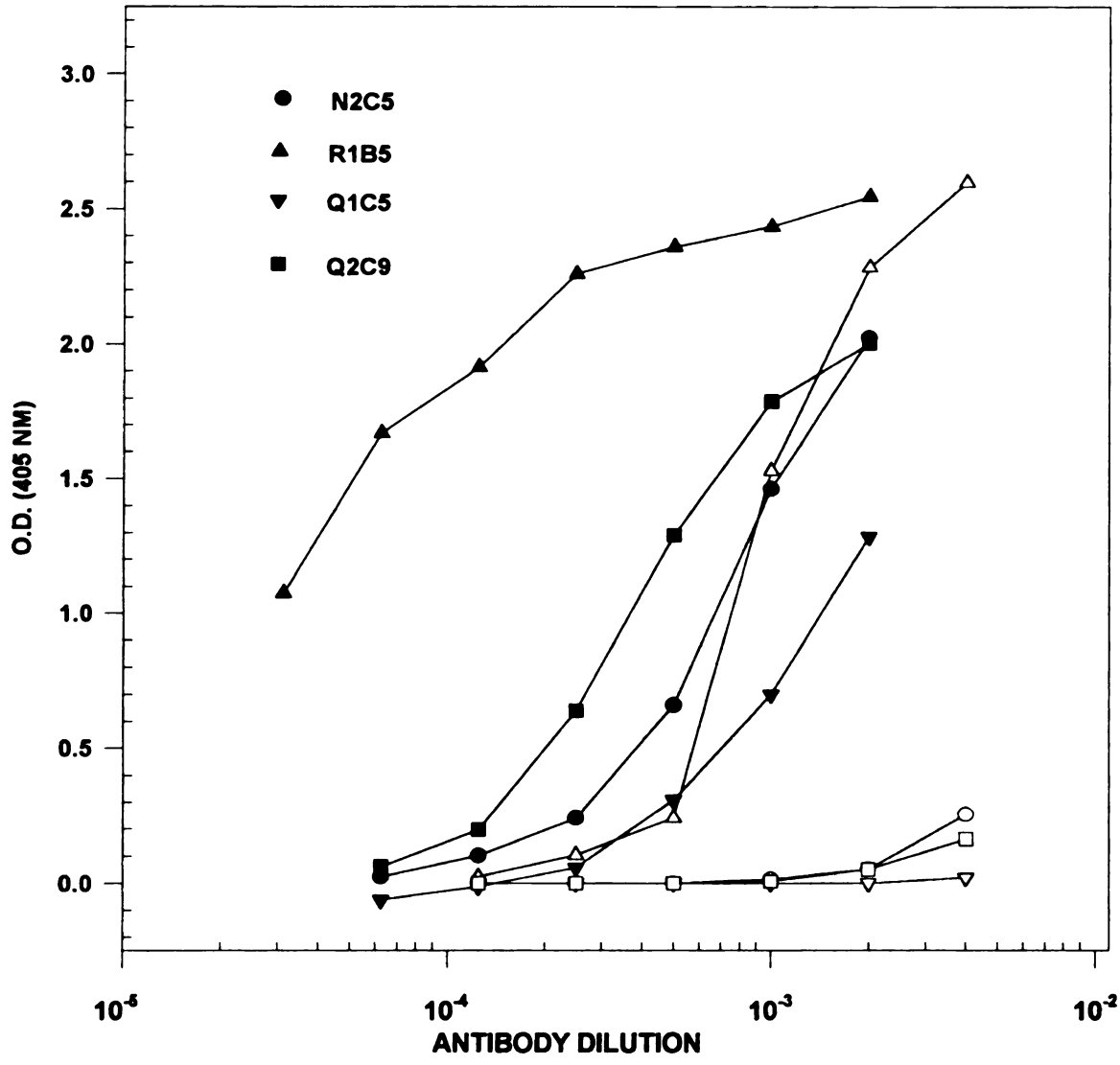


Figure 2.6. ELISA titration of monoclonal FB_1 antibodies prepared from ascites fluid. Each data point represents the mean value of duplicate measurements. Filled symbols indicate CI-ELISA. Open symbols are CD-ELISA. Q1C5 clone had no titer when determined with CD-ELISA.

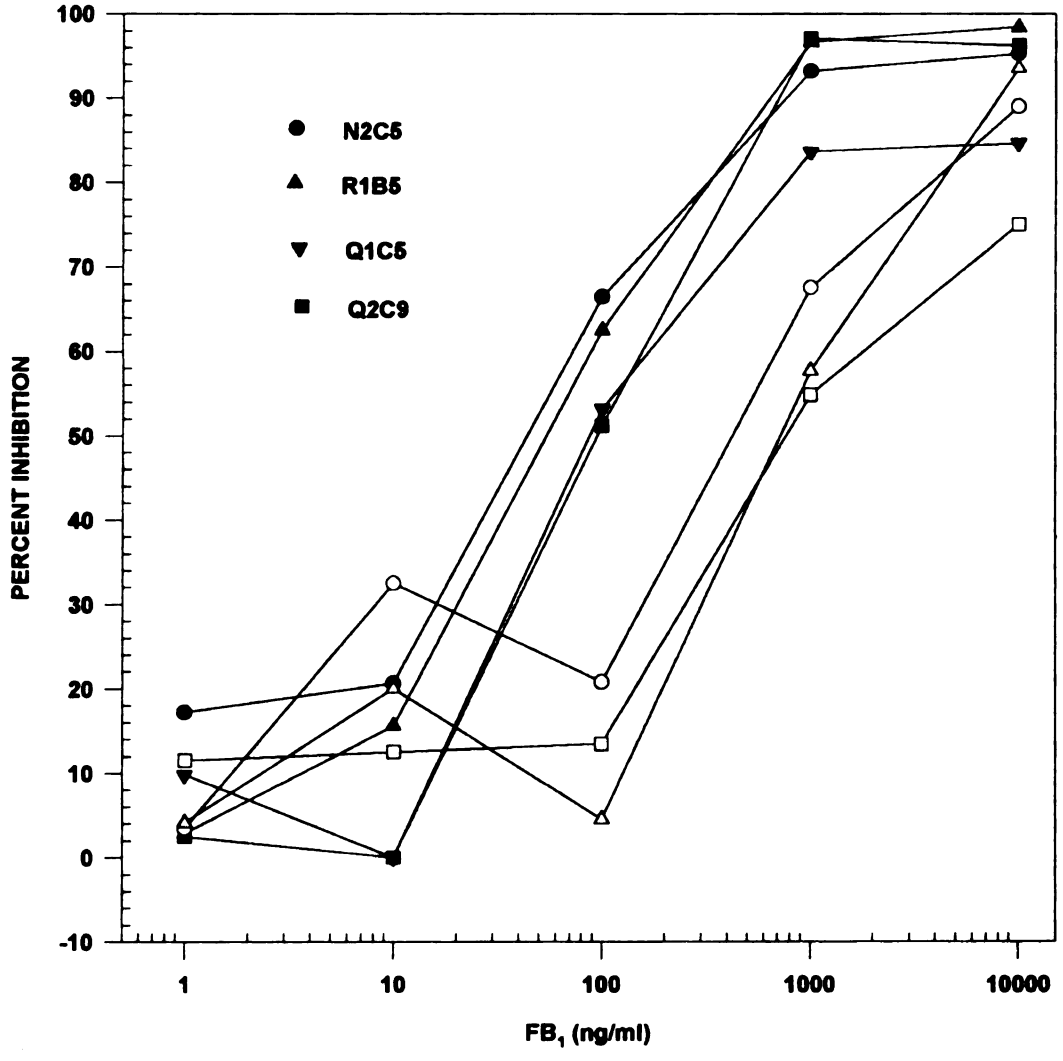


Figure 2.7. Competitive inhibition ELISA for FB_1 using monoclonal antibodies prepared from ascitic fluid. Data points represent the mean value of duplicate measurements. Filled symbols were CI-ELISA. Open symbols were CD-ELISA. Q1C5 clone had no titer when determined with CD-ELISA.

Table 2.1. Sensitivity of CI-and CD-ELISAs for FB_1 using monoclonal antibodies prepared from ascites fluid

	Amount of FB_1 required for 50% inhibition[a] (ng/ml)	
Clone	CI-ELISA	CD-ELISA
N2C5	50	400
R1B5	60	700
Q1C5	90	-[b]
Q2C9	100	800

[a] Data from Figure 2.7 [b] No titer in this assay.

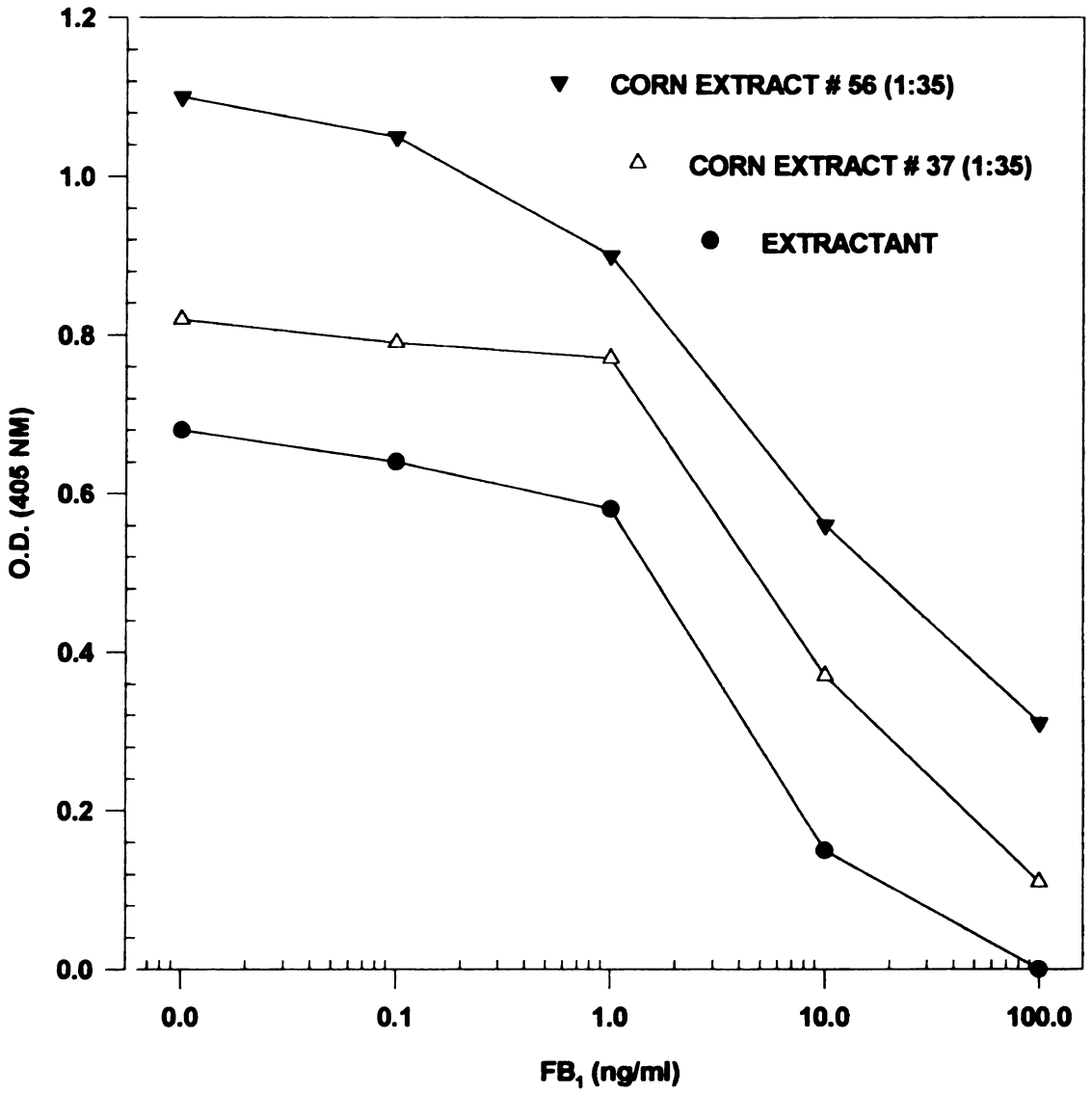


Figure 2.8. Competitive direct ELISA curves for FB_1 using FB_1 sheep anti sera (Neogen Corp., Lansing, MI). FB_1 was dissolved in extractant [10% methanol (vol./vol.) in distilled water], fresh corn extract numbers 37 and 56 at a dilution of 1:35). Each data point represents the mean value of triplicate measurements.

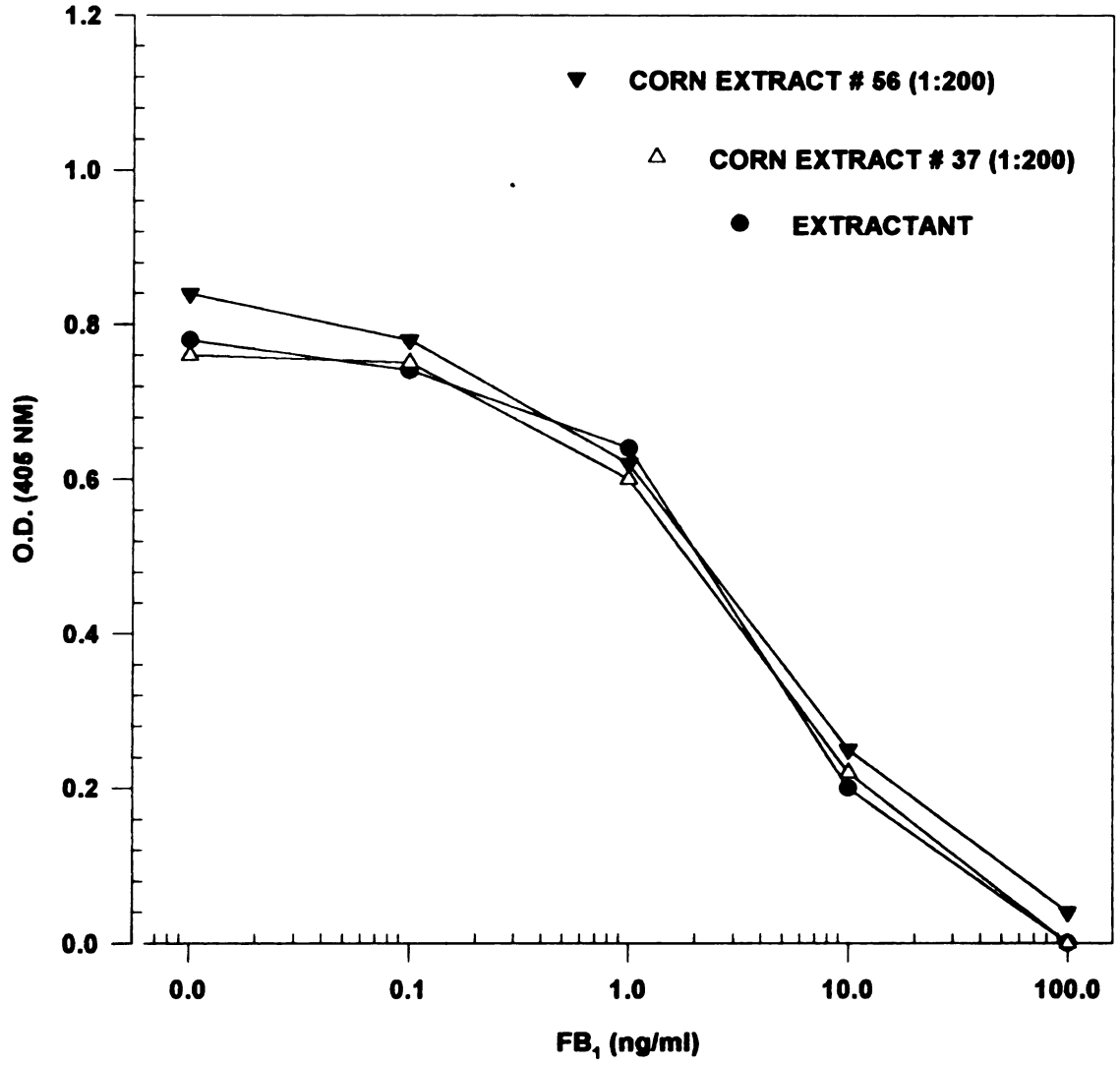


Figure 2.9. Competitive direct ELISA curves for FB_1 using FB_1 sheep anti sera (Neogen Corp., Lansing, MI). FB_1 was dissolved in extractant [10% methanol (vol./vol.) in distilled water], fresh corn extract numbers 37 and 56 at a dilution of 1:200). Each data point represents the mean value of triplicate measurements.

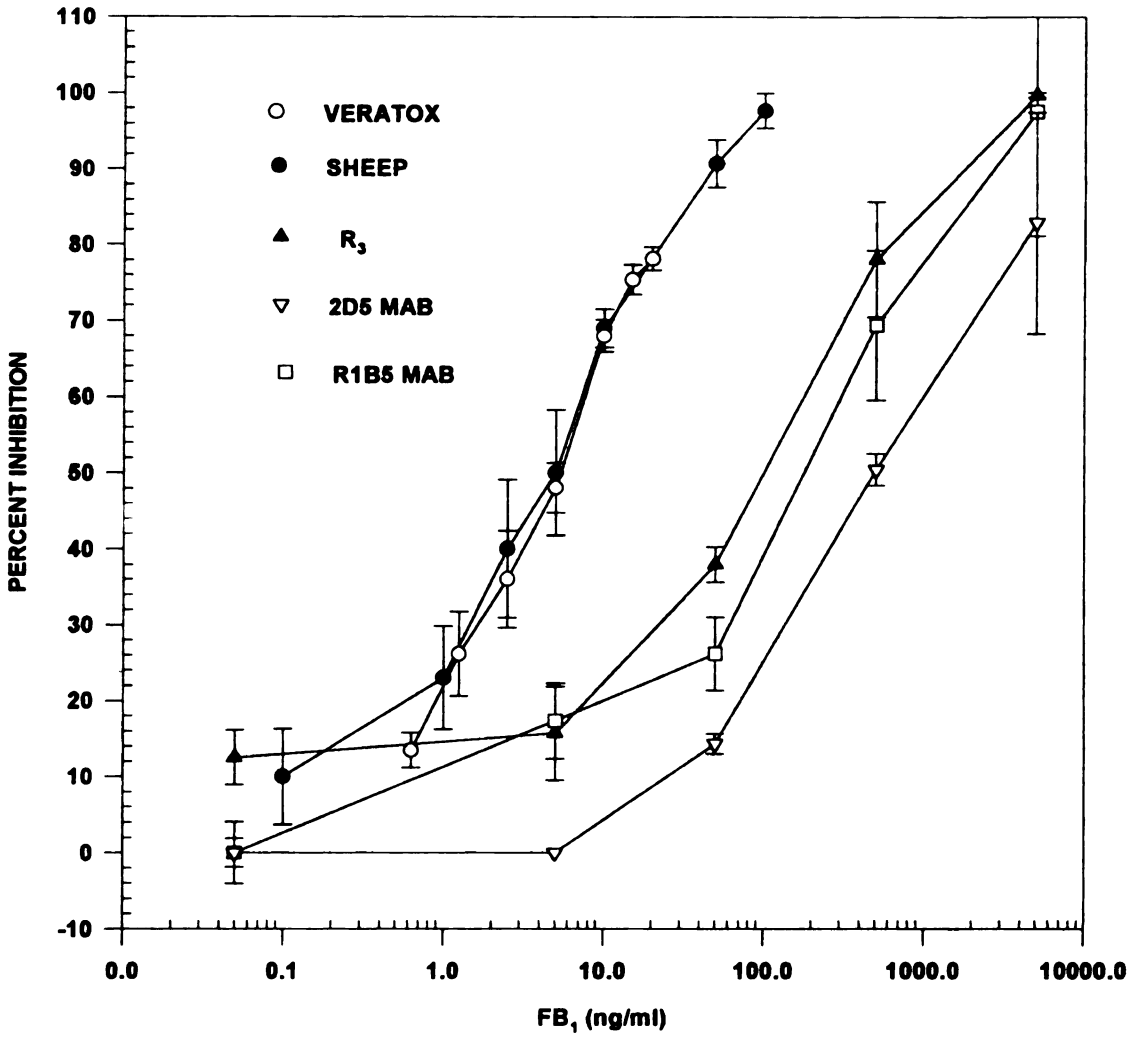


Figure 2.10. Comparison of competitive inhibition ELISA for FB_1 mouse-monoclonal antibodies, rabbit-and sheep-polyclonal antibodies, and Veratox®. Monoclonal antibodies (N2C5 MAB and 2D5 MAB) were prepared from ascitic fluids. N2C5 MAB was generated in the current study using FB_1-KLH immunogen, while 2D5 was produced by Azcona-Olivera (1992a) using FB_1-Cholera toxin immunogen. R_3 refers to rabbit number 3. Sheep antiserum was from a single lot supplied by Neogen Corp. (Lansing, MI). Veratox® is an ELISA kit for FB_1 based on CD-ELISA and is produced and supplied by Neogen Corp. (Lansing, MI). Monoclonal antibodies were determined with CI-ELISA, but polyclonal antibodies were measured with CD-ELISA. Each data point represents the mean ± standard errors of the mean (n = 4, two of duplicate measurements). Absorbencies at 0 ng/ml FB_1 ranged from 0.675 to 1.250.

Table 2.2. Comparison of competitive FB_1 ELISA using mouse-monoclonal antibodies prepared from ascites fluid, rabbit-and sheep-polyclonal antibodies

Antibody	FB_1 concentration required for 50% inhibition[a] (ng/ml)	Absorbance value for 0 ng FB_1 /ml	ELISA format
2D5[b]	200	1.10	CI-ELISA
N2C5[c]	100	1.25	CI-ELISA
R_3[d]	80	0.79	CD-ELISA
Sheep[e]	6	0.74	CD-ELISA
Veratox[f]	6	0.68	CD-ELISA

[a] Data from Figure 2.8.

[b] Monoclonal antibodies produced by Azcona-Olivera (1992a) using FB_1-Cholera toxin immunogen.

[c] Monoclonal antibodies generated in the current study using FB_1-KLH immunogen .

[d] Rabbit polyclonal antibodies from rabbit number 3.

[e] Sheep polyclonal antibodies from Neogen Corp. (Lansing, MI).

[f] Veratox is an ELISA kit for FB_1 based on CD-ELISA and is produced and supplied by Neogen Corp. (Lansing, MI).

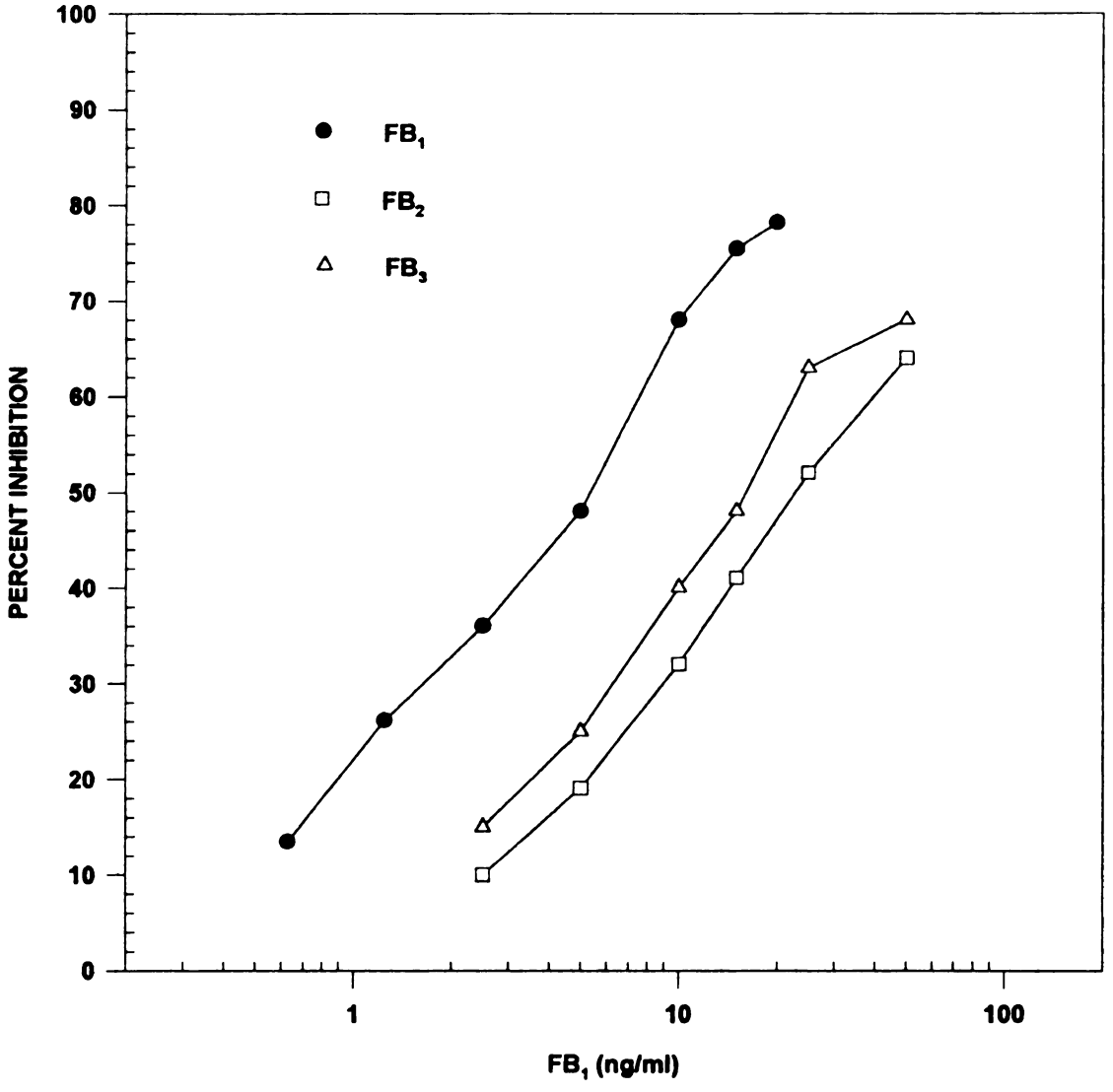


Figure 2.11. Competitive direct ELISA standard curves for FB_1 , FB_2 and FB_3 , using FB_1 sheep antisera. Data for sheep antisera were provided by Dr. Mohamed M.Abouzied (Neogen Corp., Lansing, MI). Each data point represents the average value of triplicate determination.

Table 2. 3. Cross reactivity of FB_1 sheep antisera toward fumonisin analogues.

Fumonisin analogue	Fumonisin concentration required for 50% inhibition[a] (ng/ml)	Cross reactivity (%)[b]
FB_1	5.5	100
FB_2	22.9	24
FB_3	18.3	30

[a] Data from Figure 2.9.

[b] Cross reactivity defined as (ng/ml of FB_1 required for 50% inhibition)/(ng/ml of fumonisin analogue required for 50% inhibition) x 100.

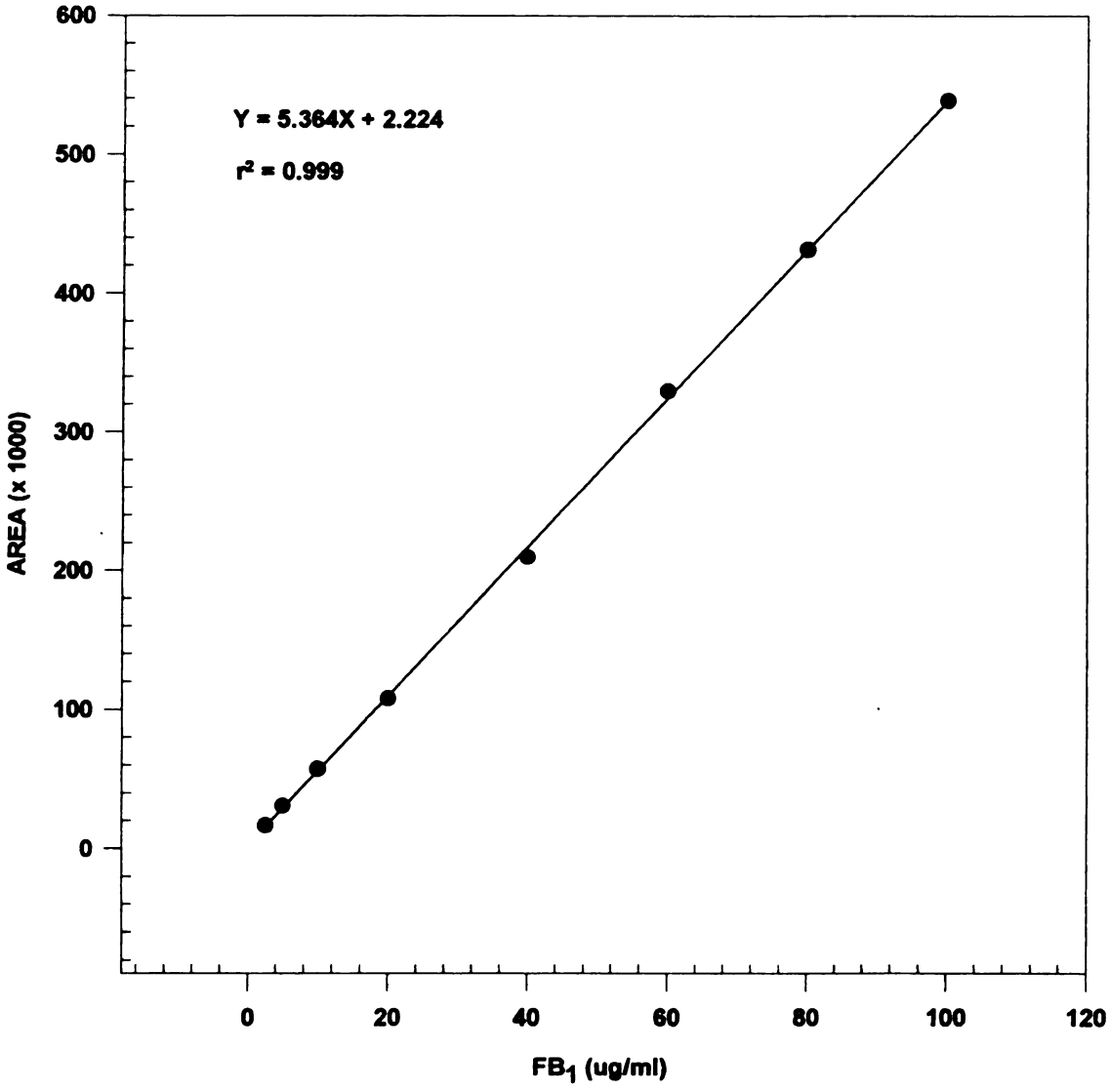


Figure 2.12. A typical "broad range " standard curve used for HPLC analyses of *Fusarium* corn cultures. Each data point represents the average value of two determinations.

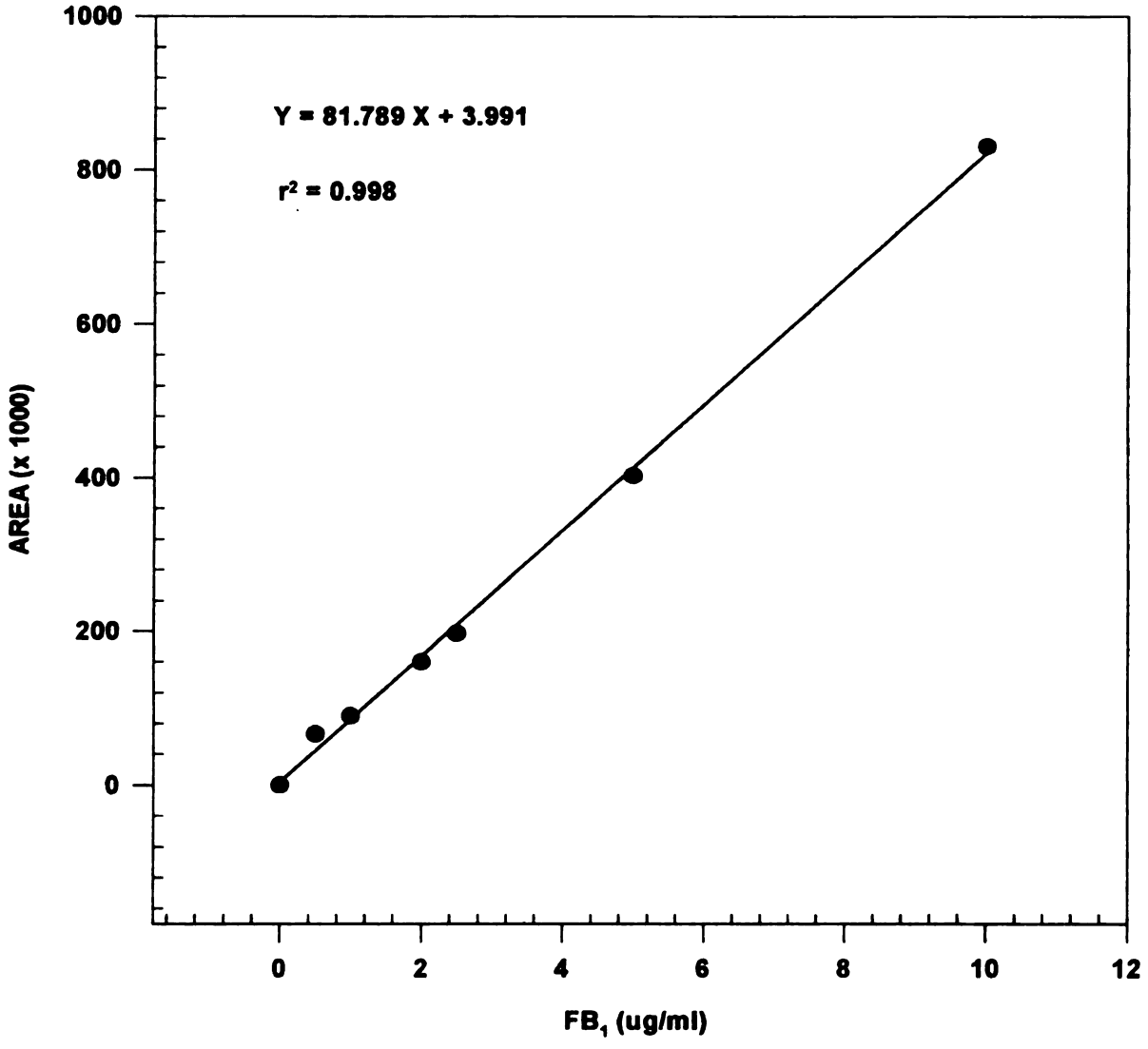


Figure 2.13. A typical "low range " standard curve used for HPLC analyses of corn and corn products. Each data point represents the average value of two determinations

Table 2.4. Comparison of FB_1 concentrations in *Fusarium* corn cultures detected by HPLC and CD-ELISA methods

SAMPLE	HPLC (µg/g)	CD-ELISA (µg/g)[a]	RATIO OF ELISA TO HPLC
Control[b], wk. 2	<0.04[g]	<0.02[h]	-
Control, wk. 3	<0.04	<0.02	-
Control, wk. 4	<0.04	<0.02	-
Control, wk. 5	<0.04	<0.02	-
FW-8[c], wk. 2	<0.04	<0.02	-
FW-8, wk. 3	<0.04	<0.02	-
FW-8, wk. 4	<0.04	<0.02	-
FW-8, wk. 5	<0.04	<0.02	-
M-5956[d], wk. 2	7.0	19.3	2.8
M-5956, wk. 3	33.9	30.3	0.9
M-5956, wk. 4	9.7	26.1	2.7
M-5956, wk. 5	21.4	39.7	1.9
M-5958[e], wk. 2, R1	23.8	58.1	2.4
M-5958, wk. 3, R1	9.8	27.4	2.8
M-5958, wk. 4, R1	17.9	58.1	3.2
M-5958, wk. 5, R1	24.3	76.2	3.1
M-5958, wk. 2, R2	8.9	24.2	2.7
M-5958, wk. 3, R2	220.0	759.0	3.5
M-5958, wk. 4, R2	<0.04	<0.02	<0.02-
M-5958, wk. 5, R2	25.7	109.0	4.2
MEAN ± SEM[f]			2.8 ± 0.9

[a] CD-ELISA using FB_1 sheep antisera (Neogen Corp., Lansing, MI); Linear regression analyses between HPLC (X) and CD-ELISA (Y) at $P<0.05$ yielded an equation line of $Y = 3.428X - 7.640$ and linear correlation coefficient (r)=0.992.

[b] Uninoculated samples.

[c] *Fusarium graminearum* cultures(FW-8).

[d] *F. proliferatum* cultures (M-5956).

[e] *Fusarium moniliforme* cultures (M-5958).

[f] Standard errors of the means.

[g] HPLC detection limit (0.04 ng/g)

[h] CD-ELISA detection limit (20 ng/g)

wk. 2, 3, 4, and 5 refer to week number 2, 3, 4, and 5 respectively at which *Fusarium* cultures were harvested.

R1 and R2 refer to replication number 1 and 2, respectively.

Table 2.5. Comparison of FB_1 recoveries from spiked-corn samples containing FB_1 of 100 to 3,000 μg/g using raw methanolic extracts and SAX-cleaned extracts as determined by HPLC, Veratox®[a], and CD-ELISA using FB_1 sheep antisera (Neogen Corp., Lansing, MI)

	PERCENT RECOVERIES[b] of FB_1 in				
FB_1 ADDED	RAW METHANOLIC EXTRACTS		SAX-CLEANED EXTRACTS		
ng/g	Veratox®	CD-ELISA	HPLC	Veratox®	CD-ELISA
100	86.3 ± 24.0	nd	68.8 ± 4.2	nd	nd
200	127.2 ± 31.8	48.5 ± 3.3	71.2 ± 12.4	nd	nd
600	80.5 ± 12.4	70.3 ± 13.3	84.7 ± 7.2	56.5 ± 6.4	50.2 ± 9.1
1,000	71.5 ± 6.8	70.0 ± 2.3	73.6 ± 9.9	50.2 ± 10.7	58.7 ± 9.6
3,000	60.5 ± 6.4	50.1 ± 4.5	72.1 ± 5.4	51.6 ± 9.1	38.8 ± 1.7
MEAN[c]	85.2 ± 16.3	59.7 ± 5.9	74.1 ± 7.8	45.2 ± 16.3	49.2 ± 6.8

[a] Veratox® Fumonisin Test, Neogen Corp.(Lansing, MI).

[b] (FB_1 found/FB_1 added) x 100%.

[c] Calculated from positive recoveries.

nd indicates no FB_1 detected (Detection limits of Veratox® and CD-ELISA were 0.02 μg/g) ; Thus, recovery could not be calculated.

Each data point represents the mean ± SEM (n = 3).

SEM = standard errors of the means.

Table 2.6. Comparisons of FB_1 concentrations in 14 corn-food samples by HPLC, Veratox®[a], and CD-ELISA using FB_1 sheep antisera (Neogen Corp., Lansing, MI) and the ratios of Veratox®[a] and CD-ELISA to HPLC results

SAMPLE	FUMONISIN B_1 (µg/g) FOUND[b] BY			RATIOS TO HPLC[c]	
NUMBER[d]	HPLC	Veratox®[a]	CD-ELISA	Veratox®[a]	CD-ELISA
41	<0.04[e]	0.13	0.02[f]	-	-
38	<0.04	0.15	0.29	-	-
43	<0.04	0.17	0.12	-	-
48	<0.04	0.14	0.07	-	-
33	0.34	0.44	0.41	1.3	1.2
32	0.42	0.58	0.59	1.4	1.4
25	0.64	0.94	0.85	1.5	1.3
10	0.75	0.96	1.16	1.3	1.5
8	0.92	0.97	1.28	1.1	1.4
7	1.10	1.24	1.08	1.1	1.0
22	1.21	1.61	1.20	1.3	1.0
6	1.79	2.35	2.25	1.3	1.3
21	2.27	3.21	2.40	1.4	1.1
H9	7.11	9.59	10.16	1.3	1.4
MEAN	1.18	1.61	1.56	1.31	1.26
STD[g]	1.84	2.47	2.59	0.12	0.20

[a] Veratox® Fumonisin Test, Neogen Corp. (Lansing, MI).

[b] Linear regression analyses between HPLC (X) and Veratox® (Y_1) and between HPLC and CD-ELISA (Y_2) at $P < 0.05$ yielded equation lines of $Y_1 = 1.473X - 0.485$; $r = 0.992$ and $Y_2 = 1.370X - 0.356$; $r = 0.983$.

[c] Veratox® or CD-ELISA results divided by HPLC results.

[d] Ranked according to HPLC results; the sample descriptions were on Appendix (Table 5.1).

[e] HPLC detection limit (40 ng/g).

[f] CD-ELISA detection limit (20 ng/g).

[g] Standard errors of the means.

Table 2.7. Comparisons of FB_1 concentrations in 28 Italian feed samples by HPLC, Veratox®[a], and CD-ELISA using FB_1 sheep antisera (Neogen Corp., Lansing, MI) and the ratios of Veratox®[a] and CD-ELISA to HPLC results

SAMPLE	FUMONISIN B_1 (μg/g) FOUND[b] BY			THE RATIOS OF HPLC[c]	
NUMBER[d]	HPLC	Veratox®[a]	CD-ELISA	Veratox®[a]	CD-ELISA
F18	<0.04[e]	0.34	0.51	-	-
F20	<0.04	0.49	0.27	-	-
F8	<0.04	0.51	0.87	-	-
F25	0.24	0.62	0.41	2.6	1.7
F3	0.11	0.40	0.38	3.6	3.4
F22	0.21	0.43	0.15	2.1	0.7
F1	0.22	0.95	0.61	4.3	2.7
F16	0.24	0.56	0.68	2.3	2.8
F11	0.34	0.90	0.74	2.6	2.2
F24	0.36	0.72	0.88	2.0	2.5
F23	0.38	1.25	0.74	3.3	2.0
F30	0.42	0.67	0.32	1.6	0.8
F13	0.44	1.01	0.58	2.3	1.3
F10	0.50	1.07	0.74	2.2	1.5
F29	0.62	1.12	0.79	1.8	1.3
F4	0.67	2.23	1.87	3.3	2.8
F7	0.79	1.62	2.85	2.0	3.6
F28	0.94	1.16	1.44	1.2	1.5
F9	0.94	3.05	2.30	3.2	2.4
F2	1.00	1.69	0.88	1.7	0.9
F14	1.07	2.06	1.73	1.9	1.6
F27	1.16	2.37	1.66	2.0	1.4
F19	1.24	2.40	1.93	1.9	1.6

Table 2.7. (Con'd)

SAMPLE	FUMONISIN B_1 (μg/g) FOUND[b] BY			THE RATIOS OF HPLC[c]	
NUMBER[d]	HPLC	Veratox®[a]	CD-ELISA	Veratox®[a]	CD-ELISA
F17	1.31	2.37	2.60	1.8	2.0
F31	1.39	2.17	1.96	1.6	1.4
F26	1.67	2.96	3.84	1.8	2.3
F15	3.71	5.01	8.43	1.3	2.3
F12	4.99	7.63	7.87	1.5	1.6
MEAN[f]	1.00	1.86	1.86	2.24	1.93
SEM[g]	1.11	1.60	2.10	0.77	0.77

[a] Veratox® Fumonisin Test, Neogen Corp. (Lansing, MI).

[b] Linear regression analyses between HPLC (X) and Veratox ® (Y_1) and between HPLC (X) and CD-ELISA (Y_2) at $P < 0.05$ yielded equation lines of $Y_1 = 1.010X + 0.532$; $r = 0.961$ and $Y_2 = 1.300X + 0.160$; $r = 0.951$).

[c] Veratox® or CD-ELISA results divided by HPLC results.

[d] Ranked according to HPLC results, and the sample descriptions were on Appendix (Table 5.2).

[e] HPLC detection limit (0.04 ng/g).

[f] Mean of positive samples according to HPLC results.

[g] Standard errors of the means

Table 2.8. Comparisons of FB_1 concentrations in fresh corn [a] samples by HPLC, Veratox® [a], and CD-ELISA using FB_1 sheep antisera (Neogen Corp., Lansing, MI) and the ratios of Veratox® [b] and CD-ELISA to HPLC results

SAMPLE	FUMONISIN B_1 (μg/g) FOUND[c] BY			THE RATIOS OF HPLC[d]	
NUMBER[e]	HPLC	Veratox® [b]	CD-ELISA	Veratox® [b]	CD-ELISA
P14	<0.04[f]	<0.02[g]	<0.02[h]	-	-
P15	<0.04	<0.02	<0.02	-	-
P19	<0.04	<0.02	<0.02	-	-
P21	<0.04	<0.02	<0.02	-	-
P25	<0.04	<0.02	<0.02	-	-
P27	<0.04	<0.02	<0.02	-	-
P28	<0.04	<0.02	<0.02	-	-
P31	<0.04	<0.02	<0.02	-	-
P32	<0.04	<0.02	<0.02	-	-
P37	<0.04	<0.02	<0.02	-	-
P46	<0.04	<0.02	<0.02	-	-
P5	<0.04	<0.02	<0.02	-	-
P50	<0.04	<0.02	<0.02	-	-
P51	<0.04	<0.02	<0.02	-	-
P52	<0.04	<0.02	<0.02	-	-
P53	<0.04	<0.02	<0.02	-	-
P54	<0.04	<0.02	<0.02	-	-
P55	<0.04	<0.02	<0.02	-	-
P56	<0.04	<0.02	<0.02	-	-
P58	<0.04	<0.02	<0.02	-	-
P59	<0.04	<0.02	<0.02	-	-
P62	<0.04	<0.02	<0.02	-	-
P67	<0.04	<0.02	<0.02	-	-
P7	<0.04	<0.02	<0.02	-	-
P74	<0.04	<0.02	<0.02	-	-
P9	<0.04	<0.02	<0.02	-	-
P17	<0.04	<0.02	<0.02	-	-
P26	<0.04	<0.02	<0.02	-	-
P63	<0.04	<0.02	<0.02	-	-
P42	0.04	0.27	0.26	7.1	6.8
P61	0.05	0.26	0.16	5.2	3.1
P80	0.05	0.25	<0.02	4.6	0.0

Table 2.8. (Con'd)

SAMPLE	FUMONISIN B_1 (μg/g) FOUND[c] BY			THE RATIOS OF HPLC[d]	
NUMBER[e]	HPLC	Veratox®[b]	CD-ELISA	Veratox®[b]	CD-ELISA
P10	0.08	0.16	<0.02[h]	2.0	0.0
P12	0.10	0.28	<0.02	2.8	0.0
P57	0.10	0.23	<0.02	2.2	0.0
P8	0.12	0.21	<0.02	1.7	0.0
P1	0.15	0.31	0.15	2.0	1.0
P66	0.25	0.75	0.60	3.0	2.4
P29	0.28	0.52	0.34	1.9	1.2
P45	0.30	0.47	0.86	1.5	2.8
P20	0.47	0.76	0.88	1.6	1.9
P44	0.70	0.92	0.56	1.3	0.8
MEAN[i]	0.20	0.41	0.29	2.85	1.54
SEM[j]	0.21	0.23	0.33	1.75	1.95

[a] Harvested in five counties of Michigan in Summer 1994. Samples were supplied by Dr. Patrick L. Hart (105 Pesticide Research Center, Michigan State University, MI, 48824). All samples were first analyzed with CD-ELISA; and then all positive samples as well as randomly selected negative samples were analyzed with HPLC, Veratox®, and CD-ELISA.

[b] Veratox® Fumonisin Test, Neogen Corp. (Lansing, MI).

[c] Linear regression analyses between HPLC (X) and Veratox® (Y_1) and between HPLC (X) and CD-ELISA (Y_2) at $P < 0.05$ yielded equation lines of $Y_1 = 1.127X + 0.039$; $r = 0.933$ and $Y_2 = 0.973X + 0.008$; $r = 0.836$.

[d] Veratox® or CD-ELISA results divided by HPLC results.

[e] Ranked according to HPLC results, and the sample descriptions were on Appendix (Table 5.3).

[f, g, h] Detection limit of HPLC (40 ng/g), Veratox® (20 ng/g), CD-ELISA (20 ng/g), respectively.

[i] Mean of positive samples.

[j] Standard errors of the means.

PART III

PRODUCTION OF DEOXYNIVALENOL (VOMITOXIN) ANTIBODIES

ABSTRACT

PRODUCTION OF DEOXYNIVALENOL (VOMITOXIN) ANTIBODIES

By

Sutikno

Deoxynivalenol (DON) was reacted with hemisuccinate (HS) for 8 hours to form DON-HS derivatives. These derivatives were conjugated to bovine serum albumin (BSA) and then used as an immunogen for producing DON specific antibodies. Twenty female BALB/c mice were immunized intrasplenically, intraperitoneal, or subcutaneously and 6 female New Zealand rabbits were injected intramuscularly with the immunogen. All animals apparently produced high titer antisera. However, these antisera could not be used in competitive ELISAs for DON and also did not cross react with either 3-acetyl-DON, 15-acetyl-DON, nivalenol, 4,15-diacetylnivalenol or fusarenone-X.

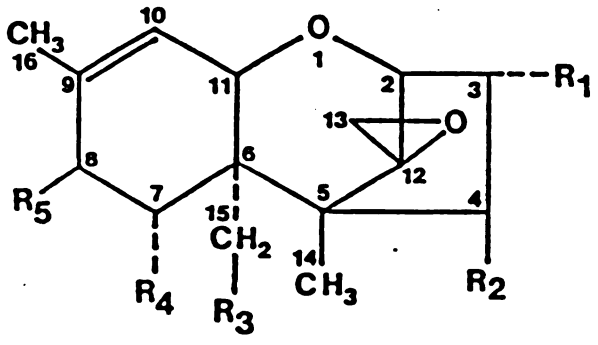
INTRODUCTION

Natural occurrence.

Deoxynivalenol (DON, vomitoxin) is a trichothecene mycotoxin, produced by *Fusarium graminearum* and chemically determined to be 3α, 7α, 15-trihydroxy-12, 13-epoxytrichothec-9-en-8-one (Table 3.1)(Yoshizawa and Morooka, 1973). DON was first isolated in Japan from *Fusarium*-infected barley (Morooka et al., 1972). This toxin was also found in the United States by Vesonder et al. (1973) who analyzed Northwest Ohio corn infected with *Fusarium*. These investigators tested the presence of the emetic compound by intubation of each fraction of their isolation procedures into pigs to induce vomiting at which they isolated and characterized DON as an active compound. Because of its emetic effect on swine, DON was also named vomitoxin (Vesonder et al., 1973). As a consequence of these findings, surveys for the presence of DON in foods and feeds as well as testing for its toxicological and immunosuppresive effects began.

Surveys for the presence of DON in foods especially grain and grain products have been done by several investigators in several countries and the results are summarized in Table 1.3. DON has been found worldwide and is a major contaminant in cereal grains (Tanaka et al., 1988; Hietaniemi and Kumpulainen, 1991). DON production occurs when environmental conditions in

Table 3.1. Structures of deoxynivalenol (DON) analogs (Adapted from Ueno, 1983)



Compound	R1	R2	R3	R4	R5
DON	OH	H	OH	OH	=O
DON-3-HS	OHS[a]	H	OH	OH	=O
DON-15-HS	OH	H	OHS[a]	OH	=O
3-Acetyl-DON	OAc[b]	H	OH	OH	=O
15-Acetyl-DON	OH	H	OAc[b]	OH	=O
Nivalenol	OH	OH	OH	OH	=O
4,15-Diacetylnivalenol	OH	OAc[b]	OAc[b]	OH	=O
Fusarenon-X	OH	OAc[b]	OH	OH	=O
T-2 toxin	OH	Oac[b]	Oac[b]	H	Olp[c]

OHS[a], OAc[b], Olp[c] indicate $OOC(CH_2)_2COOH$, $OOCCH_3$, and $OCCH_2CH(CH_3)_2$, respectively.

the field are low temperature and high humidity (Cote et al., 1984; Vesonder et al.,1978; Mills, 1982), although this toxin has also been found in high temperature regions (Richardson et al., 1985). Vesonder et al. (1978) reported that cold and wet weather tends to delay harvest and subsequently molds on the crop grow continuously, and thus produce high quantities of DON. In some cases, however, prolonged delay of harvest can yield a decline in DON concentration in grains left in the field, possibly due to reaction with plant components or metabolism by plant/ fungal enzymes (Scott et al., 1984). In North America, the areas most often contaminated by DON are the midwestern region of the United states (Vesonder, 1983) and the eastern region of Canada (Seaman, 1982).

Toxicity

DON is much less toxic than other trichothecene mycotoxins such as T-2 toxin, HT-2 toxin, diacetoxyscirpenol, nivalenol, and fusarenon-X (Scott et al., 1980). DON was found to be 60-fold less toxic than T-2 toxin and two fold less toxic than nivalenol when the trichothecene toxicity is measured using lethal dose 50 (LD_{50}, an amount of toxin required to kill 50% population) of brine shrimp larvae (Scott et al., 1980). In mice, the LD_{50} of DON is 49 mg/kg body weight (intraperitoneal) and 78 mg/kg body weight (oral) (Forsell et al., 1987). The toxicity of this toxin may be due to protein synthesis inhibition (Ueno, 1983).

Consumption of high quantities of DON (at LD_{50}, or higher) results in classical acute symptoms of trichothecene toxicity ranging from necrosis of the intestinal tract, bone marrow and lymphoid tissues as well as lesions in kidney

and heart, to death (Forsell et al., 1987; and Robbana-Barnat et al., 1987). Pathological changes include lesions and degeneration of the stomach and small intestine mucosa, enlargement and edema of mesenteric lymph nodes, vascular congestion and depletion of all lymphoid organs and liver (Cote et al., 1985; Robbana-Barnat et al., 1987). At lower doses ($<LD_{50}$), ingestion of DON can cause feed refusal, reduced growth rate, reduced milk production, reproductive problems and cause immune alteration (Vesonder et al., 1973;1979; Cote et al., 1984; 1985; Morrissey and Vesonder,1985; Whitlow and Hagler, 1987). The toxicological effects of DON are gender-related with male or castrated male animals being more sensitive to DON than female animals (Cote et al., 1985; Greene et al., 1994).

In order to elucidate the immunotoxicity of DON on animal, Pestka et al. (1987) fed mice with DON-containing diets and then challenged the mice with *Listeria monocytogenes*. They found that mouse resistance to the pathogen decreased as indicated by the lower splenic clearance of *Listeria*. Increased splenic *Listeria* counts following exposure to DON may be partly due to the toxin-induced feed refusal rather than directly as a consequence of immune function alteration of the animals (Pestka and Bondy, 1990).

Oral exposure of DON induces a dose dependent leukopenic effect in mice in which white blood cells, lymphocyte and monocyte numbers are decreased (Forsell et al., 1986; Robbana-Barnet et al., 1988) but neutrophil cells are increased (Forsell et al., 1986). Increase of an IgA-secreting cell number and decrease of an IgG-secreting cell number in the Payer's patch (PP) were also

reported in mice fed with DON (Pestka et al., 1990). In addition, ingestion of DON can also cause glomerular IgA deposition (Pestka et al., 1989) and a dysregulation of serum immunoglobulin production by decreasing IgG and IgM and increasing IgA and IgE serum levels (Forsell et al., 1986; Dong et al., 1991; Pestka and Dong, 1993; 1994).

The presence of DON in both feed and food supplies is of concern as this toxin occurs worldwide (Table 1.3) and consumption of corn and mixed feeds contaminated with DON have been associated with animal health problems. Furthermore, food preparation and processing such as heating at 350^{0}C, cleaning, milling and baking can not eliminate DON from food products (Young et al., 1984). Reduced DON concentration after food preparation results solely from the dilution effect with clean wheat (Young et al., 1984). Thus, a combination of worldwide occurrence of DON in grain and grain products and its resistance to processing enable this mycotoxin to persist in the food and feed supplies.

In order to protect human and animal health, DON regulation has been established in Canada and Russian Federation. The official DON tolerance limit is 2.0 ppm in uncleaned soft wheat in Canada and 0.5-1.0 ppm for foods in USSR (Hietaniemi and Kumpulainen, 1991). However, DON has not been regulated yet in the United States even though DON can be present in up to 50% of cereal and cereal products tested (Abouzied et al., 1991). In the United States, the only limitation of DON concentration in foods is an "advisory tolerance limit" suggesting that wheat should contain not more than 2.0 ppm and finished products for human consumption should contain no more than 1.0 ppm

(Hietaniemi and Kumpulainen, 1991). The advisory levels for DON in wheat and wheat products has been revised by the U.S. Food and Drug Administration during 1993-1994 (Trucksess, 1995). The new levels are: (1) 1 ppm for brand, flour, and germ for human consumption; (2) 10 ppm for grain and grain by-products destined for ruminating beef cattle and feedlot cattle older than 4 months and for chickens, with the added recommendation that the maximum level of these ingredients in cattle or chicken diet is 50%; and (3) 5 ppm in grains and grain by-products destined for pigs, and all other animals, with the added recommendation that the maximum levels of these ingredients are 20% in the pig diet and 40% in all other animal diets.

DON detection

Detection of DON in foods and feeds can be achieved with conventional detection methods such as TLC, GC, HPLC and MS or with immunoassay methods such as RIA and ELISA (Pestka et al., 1994a). These detection methods have been reviewed in Part I. Notably, a monoclonal antibody against DON was generated by Casale et al. (1988) who derivatized DON to 3-O-hemisuccinyl-DON after blocking two of the three available hydroxyl moieties with a cyclic boronate ester. The detection limit of both direct and indirect ELISAs employing the DON monoclonal antibodies in buffer solution was 200 ng/ml (Casale et al., 1988). When these antibodies were employed in indirect ELISAs, detection limit for DON in grain-based foods after dilution was 1000 ppb (Abouzied et al., 1991). Later, higher affinity polyclonal antibodies against DON

were reported by Usleber et al. (1991) who derivatized DON to hemisuccinyl-DON (HS-DON) without the blocking reaction. When the DON polyclonal antibodies were used in the direct ELISA, it could detect DON in buffer solution as low as 1.0 ng/ml (Usleber et al., 1991). This suggested that either the use of rabbits or the type of immunogen used by Usleber's group resulted in an antibody with improved affinity.

Rationale

Although conventional analytical methods such as TLC, GC, HPLC and MS are very sensitive and selective, they generally require extensive and time-consuming sample clean up prior to DON detection (Pestka et al., 1994a). As an alternative, ELISAs, which require simpler, quicker and easier sample preparation, have also been developed (Casale et al., 1988; Usleber et al., 1991; Abouzied et al., 1991). ELISAs using DON monoclonal antibodies, which were developed in our laboratory, had high (1000 ppb) detection limit when applied to food samples (Abouzied et al., 1991). Much lower detection limits of ELISA approaches using polyclonal antibodies have been demonstrated by Usleber et al. (1991). They suggested that reacting DON directly (without blocking reaction) with succinic anhydride prior to coupling with protein carriers resulted in higher affinity antibodies against DON.

In their attempts to produce DON antibodies, Zhang et al. (1986), Casale et al. (1988) and Usleber et al. (1994) immunized animals either intraperitoneally, subcutaneously or intradermally. Recently, intrasplenic immunization has been

proposed as a highly efficient technique for producing antibodies against small amount of matrix bound antigen (Nilsson et al., 1987). This procedure is applicable to the production of antibodies directed against highly conserved or weakly immunogenic determinants (Van Ness et al., 1984). Thus, the intrasplenic immunization technique may be capable of eliminating the difficulties of producing DON antibodies (Zhang et al., 1986; Casale et al., 1988, Usleber et al.,1994).

Based on the above findings, I hypothesized that the use of DON-HS-BSA conjugates prepared with the procedure of Usleber et al. (1991) for immunizing either mice or rabbits via either intrasplenic, subcutaneous, intraperitoneal, or intradermal injections would result in high affinity and specificity antibodies against DON. The objectives of this study were to produce polyclonal or monoclonal DON antibodies which have high affinity and specificity via intrasplenic, subcutaneous, intraperitoneal, and intradermal immunizations with DON derivative-BSA conjugation as described by Usleber et al. (1991), and then compare the applicability of these antibodies in ELISA.

MATERIALS AND METHODS

Chemical and reagents

All organic solvents and inorganic chemicals were of reagent grade or better. Ovalbumin (OA) (chicken egg albumin grade III; fraction VII), Tween 20, 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)(ABTS), hydrogen peroxide, horseradish peroxidase (HRP)(fraction VI), N-hydroxysuccinimide and 1,3-dicyclohexyl-carbodiimide were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA)(Albumin, bovine fraction V) was obtained from Amresco (Solon, Ohio). Goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate and goat anti-rabbit IgG-horseradish peroxidase conjugate were obtained from Cappel Laboratories (West Chester, PA). DON, 3-acetyl-DON, 15-acetyl-DON, nivalenol, and fusarenone-X were purchased from Romer Laboratories, Inc. (Washington, MO). Methofane (methoxyflurane, inhalation anesthetic for veterinary) was purchased from Pitman-Moore Inc. (Mundelein, IL).

Derivation of DON

DON was derivatized by reacting with succinic anhydride according to the procedures of Usleber et al. (1994) with slight modification. Briefly, DON (10 mg) was dissolved in 1.0 ml of pyridine and then succinic anhydride (200 mg) was added. This mixture was slowly stirred with a magnetic stirrer and heated at 100^0C in a

steam bath for 8 hours. Pyridine was removed under N_2 pressure and the residue was dissolved in $CHCl_3$ (15 ml). This solution was extracted with 0.1 M HCl (3x10 ml). The organic phase was dried under vacuum in a nitrogen flushing evaporator and the remaining substance was re-dissolved in 2 ml of methanol. Results of the derivatization reaction were detected with TLC methods using silica gel TLC plates (Linear K High Performance Silica Gel # 4805-411, Whatman International Ltd., Maidstone, England) developed in a solvent system of ethylacetate/ n-hexane/acetic acid at ratio of 75 : 25 : 5 (vol./vol./vol.). Unreacted DON and its derivative (HS-DON) were visualized by spraying the developed TLC plates with 15% $AlCl_3$ (15 g of $AlCl_3$ dissolved in 85% alcohol in distilled water), heating at 110^{0}C in a conventional oven for 10 minutes, cooling in room temperature, and then observing the toxin spots under 336 nm UV light (Kamimura et al., 1981). The toxin and its derivatives were also visualized by spraying the TLC plates with 1% (vol./vol.) of 4(p-nitrobenzyl)-pyridine in a mixture solution of chloroform:carbon tetrachloride at a ratio of 2:3 (vol./vol.), heating at 150^{0}C, cooling at room temperature, spraying again with 10% (vol./vol.) of tetraethylene pentamine in the mixture solution and then observing the toxin spot under 336 nm UV light (Takitani et al., 1979). The toxins appeared as blue fluorescent spots. After a reaction of 8 hours, all DON was converted to HS-DON which was then quantified with TLC methods, dried under nitrogen at room temperature and stored in 4^{0}C until conjugation reaction.

Conjugation of DON derivatives to protein

The HS-DON was coupled to protein [to bovine serum albumin (BSA) for use as immunogen, to ovalbumin (OA) (fraction VII) for use as a solid-phase antigen in indirect ELISA, and to horseradish peroxidase (HRP) for use as a labeled toxin in direct ELISA] at a DON:protein ratio of 2:5 (w/w) by using an activated ester method (Kitagawa et al., 1981). Briefly, HS-DON (5.0 mg) was dissolved in 0.5 ml N,N-dimethyl formamide (DMF) and slowly stirred with a magnetic stirrer. Fifty μl of a mixture containing 70 mg of N-hydroxysuccinimide and 60 mg of 1,3-dicyclohexyl-carbodiimide dissolved in 1 ml DMF was added drop by drop and the mixture was continuously stirred at room temperature for 30 minutes. Meanwhile, protein (12.5 mg) was dissolved in 1 ml sodium bicarbonate buffer (0.821 g of sodium bicarbonate buffer dissolved in 100 ml distilled water), pH 7.2. The HS-DON solution was added slowly to the protein solution and stirred for 2 hours at 4^0C. This mixture was dialyzed overnight three times against 4 liters of 0.01M phosphate buffer solution (PBS) pH 7.2, aliquoted into 2 mg protein/tube, lyophilized and stored at -80^0C.

Mouse immunization

Twelve female BALB/c mice (6-8 week old, Charles River, Laboratories, Wilmington, MA) were injected subcutaneously (SC) and intraperitoneally (IP) with DON-HS-BSA conjugates at two week intervals up to five times. The first immunization was given at a dose of 50 and 100 μg conjugate/animal. In this immunization, "complete " Freund's adjuvant (Difco Laboratory, Detroit, Michigan) was used to emulsify the conjugate at a ratio (conjugates:adjuvant) of 1:1 (vol./vol.).

The subsequent (booster) injections were at a dose of 25 and 50 μg of the conjugate/ mouse. For intraperitoneal injection, the conjugates were emulsified with an equal volume of "incomplete" Freund's adjuvant (Difco Laboratory, Detroit, Michigan) and for subcutaneous injection, the conjugates were mixed with an equal volume of 85% of saline solution (85 g of NaCl dissolved in 100 ml of distilled water). Ten days after every booster injection, methoxyflurane-anesthetized mice were bled from their tail vein and the blood was collected within a heparinized tube. The blood was incubated at 4^0C over night and centrifuged at 1000 x g for 15 minutes to obtain mouse plasma. Serum titer and serum specificity were then determined by indirect ELISA methods.

In addition to the above, 8 female BALB/c (6-8 week old, Charles River, Laboratories, Wilmington, MA) were given an intrasplenic immunization at a dose of 20 μg/mice by the procedure of Nilsson et al. (1987). Briefly, mice were anaesthetized using methoxyflurane before surgery. The animal was placed on its right side and its left side fur was shaved. After swabbing the left abdominal with 70% ethanol, a cutaneous incision, about 10 mm long, was cut in the left midscapular line followed by incision of the abdominal wall and peritoneum. The caudal end of the spleen was carefully exposed, lying on fat and pancreatic tissue. After a sterile nitrocellulose strip (1 x 2 mm) containing 20 μg of DON-HS-BSA was deposited into the spleen by using a 20 gauge needle, the spleen was returned to the abdominal cavity. The abdominal wall and the skin were sutured separately with sterile thread. After two and four weeks, the mice were given the second and third immunizations by IP and SC injections at a dose of 10 and 50 μg of the

same conjugate/mouse, respectively. Ten days after each injection, the mice were bled and the serum titers as well as specificity were determined by indirect ELISA.

Rabbit immunization

Three female New Zealand rabbits (Charles River Laboratories, Wilmington, MA) were given a ten site subcutaneous (sc) injection with 500 μg/rabbit DON-HS-BSA conjugate which has been emulsified with Freund's "complete" adjuvant (Difco Laboratory, Detroit, Michigan) at a ratio of 1:1 (vol./vol.). Four and ten weeks later, the rabbits were boosted with the same immunogen but at half the dose. Freund's "complete" adjuvant was replaced with Freund's "incomplete" adjuvant (Difco Laboratory, Detroit, Michigan) for boosting. Another three female New Zealand rabbits were given a two site intramuscular injection with 100 μg of BSA-HS-DON conjugate per rabbit using Hunter's Titer Max™ (CytRx® Corporation, Norcross, GA) as an adjuvant. Four weeks later, a booster injection was performed exactly the same as the first injection. Every other week after the booster injection, rabbits were bled from lateral marginal ear vein. Serum was obtained after overnight incubation of the blood at 4^0C and centrifugation at 1,000g for 15 minutes. Rabbit immunoglobulins were purified by ammonium sulfate precipitation according to the procedure of Hebert et al. (1973) prior to determination of serum titer and antibody specificity with direct ELISA techniques.

ELISA

Both direct and indirect ELISA were similarly performed as described in Materials and Methods of Part II with the exception of toxin-OA conjugate, toxin-HRP conjugate and blocking solution. In DON ELISAs, DON-HS-OA conjugate, instead of FB_1 -OA conjugate, was used for coating microtiter wells in indirect ELISA; DON-HS-HRP conjugate, instead of FB_1 -HRP conjugate, was used as a labeled mycotoxin in direct ELISA; and 1% (wt/vol) of ovalbumin, instead of 1% (wt/vol) BSA, in PBS 0.01 M, pH 7.2 was used to block non specific binding in both direct and indirect ELISAs.

The titer of each serum was arbitrarily chosen as the maximum dilution that yielded twice or greater absorbance as the same dilution of nonimmune control serum. Each serum was tested for cross-reactivity with 3-acetyl-DON, 15-acetyl-DON, nivalenol, 4, 15-diacetylnivalenol, and fusarenone-X.

RESULTS

DON derivatization

To introduce a carboxyl group into DON molecules, DON was reacted with succinic anhydride. The end point of DON derivatization was monitored by TLC methods after 2, 4, 6, and 8 hours of derivatization reaction. DON and its derivatives were visualized with $AlCl_3$ and 4(p-nitrobenzyl)-pyridine reagents, and both compounds were positive (appeared as a blue fluorescence spot) on the TLC plates. This indicated that the derivative compound still possessed both keto and epoxy moieties because $AlCl_3$ is specific for trichothecenes having 7-hydroxy-8-keto side-chain residues (Kamimura et al., 1981) and 4(p-nitrobenzyl)-pyridine was specific to compounds containing an epoxy group (Takitani et al., 1979). The DON derivative ran as a single spot on the silica gel TLC plates developed in a solvent system of ethylacetate/ n-hexane/acetic acid at ratio of 75 : 25 : 5 (vol./vol./vol.). Retention fraction (Rf) values for DON and its derivative (HS-DON) were 0.67 and 0.33, respectively. After heating for 8 hours, almost all DON was already converted to HS-DON as indicated by a single spot on TLC plates at a Rf value of 0.33.

Mouse immunization

Twelve mice were intraperitoneally (IP) and subcutaneously (SC) immunized with BSA-HS-DON conjugate at a dose of 50 and 100 μg/mouse. The mice were then given four booster injections with the same conjugate. Ten days after each booster injection, the mice were bled from their tail vein and the serum titer and specificity were determined by indirect ELISA. All mice produced apparent high titers of antibodies (Figure 3.1) but their binding to solid-phase bound DON could not be inhibited by as much as 20μg/ml of DON. These antibodies also did not cross-react with either DON, 3-acetyl-DON, 15-acetyl-DON, nivalenol, 4, 15-diacetylnivalenol, or fusarenone-X.

Another 8 mice were immunized with 20 μg of BSA-HS-DON conjugate per mouse via intrasplenic deposition. Two and four weeks later, the mice were given the second and third immunizations by IP and SC injections at a dose of 10 and 50 μg of the same conjugate/mouse, respectively. Ten days after each injection, the mice were bled and the serum titers as well as specificity were determined by indirect ELISA. All of the animals could also produce relatively high titer antibodies (Figure 3.2). However, when these antibodies were used in indirect ELISAs, they could not detect DON although at high concentration (up to 20μg/ml). In addition, these antibodies were neither cross-reactive with 3-acetyl-DON, 15-acetyl-DON, nivalenol, 4, 15-diacetylnivalenol, nor with fusarenone-X.

Rabbit immunization

Three rabbits were given a two site intramuscular injection with 100μg of BSA-HS-DON conjugate/animal using Hunter's Titer Max™ (Hunter's adjuvant). Another three rabbits were given a ten site subcutaneous injected with BSA-HS-DON immunogen using Freund's adjuvant. The animals were then given two booster injections. Every two weeks after each booster, the rabbits were bled and the antibodies were analyzed by direct ELISA to determine their titers and specificities. All rabbits produced antibodies, but use of Freund's adjuvant resulted in higher titer antibodies than that of Hunter's adjuvant (Figure 3.3). Similar to mouse antisera, these rabbit antisera could not recognize free DON. They were also not cross reactive with 3-acetyl-DON, 15-acetyl-DON, nivalenol, 4,15-diacetylnivalenol or fusarenone-X.

DISCUSSION

DON derivatization

The results of DON derivatization were similar to that of Usleber et al. (1994) who also reported that all DON was already converted to its derivatives after a reaction of 8 hours with succinic anhydride. Casale et al. (1988) have analyzed the toxin derivatives by TLC and FAB-MS methods. They did not detect DON, a Rf value of 0.84, but found at least three compounds (Rf values of 0.33, 0.60, and 0.68) when the derivative compounds were spotted on silica gel TLC plates developed in a solvent system of chloroform:methanol (1:1) and visualized with 4(p-nitrobenzyl)-pyridine reagents. The slowest migrating compound (Rf value of 0.33) gave the most intense spot of the three. After analyzing the derivatives with FAB-MS methods, the authors concluded that the three compounds (Rf values of 0.33, 0.60, and 0.68) were dihemisuccinic-DON, 3-hemisuccinic-DON, and other isomer of hemisuccinic, respectively. These derivative compounds were then conjugated to BSA and used as immunogen.

Antibody production

Despite use of Hunter's and Freund's adjuvants and intrasplenic, intraperitoneal, subcutaneous and intramuscular immunization techniques, all of 20

female BALB/c mice and 6 female New Zealand rabbits that were immunized with BSA-HS-DON conjugate were not able to produce antisera that could be used in a competitive ELISA for DON. Difficulty producing DON antibodies was also reported by several authors. Zhang et al. (1986) were also unsuccessful in producing DON antibodies after immunizing rabbits with BSA-3-O-hemisuccinyl-DON conjugate as well as with BSA-8-O-carboxymethyl-oxime-DON conjugate (Figure 3.4). Initially, Usleber et al. (1994) were also unable to produce DON antibodies after they immunized rabbits, mice and guinea pigs with human serum albumin-8-O-carboxymethyl-oxime-DON conjugate and keyhole limpet hemocyanin-hydrazone-DON conjugate. However, after injecting three female Chinchilla bastard rabbits with human serum albumin-HS-DON conjugate, Usleber et al. (1994) found one rabbit which produced specific DON antibodies. Thus, it is possibly antibodies to DON may be produced in rabbits other then the New Zealand White strains.

Previously, Casale et al. (1988) successfully produced DON monoclonal antibodies after they immunized mice with BSA-3-HS-DON which was derivatized by protecting C7 and C15 during esterification with succinic anhydride (Figure 1.5). It seemed that the conjugation site to the carrier protein, rather than the immunization techniques (intrasplenic, intraperitoneal, subcutaneous, or intramuscular immunizations) was very critical for the production of DON specific antibodies. Thus, the results of this study did not support this my hypothesis.

CONCLUSION

DON derivatives having epoxy and keto functional groups were readily prepared by reacting DON with hemisuccinate. After a reaction of eight hours, DON was completely converted to its derivatives. These toxin derivatives were conjugated to BSA, and then emulsified with Hunter's or Freund's adjuvants prior to animal immunization. Both adjuvants could induce the production of high titer antisera. However, these antisera could not be used in competitive ELISAs for DON and did not cross react with either 3-acetyl-DON, 15-acetyl-DON, nivalenol, 4,15-diacetylnivalenol or fusarenone-X.

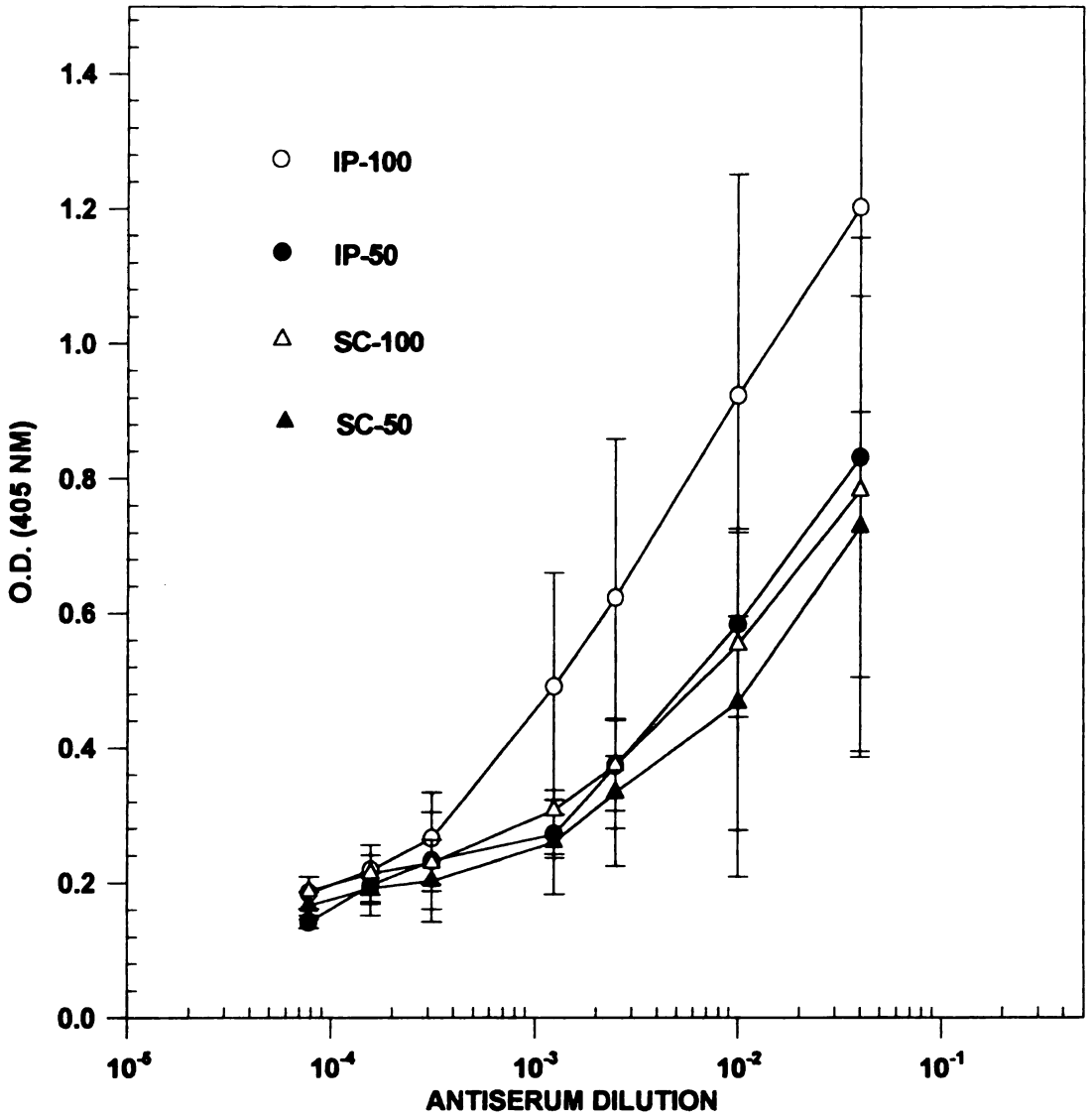


Figure 3.1. Indirect ELISA titration of mouse antibodies obtained ten days after the third injection with BSA-HS-DON conjugate. The first injection was given at a dose of 50 and 100 μg/mouse. The second and third injections were given at a dose of 25 and 50 μg/mouse. Each data point represents the mean ± standard error of the mean (n = 6, duplicate measurements from three mice). IP indicates peritoneal injection. SC indicate subcutaneous injection. 50 and 100 indicate dose (μg) of the first injection.

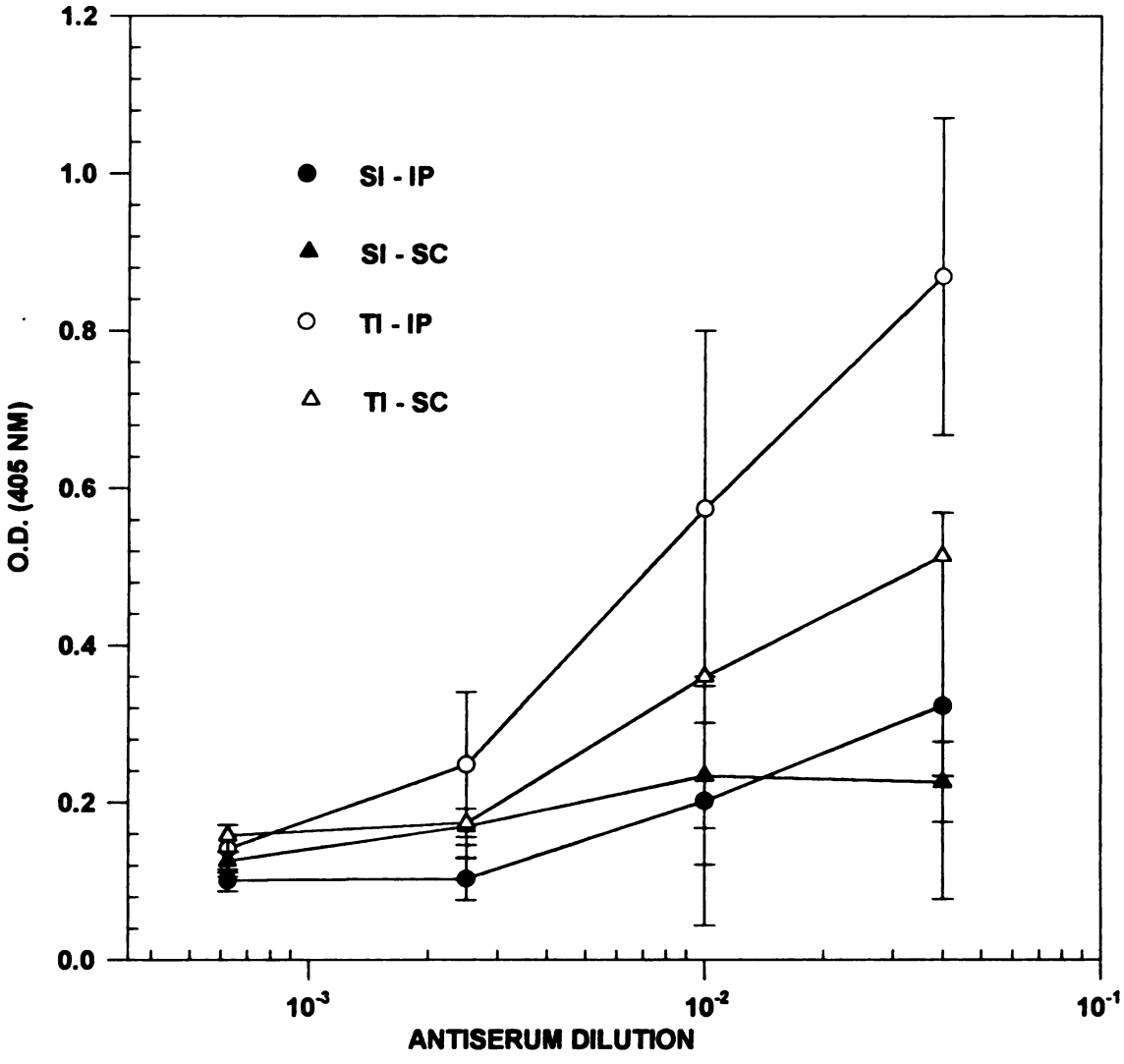


Figure 3.2. Indirect ELISA titration of mouse antibodies obtained ten days after the second (SI) and third immunizations (TI) with BSA-HS-DON conjugate. The first immunization was given via intrasplenic deposition at a dose of 20 μg/mouse. The second and third immunization were given via intraperitoneal (IP) and subcutaneous (SC) injections at a dose of 10 and 50 μg, respectively. Each data point represents the mean ± standard error of the mean (n = 8, duplicate measurements from four mice).

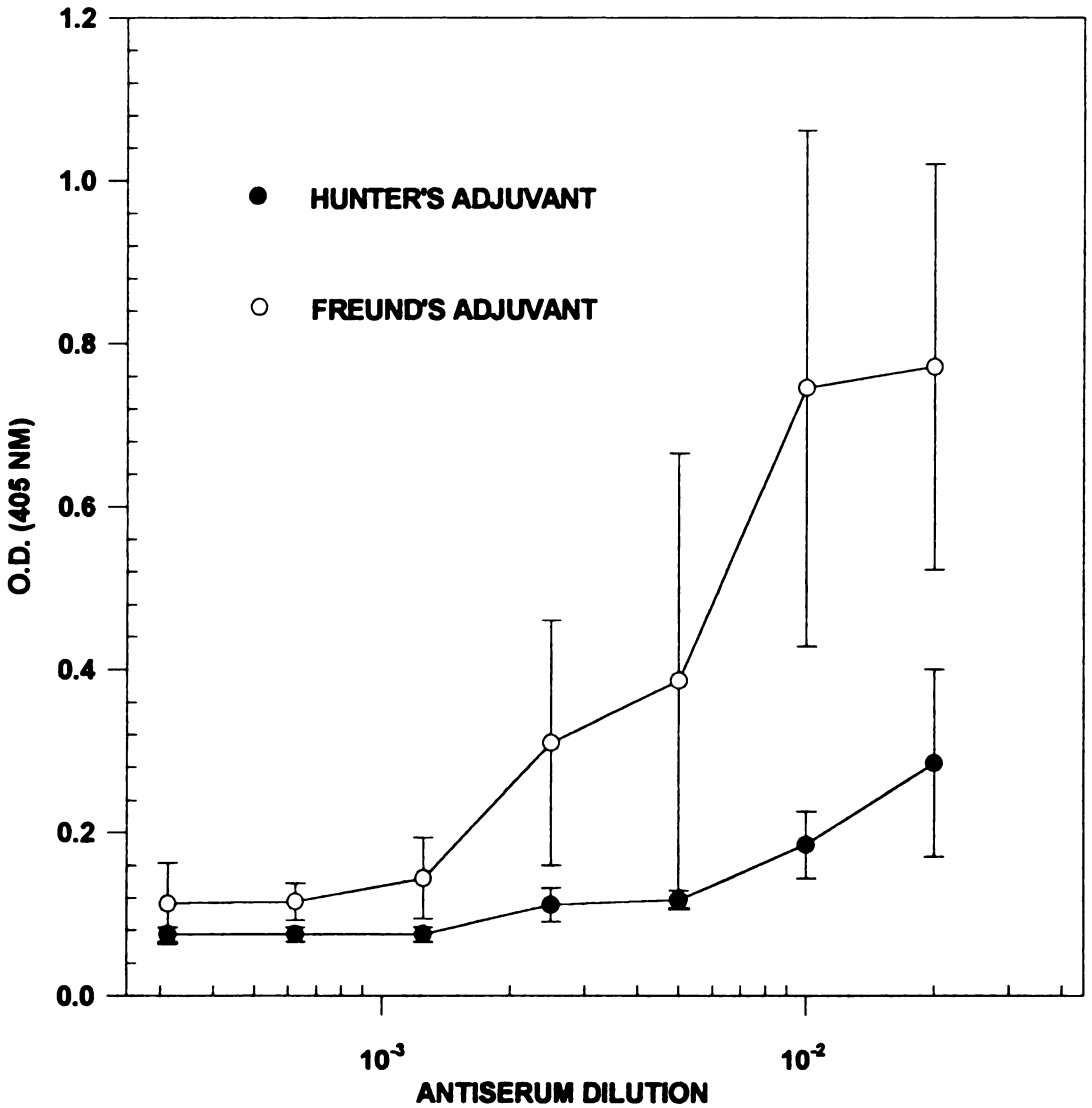
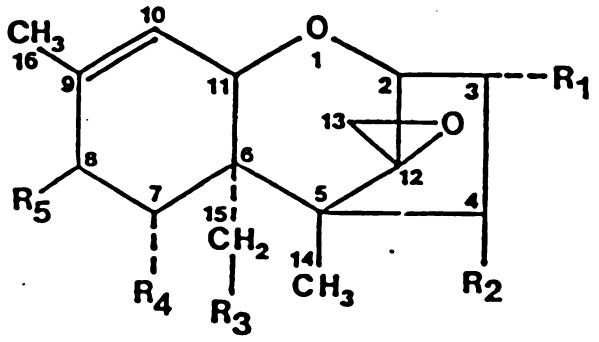


Figure 3.3. Direct ELISA titration of rabbit antisera. Hunter's adjuvant indicates that rabbits were given a two sites intramuscular injection with an emulsion of Hunter Titer Max and BSA-HS-DON conjugate at a dose of 100 μg per rabbit for both the first and second injections. Freund's adjuvant indicates that rabbits were given a ten site subcutaneous injection with an emulsion of Freund's adjuvant and BSA-HS-DON conjugate at a dose of 500 μg and 250 μg per animal for the first and second immunization, respectively. Antisera were obtained four weeks after the second injection with BSA-HS-DON conjugate. Each data point represents the mean ± standard error of the mean (n = 6, duplicate measurements from three rabbits).



Compound	R1	R2	R3	R4	R5
DON	OH	H	OH	OH	=O
A	O-HS-BSA	H	OH	OH	=O
B	OH	H	OH	OH	-O-CMO-BSA

Figure 3.4. Deoxynivalenol (DON) and its conjugates. BSA-3-O-hemisuccinyl-DON (A) and BSA-8-O-carboxymethyl-oxime-DON (B). BSA, HS, and CMO indicate bovine serum albumin, hemisuccinyl, and carboxymethyl-oxime, respectively.

PART IV.

PRODUCTION OF ANTIBODIES AGAINST TRICHOTHECENE-YEAST RIBOSOMAL BINDING SITE

ABSTRACT

PRODUCTION OF ANTIBODIES
AGAINST TRICHOTHECENE-YEAST RIBOSOMAL BINDING SITE

By

Sutikno

Both pure 60S and 80S ribosomal subunits were isolated from trichodermin-sensitive yeast and used for the production of antibodies against trichothecene ribosomal binding site. Twelve female BALB/c mice were immunized with either the 60S or 80S ribosomal subunits. Another ten female BALB/c mice were injected with the mixture of the 80S subunits with different adjuvants. All mice apparently produced high titer antisera. These antisera were used to develop a competitive indirect enzyme-linked immonosorbent assay (CI-ELISA) for detection of trichothecenes. However, this CI-ELISA could not detect trichothecene because the antisera were not specific to the trichothecene ribosomal binding site.

INTRODUCTION

Trichothecene mycotoxins

Trichothecenes are a group of structurally similar sesquiterpenoid metabolites produced mainly by toxigenic strains of *Fusarium,* and by some toxigenic strains of *Trichothecium, Trichoderma, Acremonium, Cylindrocarpon, Myrothecium and Stachybotrys* (Bamburg, 1983; Ueno, 1983; Committee on Protection Against Mycotoxin, 1983; Betina, 1989). These mycotoxins, especially deoxynivalenol (DON) and nivalenol (NIV) are found as natural contaminants in agricultural commodities throughout the world (Tanaka et al., 1988a). The presence of DON and NIV trichothecenes in cereal and cereal products in several countries has been surveyed and the results were summarized in Table 1.3. Trichothecenes can cause a wide range of toxicoses to both animals and human (Table 1.4) and cause large economic losses to farmer and livestock producers (Table 1.1).

The presence of trichothecenes in agricultural products in the field and during storage is mainly dictated by environmental factors and less importantly by genetic factors (Figure 1.1). Controlling the climatic and biological factors that contribute to the presence of the mycotoxins in agricultural commodities is almost impossible (Pestka, 1988). Thus, detection and diversion are the most important means for preventing the mycotoxin entry into food chains.

Immunoassay methods to detect the presence of mycotoxins in agricultural commodities have recently gained wide acceptance because of sensitivity, specificity, simplicity, and rapidity (Chu, 1991; Pestka et al., 1994a). Immunoassays for individual trichothecenes, such as T-2 toxin, DON, diacetoxyscirpenol, NIV, and roridin A, have been developed to date (Table 1.5). Immunoassay kits for T-2 toxin, and DON have also been produced and marketed in the United States (Table 1.6). However, immunoassays that are capable of detecting trichothecenes as a group in agricultural commodities have not yet developed.

Ribosomal binding as a toxicity mechanism

Trichothecenes are potent inhibitors of eukaryotic protein synthesis (Bamburg and Strong, 1971) because they bind to a common site on the 60S ribosomal subunit (Carrasco et al., 1973; Barbacid, and Vazquez, 1974; Cundliffe et al., 1974; Schindler et al., 1974; Cannon et al., 1976; McLaughlin et al., 1977). Occupancy of this binding site by some trichothecenes, such as T-2 toxin and NIV results in inhibition of protein synthesis at an initiation stage (Smith et al., 1975), whereas other trichothecenes including trichodermin lead to block elongation and termination stages by interfering with the peptidyl transferase on the ribosomes (Cundliffe et al., 1974).

In order to elucidate the binding site, Schindler et al. (1974) induced mutations in a trichodermin-sensitive yeast strain (*Saccharomyces cerevisiae* A224A) and then isolated a trichodermin-resistant strain (S. *cerevisiae* CLP-1). By comparing the ribosomal components from both strains, these authors reported that

a mutant, which was resistant to the action of trichodermin, has an altered component on its 60S ribosomes. The gene determining the altered 60S ribosomal component is a single, recessive nuclear gene located on the right arm of chromosome XV and named *tcm1* (Grant et al., 1976). By cloning yeast genes for trichodermin resistance and ribosomal protein L3 (the largest ribosomal protein), Fried and Warner (1981) isolated *tcm1* gene and found that the gene coded for ribosomal protein L3. The nucleotide sequence of this *tcm1* gene (ribosomal protein L3) has also been determined (Schultz and Friesen, 1983).

The binding characteristics between trichothecenes and eukaryotic ribosomes have been studied by several investigators. Jimenez and Vazques (1975) used [acetyl-^{14}C] trichodermin to study quantitative binding of trichodermin to ribosomes from trichodermin sensitive(Y_{166}) and trichodermin resistant (TR_1) yeasts. They found that Y_{166} ribosomes bound to trichodermin with a dissociation constant (Kd) of 0.99 μM while those from the resistant one (TR_1) bound to trichodermin with a Kd of 15.4 μM. Using similar techniques, Barbacid and Vazquez (1974) found that trichodermin bound to the peptidyl transferase center of trichodermin-sensitive yeast ribosomes with a Kd of 1.8 μM, and to 60S subunit of the yeast ribosome with Kd of 1.4 μM. Cannon et al. (1976) studied the binding affinity of the same toxin to different states of the sensitive yeast ribosomes (polyribosome and "run off" ribosome). They found that the binding affinity between trichodermin and the "run off ribosome (Kd = 0.72 μM) is approximately threefold higher than that between trichodermin and the polyribosome (Kd = 2.1 μM).

Factors affecting binding of trichothecenes and eukaryotic ribosomes have also been studied. Changes in ionic concentrations (from 0 to 300 mM of ammonium ion; from 0 to 25 mM of $MgCl_2$), pH (from 6 to 8), and ethanol concentration (from 0 to 40% vol./vol) do not significantly affect the binding between trichodermin and the peptidyl transferase center of yeast ribosomes although the activity of the center is much affected by ionic concentrations (Barbacid and Vazquez, 1974). Time and temperature of the reaction greatly affect the binding between T-2 toxin trichothecene and the yeast ribosomes (Middlebrook and Leatherman, 1989). When incubated at 37^0C, the association of ^{3}H-T-2 with ribosome is biphasic. In the initial phase, binding is rapidly increased and by 30 minutes a plateau equilibrium state is achieved. After that a second phase occurs in which the binding gradually decreases to the base line value at a half-time of 2.5 hours. However, ^{3}H-T-2-ribosome binding at 4^0C is much slower, taking 24-28 hours to reach completion, and very stable, requiring 3 weeks to decay 50%. When T-2 is prebound to ribosomes at 4^0C and then incubated at 37^0C, a significant degradation process begins after a time lag of approximately 4 hours (Middlebrook and Leatherman, 1989).

A ribosome has only one binding site and can bind to only one molecule of trichothecene (Barbacid and Vazquez, 1974; Middlebrook and Leatherman, 1989). Trichothecenes as well as anisomycin compete with each other for the ribosomal binding site (Barbacid and Vazquez, 1974; Cannon et al., 1976; Middlebrook and Leatherman, 1989). Interestingly, competition among trichothecenes for the binding

site appears to be proportional to their toxicity (Committee on Protection Against Mycotoxin, 1983; Middlebrook and Leatherman, 1989).

Rationale and objective

Trichothecenes bind specifically to yeast ribosomes. These toxins compete with each other for the ribosomal binding site in proportion to their toxicity. Production of antibodies specific to the binding site would enable researchers to develop ELISAs which can assess total "trichothecene load" in agricultural commodities and , thus would enhance food safety.

Maximum binding between trichothecenes and yeast ribosomes is achieved by 30 minutes when incubated at 37^0C. Thus, developing indirect ELISA using yeast ribosomes for coating microtiter plates (Figure 4.1) might be feasible. In such an assay, trichothecenes and trichothecene-ribosomal binding site antibodies would be incubated together over a solid-phase ribosomal binding site. Trichothecenes would compete with the antibodies for the ribosomal binding site. The total bound antibodies could be then detected by a second antibody which has been labeled with an enzyme. After the addition of an appropriate enzyme substrate, the bound antibody could be calculated based on the intensity of color development which is measured spectrophotometrically. The "trichothecene load" would be inversely related to a complex of bound enzyme-labeled antibodies.

Based on the above literature study, I hypothesized that antibodies against trichothecene-yeast ribosomal binding site could be generated by immunization mice with either 60S or 80S yeast ribosomes. The specific objectives of this study were to

produce antibodies against trichothecene-yeast ribosomal binding site and use these antibodies to develop indirect ELISA for trichothecenes as a group.

MATERIALS AND METHODS

Chemicals and reagents

All organic solvents and inorganic chemicals were of reagent grade or better. Trichodermin sensitive *S. cerevisiae* (ATCC 46913) was obtained from American Type Culture Collection (Rockville. Maryland). This strain was maintained at 30^0C on YEPD plates and grown in liquid medium at this temperature. Liquid YEPD media were prepared by autoclaving of a mixture solution of 10 g of yeast extract (Difco Laboratories, Detroit MI), 20 g of bacto-peptone (Difco 0118, Difco Laboratories, Detroit MI), and 20 g of dextrose in 1 liter of distilled water. Ovalbumin (OA) (chicken egg albumin grade III; fraction VII), Tween 20, 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)(ABTS), hydrogen peroxide, T-2 toxin, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). Goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate was obtained from Cappel Laboratories (West Chester, PA). Tritium labeled T-2 toxin (3H-T-2) was kindly supplied by Dr. Fun Sun Chu (University of Wisconsin, Madison, Wisconsin). Methofane (methoxyflurane, inhalation anesthetic for veterinary) was purchased from Pitman-Moore Inc. (Mundelein, IL).

Yeast production

Yeast was grown by the procedure of Treadgill et al. (1986). Briefly, colonies were picked from plates and inoculated into several 10 ml liquid YEPD media (cultures). After 10-18 hours of growth, four of these cultures were used to inoculate four separate 150 ml cultures, which were incubated at 30^0C with slow (approximately 150 rpm) shaking overnight. These cultures were examined microscopically for possible bacterial contamination and two were selected and used to inoculate two separate two liter cultures. These cultures were incubated at 30^0C with slow shaking until the cell solution reached an A_{600} of 2.0. The grown culture was then cooled slowly to 8^0C to induce ribosome run-off. Cells were harvested and washed twice in distilled water with centrifugation at 10,000 g for 5 minutes at 2^0C.

Ribosome preparation

Yeast ribosomes were prepared by the procedure of Battaner and Vazquez (1971) with slight modification. Briefly, each yeast preparation (approximately 100 g wet weight of yeast cells) was resuspended in 300 ml of buffer containing 100 mM-Tris/HCl. pH 7.4, 12.5 mM-magnesium acetate, 80 mM KCl, and 5 mM dithiothreitol. Cells were then disrupted by four passages through a French Pressure Cell (Aminco) at 700 lbf/in^2 (48.3 MPa), and the lysates were centrifuged at 20,000g for 15 minutes at 2^0C. The supernatant fractions were then centrifuged again at 20,000g for 30 minutes at 4^0C to clear any remaining debris and kept at 4^0C before ultracentrifugation.

Clean supernatant fractions were sedimented by centrifugation in a Sorvall RC-60 ultracentrifuge (Du Pont Company, Willington, Delaware) using T865.1 rotor at 45,000 rpm (150,000g) for 120 minutes. For washing, the pellet (crude ribosomes) was suspended in 24 volumes of washing buffer (10 mM Tris-HCl buffer, pH 7.4, 500 mM NH_4Cl, 100 mM Mg acetate, 5 mM dithiothreitol). An aliquot (5 ml) of ribosomes was layered on 6 ml of a discontinuous sucrose gradient in washing buffer (3 ml 20%, w/v sucrose in the bottom and 3 ml 5%, w/v in the top) and then sedimented by centrifugation at 150,000g for 8 hours. The ribosome pellet was resuspended in about 25 volumes of the standard buffer (10 mM Tris-HCl buffer, pH 7.4, 50 mM NH_4Cl, 5 mM Mg acetate, 5 mM dithiothreitol). After centrifugation at 10,000g for 10 minutes, the pellet was discarded, and the supernatant containing the 80S ribosomes was finally adjusted with the standard buffer to give a concentration of approximately 30 mg ribosomes/ml, aliquoted, and stored at -80^{0}C.

For preparation of 60S ribosome subunits, 1 ml of the 80S ribosomes was dialyzed for 2 hours against two liter of dialyzing buffer (10 mM Tris-HCl buffer, pH 7.4, 50 mM NH_4Cl, 0.2 mM magnesium acetate, 5 mM dithiothreitol) at 4^{0}C. Linear sucrose gradients were prepared by dissolving sucrose in the dialyzing buffer and slowly layered into 11-ml tubes of the T865.1 rotor (Du Pont Company, Willington, Delaware). The linear gradient consisted of 1 ml of each (from bottom to top) 25%, 20.5%, 17.0%, 13.0%, 9.5% and 7% sucrose (% sucrose was measured with ABBEL-3L refractometer, Milton Roy Co., Rochester, NY). Three ml of 4% sucrose in dialyzing buffer, 1 ml of ribosome solution (15 mg/ml), and then 1 ml of the dialyzing buffer were gently layered onto this linear gradient.

After 4 hours of centrifugation at 122,000g (40,000 rpm of the T865.1 rotor), the supernatant was fractionated and their absorbency was measured using Spectronic 601 spectrophotometer (Milton Roy Co., Rochester, NY). The pellet (containing enriched 60S subunits) was resuspended in the dialyzing buffer (5 ml) and centrifuged at 101,800g for 3.5 hours. The resultant supernatant was discarded, and the 60S subunit pellet was rinsed and resuspended in standard buffer, aliquoted and stored at -80^{0}C.

T-2 toxin-ribosome binding assay

Ethanol precipitation techniques used to determine T-2 toxin-ribosome binding were performed based on the procedure of Fernandez-Munoz et al. (1971) with slight modification. Briefly, 100 µl of ribosome solution (0.4 µg ribosome/ml of 10 mM Tris-HCl buffer, pH 7.4, 50 mM NH_4Cl, 5 mM Mg acetate, 5 mM dithiothreitol) was mixed with 10 µl of standard buffer (10 mM Tris-HCl buffer, pH 7.4, 50 mM NH_4Cl, 5 mM Mg acetate, 5 mM dithiothreitol) and 10 µl of ^{3}H-T-2 solution (0.2 µCi/ml). After incubation at room temperature for 30 minutes, this mixture was mixed with 60 µl precooled absolute alcohol and then incubated at 4^{0}C for 30 to 90 minutes. Ribosome-bound ^{3}H-T-2 was separated from the solution by centrifugation at 3000g for 20 minutes at 4^{0}C. One hundred (100) µl of the supernatant was carefully removed and mixed with 4 ml of scintillation cocktail (Research Products International Corp.) into scintillation vials. Radioactivity was determined by liquid scintillation counting using a Packard TRICARB 4430 Liquid Scintillation System (Packard Instrument Inc., Downers Grove, IL).

The resultant value provided an estimate for the concentration of the labeled toxin in free solution at equilibrium. For control, the total radioactivity was determined in duplicate samples in which ribosomes were omitted. The difference between the total radioactivity and the radioactivity in free solution gave an estimate for the concentration of ribosome-bound compound.

Competitive binding assay was used to determine whether nonradiolabeled T-2 toxin or mouse sera competed with tritium labeled T-2 toxin for trichothecene-ribosome binding site. Briefly, 100 μl of ribosome solution (0.4 μg ribosome/ml of standard buffer) was mixed with 10 μl of serially diluted (0-1000 ng/ml) T-2 toxin standard solution or 10 μl of appropriately diluted mouse sera. After incubation at room temperature for 30 minutes, 10 μl of ^{3}H-T-2 solution (0.2 μCi/ml) was added and the mixture was incubated for another 30 minutes at room temperature. The binding assay was then completed as described above.

Mouse immunization

Twelve female BALB/c mice (6 to 8 weeks of age, Charles River Laboratories, Wilmington, MA) were immunized by intraperitoneal (i.p.) and subcutaneous (s.c.) routes. The mice were immunized four times with an emulsion of 60S or 80S subunit of yeast ribosomes with Freund's adjuvant at a ratio of 1:1 (vol./vol.) at two-week intervals. The first immunization was performed at week zero at a dose of 50 μg ribosome/mouse and the ribosome was emulsified with Freund's complete adjuvant. The second, third and fourth injections were performed at week 2, 4 and 6 at a dose of 25 μg

ribosomes/mouse and Freund's incomplete adjuvant was used to emulsify the ribosomes. Ten days after the second, third and fourth immunizations, methoxyflurane-anesthetized mice were bled from the tail vein and the blood was collected with a heparinized tube. The blood was incubated at 4^0C overnight and then centrifuged at 1000g for 15 minutes to obtain mouse plasma. Antibody titer and specificity were then determined by indirect ELISAs as described below.

Indirect ELISA.

Indirect ELISA was performed by a modification of the procedure of Azcona-Olivera et al. (1992a) and used to determine serum titers. Briefly, wells of polystyrene microtiter plates (Immunolon 4, Dynatech Laboratories, Alexandria, VA) were coated overnight (at 4^0C) with 100 µl of ribosomes (5 µg/ml) in standard buffer (10 mM Tris-HCl buffer, pH 7.4, 50 mM NH_4Cl, 5 mM Mg acetate, 5 mM dithiothreitol). Plates were washed four times by filling each well with 300 µl of the standard buffer and aspirating the contents. Nonspecific binding was blocked by filling the wells with 300 µl of 1% (wt/vol) ovalbumin in the standard buffer. After incubating for 30 minutes at 37^0C, the plates were washed four times with the standard buffer. Fifty µl of serially diluted mouse serum was added to each well and incubated at 37^0C for 30 minutes. Wells of serially diluted preimmune serum were used as control. Unbound antibodies were removed by washing four times with the same buffer and 100 µl of goat anti-mouse IgG peroxidase conjugate (2 µg/ml of the ovalbumin solution) was added to each well. The plates were incubated for 30 minutes at 37^0C, washed eight times with the standard buffer

and then rinsed twice with distilled water. Bound peroxidase was determined with ABTS substrate [1 ml of 35 mg 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)/15 ml distilled water mixed with 11 ml of citrate buffer pH 4 and 8 μl hydrogen peroxide] as described previously by Pestka et al., (1982). Absorbance at 405 nm was read with a Vmax Kinetic Microplate Reader (Molecular Devices Corporation, Menlo Park, CA). Titer of each serum was arbitrarily designed as the maximum dilution that yielded twice or greater absorbance as the same dilution nonimmune control serum.

A competitive indirect ELISA (CI-ELISA) was used to test the potential for T-2 to block the binding of antibody to yeast ribosomes. verify specificity of antibodies in sera toward T-2 toxin. Briefly, microtiter plates were coated and blocked as described in the indirect ELISA procedure, and then 50 μl of serially diluted (0-10,000 ng/ml) T-2 standard solution was simultaneously incubated with 50 μl of appropriate dilution of antibodies over the ribosome solid phase for 30 minutes at 37^0C. The assay was then completed as described above.

RESULTS AND DISCUSSION

Ribosome preparation

Four liter yeast cultures were prepared by inoculating trichothecene-sensitive yeast into 4 liter liquid medium and then incubating it at 30^0C with slow shaking for one week. These cultures were harvested by centrifugation at 10,000g for 5 minutes and resulted in approximately 80 g wet weight yeast cells. From these, approximately 50 mg of 80S ribosomal subunit and 30 mg of 60S ribosomal subunit were prepared. Ribosomal purity was checked by calculating the values of ribosomal absorbance at 260 nm (A_{260})divided by that at 280 nm (A_{280}). The A_{260}/ A_{280} values of the 80S and 60S ribosomal subunits were 2.08 and 1.95, respectively, indicating that the ribosomal subunits were pure since proteins including ribosomes are considered as pure or uncontaminated if the value of A_{260}/ A_{280} is greater than 1.80 (Spedding, 1990).

Tritium labeled T-2 toxin was used in ribosome binding assays. ^{3}H-T-2 was incubated with yeast ribosomes diluted in the standard buffer. As a control the same amount of the radiolabeled toxin and the standard buffer were mixed without the ribosomes. After separation of ribosome-bound ^{3}H-T-2 by centrifugation, radioactivity of the supernatant was measured. It was found that supernatant from ribosome supernatant had a lower radioactivity values (disintegration per minute, DPM) than that from the control solution indicating that some of the ^{3}H-T-2 bound to

the ribosomes. This suggested that the trichothecene-ribosomal binding site was still active. When these ribosomes were reacted with the radiolabeled toxin in the presence of free T-2 toxin, the association of the ^{3}H-T-2 to the ribosomes was inhibited by the free toxin (Figure 4.2). These ribosomes, which still had the capability to bind to T-2 toxin, were used to immunize female BALB/c mice.

Mice immunization

Initially, twelve mice were immunized and boosted intraperitoneally and subcutaneously with an emulsion of 80S or 60S yeast ribosomal subunits and Freund's adjuvant. Ten days after the booster injection, the mice were bled. The blood was incubated at 4^{0}C overnight and then centrifuged at 1000g for 15 minutes to obtained mouse plasma. The mouse antisera were analyzed by indirect ELISA to determine their titers and specificities. These antisera had high titers (Figure 4.3) indicating that they could recognized solid phase bound ribosomes. However, these antibodies could not inhibit the association of trichothecenes to yeast ribosomes when used in CI-ELISA. In addition, when these antibodies were incubated together with ribosomes and tritium labeled T-2 toxin during binding assay, they could not inhibit the association of the radiolabeled toxin to the ribosomes. Thus, these antibodies were not applicable for assays of "trichothecene load" in food samples.

In subsequent experiments, ten mice were immunized subcutaneously (sc) using 80S ribosomal subunit because in the previous experiments, sc immunization yielded higher titer antibodies (Figure 4.3). Percent inhibition of T-2 toxin to the association of tritium labeled T-2 toxin to 80S ribosomal subunit was similar to that to

the 60S one (Figure 4.2). Moreover, preparation of the 80S subunit was much simpler than that of the 60S one. In this immunization, the 80S ribosomal subunit was mixed either with T-2 toxin (T-2-80S), Freund's adjuvant (FA-80S), cholera toxin (CT-80S), or with a mixture of FA and CT (FACT-80S) prior to animal immunization. All mice apparently produced antisera with high titers (Figure 4.4). However, these antisera could not inhibit the reaction of trichothecenes and yeast ribosomes when they were used either in competitive indirect ELISA or in binding assays.

Animal immunizations with 80S yeast ribosomal subunits either alone or mixing with adjuvants could not induce the production of antisera that could be utilized in competitive inhibition ELISA for trichothecenes. This may relate to two possibilities. Firstly, trichothecene-ribosomal binding site might be modified during immunogen preparation or during metabolism in animal body, and thus antibodies that were specific to the binding site were not generated. Secondly, the antibodies specific to the binding site might have been produced by the immunized animals but in a small proportion as compared to non specific polyclonal antibodies. As a result, trichothecene inhibition to the specific antibodies were undetectable when these antisera were used in CI-ELISA for trichothecene detection. Thus, these results did not support the hypothesis of this current study.

Direct injection of specific proteins or specific oligopeptides for the binding site into animals, rather than "whole" ribosomes, might be capable to stimulate the desired specific antibodies against the binding site. The specific proteins for trichothecene-ribosomal binding site could be isolated by comparing ribosomal components from trichodermin-sensitive yeast to that from trichodermin-resistant

one. Schindler et al., (1974) found an alteration of 60S ribosomal subunit in the trichodermin-resistant mutant. The alteration was associated with ribosomal protein L3 which was coded by *tcm1* genes (Grant et al., 1976; Fried and Warner, 1981). Thus, injection ribosomal protein L3 from trichodermin-sensitive yeast into animals may be as an alternative to produce antibodies against the ribosomal binding site. Another alternative is immunization of specific oligopeptides for the binding site. The nucleotide sequence of *tcm1* gene that encodes ribosomal protein L3 in trichodermin-resistant yeast strains has been determined (Schultz and Friesen, 1983). By comparing this nucleotide sequence and the nucleotide sequence of a gene encoding protein L3 in trichodermin sensitive yeast strains, specific nucleotide sequence for trichothecene-ribosomal binding site could be isolated. From this sequence, polypeptides could be constructed *in vitro* and might be used for the production of specific antibodies against trichothecene-yeast ribosomal binding site.

CONCLUSION

Both pure 60S and 80S ribosomal subunits have been isolated from trichodermin-sensitive yeast and used for the production of antibodies which were specific to trichothecene ribosomal binding site. These ribosomal subunits (either alone or after mixing with different adjuvants) were used to immunize 22 female BALB/c mice. All mice apparently produced high titer antisera. However, these antisera were not able to inhibit the association of trichothecenes and yeast ribosomes when they were used either in CI-ELISAs or in binding assays for trichothecenes. Thus, the production of antibodies that were specific to trichothecene-ribosomal binding site could not be accomplished through animal immunization with either 60S or 80S subunits of yeast ribosomes

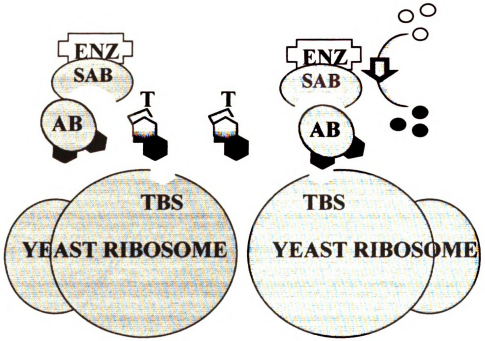


Figure 4.1. Competitive indirect ELISA for "trichothecene load" using yeast ribosomes for coating microtiter plates. Trichothecene ribosomal binding site specific antibodies (AB) compete with trichothecenes (T) for the trichothecene binding site (TBS). Second antibodies (SAB) which have been labeled with enzyme (ENZ) are then added to determined total bound antibody (AB). "Trichothecene load" is inversely related to the bound enzyme-labeled antibodies and can be measured quantitatively using spectrophotometer after color development by the addition of enzyme substrate.

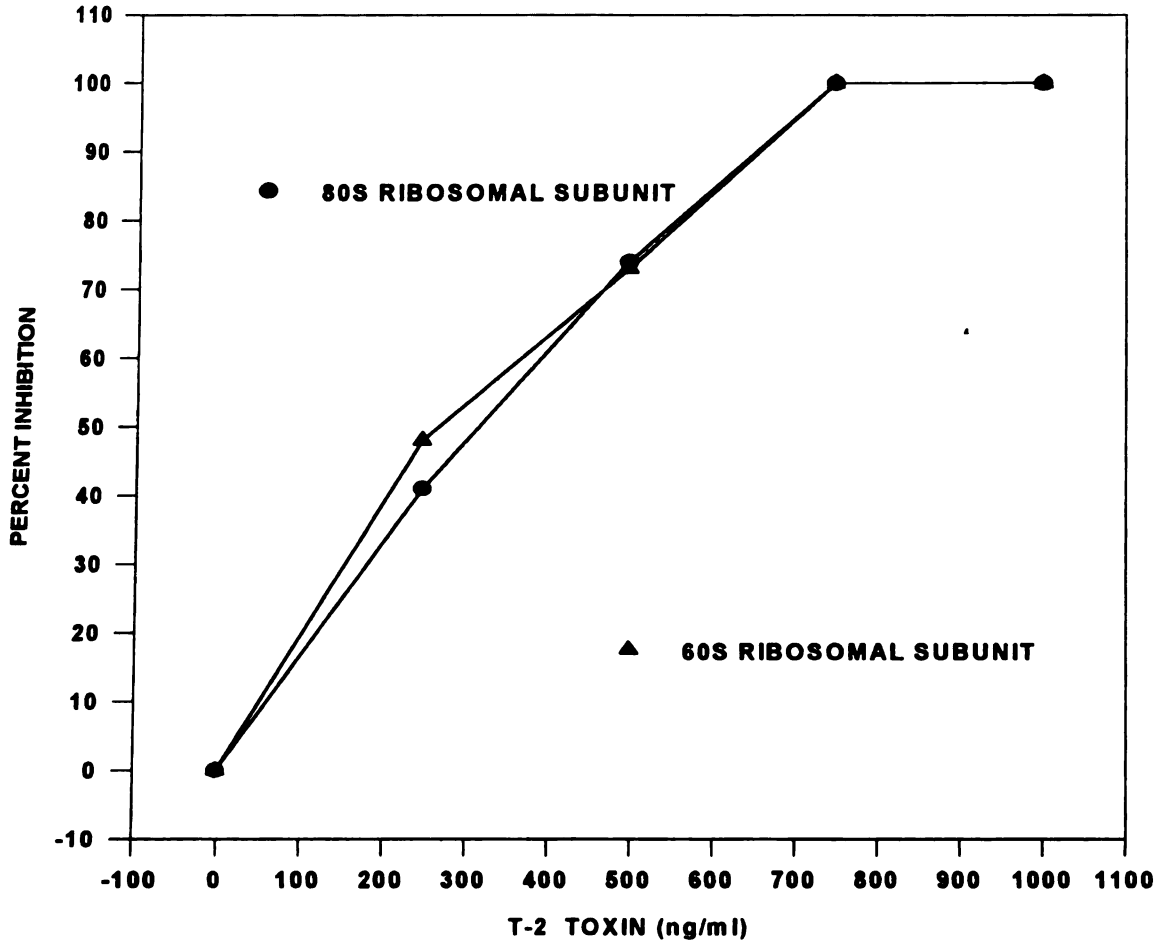


Figure 4.2. Inhibition of T-2 toxin to the association of ^{3}H-T-2 toxin to yeast ribosomes. Each data point represents the mean value of triplicate measurements. One hundred µl of yeast ribosomes (0.4 mg/ml standard buffer) were reacted with 10 µl of ^{3}H-T-2 toxin (0.2 uCi/ml) in the presence of 10 µl of serially diluted (0 to 1000 ng) non radiolabeled T-2 toxin and 60 µl of precooled alcohol. As a control the same amount of radiolabeled toxin was mixed with the standard buffer without ribosomes. After ribosome-bound ^{3}H-T-2 toxin was separated by centrifugation, radioactivity (DPM) of 100 µl of the supernatant was measured by liquid scintillation counting. DPM values of control solution and 0 ng/ml supernatant were 16,026 and 8,577, respectively. Percent inhibition = [(DPM value of certain ng T-2 toxin /ml supernatant - DPM value of 0 ng/ml supernatant)/(DPM value of control solution - DPM value of 0 ng/ml supernatant)] x 100%.

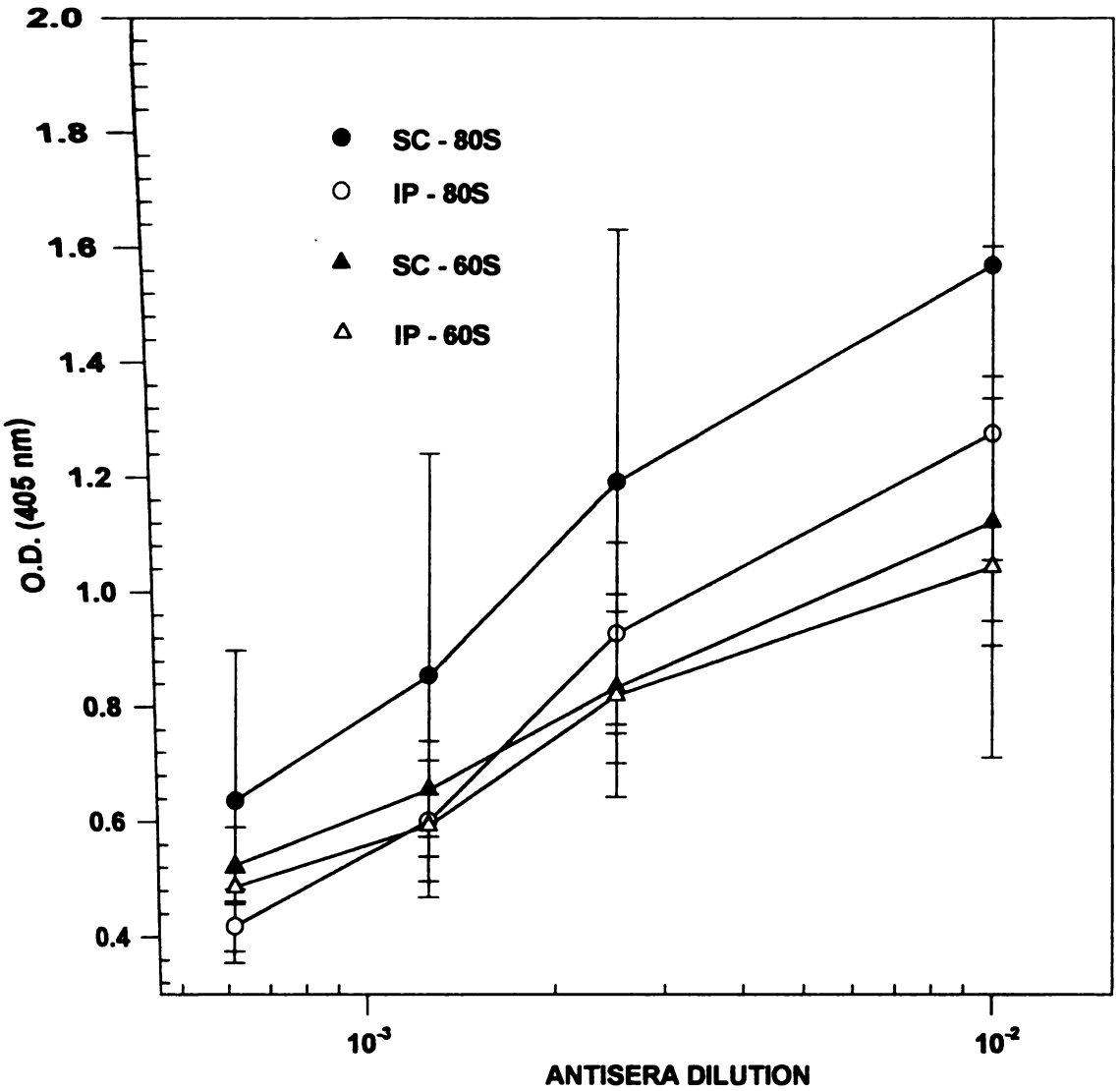


Figure 4.3. Typical indirect ELISA titration of mouse antisera. The mouse antisera were obtained ten days after the third subcutaneous or intraperitoneal injection with 50μg of 60S or 80S yeast ribosomal subunit emulsified with Freund's adjuvant. Each data point represents the mean ± standard error of the mean (n=6, duplicate measurements of three mice). IP, and SC indicate intraperitoneal and subcutaneous injections, respectively. 80S and 60S indicates 80S and 60S yeast ribosomal subunits, respectively.

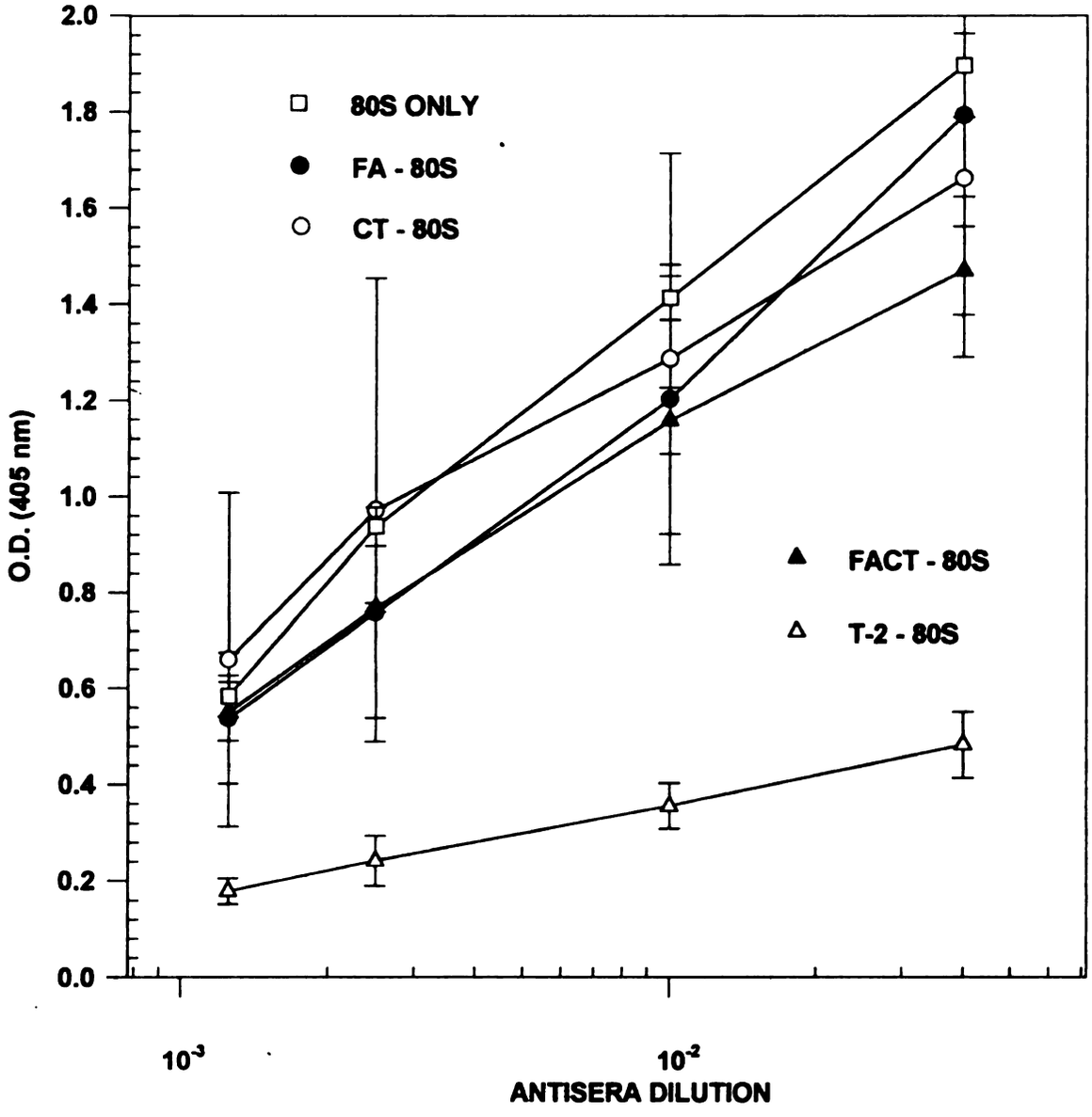


Figure 4.4. Typical indirect ELISA titration of mouse antisera. The mouse antisera were obtained ten days after the third subcutaneous injection with 50μg of 80S yeast ribosomal subunit mixed with different adjuvants. Each data points represents the mean ± standard error of the mean (n=4, duplicate measurements of two mice). 80S indicates 80S yeast ribosomal subunit. FA, CT, and T-2 indicate Freund's adjuvant, cholera toxin, and T-2 toxin, respectively.

APPENDICES

APPENDIX A:

MEDIA FOR HYBRIDOMA PRODUCING MONOCLONAL ANTIBODIES

I. Chemical and reagents

All organic solvents and inorganic chemical were of reagent grade or better. Penicillin/streptomycin solution (pen/strep) (100,000 units/ml), sodium pyruvate, polyethylene glycol (MW 1450) (PEG), hypoxanthine, aminopterin, thymidine, pristane, and dimethyl sulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM, in powder form for 1 l medium/bottle), NCTC supplemental medium, and fetal bovine serum (FBS) were obtained from Gibco Laboratories (Grand Island, NY). Tissue culture plasticware was purchased from Corning Laboratory Science Co. (Corning, NY). The myeloma cell line P3/NS1/1-Ag4-1 (NS 1) (ATCC TIB 18) was purchased from the American Type Culture Collection (Rockville, MD). Macrophage conditioned media (MCM) was prepared as described by Sugasawara et al. (1985).

II. MEDIA

Dulbecco's modified eagle's medium (DMEM)

One liter of distilled water was poured into a 2 liter Erlenmeyer flask. Powder of DMEM was added to the flash and stirred with a magnetic rod until the powder was dissolved. Sodium bicarbonate (3.7 g/l) was weighed and added so that the medium color changed from orange to red. pH of the medium was measured and adjusted to neutrality (pH 7.0) either with 1N NaOH or 1N HCl. Usually the medium was slightly basic so that 4-5 ml of 1N HCl was added. Prior to sterilization with filter sterilized (Nalgene disposable filter unit catalog number: 155-0020; volume: 150 mL; memb mat type: C.A./T.A.; color code: yellow; pore size: 0.2 mm), 10 ml of NCTC suplement and 10 ml of sodium pyruvate were added to the neutral medium so that the DMEM contained 1% NCTC and 10 mm sodium pyruvate. The sterilized DMEM medium was stored in a sterilized and labeled 500 ml bottle at 4^0C and tested for its sterility by incubating two separte 1 ml of the medium into wells of a 24-well plate at 37^0C in an incubator containing 5% CO_2 for 2 days. If the medium was contaminated by microorganims, the medium has to be refiltered, and then rechecked for its sterility again.

Maintenace medium

Twenty ml of fetal bovine serum (FBS), and 1 ml of Pen/Strep (P/S) solution were added to 80 ml of the sterilizied DMEM medium to make 20% FBS-DMEM. The 20% FBS-medium was filter sterilized (Nalgene disposable filter unit catalog number: 155-0020; volume: 150 mL; memb mat type: C.A./T.A.; color code: yellow; pore size: 0.2 mm) and stored at 4°C.

One hundred x stock solution of hypoxanthine-thymidine (HT)

One hundred x stock solution of HT was prepared by suspending 166.1 mg of hypoxanthine (H) and 38.75 mg of thymidine (T) in 50 mL of distilled water. The H and T were dissolved by adding (0.1N or 1.0N) NaOH dropwise until H and T were solubilized. The volume was adjusted to 100 ml with distilled water, filter sterilized, aliquoted in 10 ml, and stored at -20°C.

Fifty x stock solution of HT

Fifty x stock solution of HT was prepared by adding an equal volume of 100 x Stock solution HT to an equal volume of the sterilized DMEM and stored at 4°C.

Aminopterin stock solution

Aminopterin stock solution was prepared by adding 17.6 mg of aminopterin (A) to 100 ml of distilled water and stored at -20°C in aliquots of 10 ml/tube.

Fifty x Stock Solution of HAT

Fifty x Stock Solution of HAT was prepared by adding 50 ml of 100 x stock solution of HT and 5 ml of aminopterin stock solution to 45 ml of the sterilized DMEM, filter sterilized, and stored at 4°C in aliquots of 12.5 ml/tube.

HAT medium

HAT medium was prepared by adding 20 ml of FBS, 1.25 ml of P/S., and 2.4 ml of 50 x HAT stock to 100 ml of the sterilizied DMEM, filter sterilized, and stored at 4°C.

HT Medium

HT Medium was prepared by adding 20 ml of FBS, 1.25 ml of P/S, and 2.4 ml of 50 x stock solution of HT to 100 ml of the sterilized DMEM, filter sterilized, and stored at 4°C.

Macrophage Conditioned Medium (MCM)

Five female BALB/c mouse (6 to 8 weeks of age, Charles River Laboratories, Wilmington, MA) were sacrificed, dipped in 70% ethanol in water to sterilize their whole bodies, and peeled back their skin to expose their peritoneal lining. Ten ml of 20% FBS-DMEM was injected to mouse's peritoneal cavity using an 18 gauge needle. The peritoneal cavity was tapped several times to dissolve macrophage cells into DMEM which was then drew off using the same needle. The DMEM containing macrophage cells was centrifuged at 450 x g for 7 minuted. The cells (sediment) were resuspend in 40 ml of 20% FBS-DMEM, and placed into two large T-flasks (20 ml each). After incubation at 37°C in an incubator containing 5% CO_2 for 3 days, the medium was collected, and the cells were refed with 20 ml of fresh 20% FBS-DMEM medium. The medium was harvested 2 more times at 3 day intervals. The medium was stored at -20°C.

Ten or 20% macrophage-HT medium

Ten or 20% macrophage-HT medium was prepared by adding 10 or 20 ml of MCM to 90 or 80 ml of HT medium, filter sterilized, and stored at 4°C.

Cloning medium

Cloning medium was prepared by adding 20 ml of MCM to 80 ml of 20% FBS-DMEM, filter sterilized, and stored at 4°C.

Lysing buffer

Lysing buffer was prepared by adding 8.29 g of NH_4Cl, 1.0 g of $KHCO_3$ and 0.037 g of EDTA to 1 liter of double distilled water, filter sterilized, and stored at room temp.

APPENDIX B:

SAMPLE LISTS OF CORN AND CORN PRODUCTS

Table 5.1. Number and description of food samples bought in retail supermarket in Mid Michigan, in September, 1991.

SAMPLE: #	DESCRIPTION
6	Self-rising white, corn meal
7	Yellow corn meal
8	Self-rising white, corn meal mix
10	Yellow corn meal
21	Self-rising white corn meal
22	Self rising white corn meal mix
24	Corn tortilla mix
25	White yellow plain enriched corn meal
32	Yellow corn meal
33	White corn meal
38	Seven grain cereals
41	Corn cereals
43	Corn flakes
48	Corn meal
H9	Corn meal

Table 5.2. Number and description of Italian feed samples.

SAMPLE NUMBER	DESCRIPTION	SAMPLE NUMBER	DESCRIPTION
F1	Feed for goat	F17	Feed for laying chicken
F2	Feed for milk cow	F18	Feed for swine
F3	Complementary feed for horse	F19	Feed for swine (45% maize)
F4	Feed for calf	F20	Starter feed for swine
F5	Feed for chicken	F21	Feed for swine
F7	Flour for pig	F22	Prestarter feed for swine
F8	Feed for swine on phase of growth	F23	Feed for pregnant cow
F9	Feed for milk cow	F24	Feed for chicken
F10	Feed for swine	F25	Silage maize
F11	Feed for milk cow	F26	Feed for swine
F12	Flour of maize	F27	Feed for laying chicken
F13	Feed for swine	F28	Feed for swine
F14	Feed for milk cow	F29	Feed for buffalo
F15	Feed for calf (50% maize, 50% barley)	F30	Feed for calf
F16	Feed for rabbit	F31	Feed for calf

Table 5. 3. Number and description of fresh corn sample harvested from five counties in Michigan in Summer, 1994[a]

SAMPLE NUMBER	NAME	AGE (DAYS)	LOCATION
P1	Cargill 3777	98	Montcalm
P2	Cargill 4327	104	Montcalm
P3	Dekalb DK 527	102	Montcalm
P4	Pioneer 3293	113	Monroe
P5	Pioneer 3394	110	Montcalm
P6	Amcorn 5930	110	Cass
P7	Callahan C7337	97	Cass
P8	Callahan C7446X	103	Cass
P9	Cargill 3777	98	Cass
P10	Cargill 7777	115	Cass
P11	Country mard 432	100	Cass
P12	Dekalb DK 471	97	Cass
P13	Dekalb DK 560	106	Cass
P14	Mycogen 6060	107	Cass
P15	Peyco 614	98	Cass
P16	Pioneer 3293	113	Cass
P17	Pioneer 3525	106	Cass
P18	Renk 646PT	105	Cass
P19	Rusb XR-1727	106	Cass
P20	Callahan C7252	107	Huron
P21	Cargill 3777	98	Huron
P22	Cargill 4327	105	Huron
P23	Cargill 5547	106	Huron
P24	Country Mark 432	100	Huron
P25	Dairy cand Stealth 1205	105	Huron
P26	Decald DK 471	97	Huron
P27	Dekalb DK 527	102	Huron
P28	Mycogen 3440	93	Huron
P29	NK 4242	100	Huron
P30	Northrup King N K4242	100	Huron
P31	Peyco 614	98	Huron
P32	Pioneer 3394	110	Huron
P33	Pioneer 3525	106	Huron
P34	Renk RK 657	106	Huron
P35	Rupp XR 1727	106	Huron
P36	Amcorn 5930	110	Monroe
P37	Callahan C7337	97	Monroe
P38	Callahan C7446X	103	Monroe
P39	Cargill 7777	115	Monroe

Table 5.3. (Cont'd)

SAMPLE NUMBER	NAME	AGE (DAYS)	LOCATION
P40	Country Mark 432	100	Monroe
P41	Dekalb DK 569	106	Monroe
P42	Dekalb OK 471	97	Monroe
P43	Mycogen 6060	107	Monroe
P44	Northrup King N K4242	-	Monroe
P45	Peyco 614	98	Monroe
P46	Pioneer 3525	106	Monroe
P47	Renk 646 PT	105	Monroe
P48	Rupp XR 1727	106	Monroe
P49	Cargill 3777 9g	-	Montcalm
P50	Cargill 5877	108	Montcalm
P51	Country Mark 432	100	Montcalm
P52	Decald DK 471	97	Montcalm
P53	Jung 2672	108	Montcalm
P54	Mycogen 3440	93	Montcalm
P55	NK 4242	100	Montcalm
P56	Peyco 614	98	Montcalm
P57	Pioneer 3394	110	Montcalm
P58	Pioneer 3525	106	Montcalm
P59	Renk 657	106	Montcalm
P60	Renk RK 657	106	Montcalm
P61	Rupp 1727	106	Moutcalm
P62	Callaahan 7446 x	103	Saginaw
P63	Callahan C7337	97	Saginaw
P64	Cargill 3777	98	Saginaw
P65	Cargill 4277	102	Saginaw
P66	Cargill 6677	110	Saginaw
P67	Ciba 4394	107	Saginaw
P68	Country Mark 432	100	Saginaw
P69	Dekalb DK 471	97	Saginaw
P70	Dekalb DK 569	106	Saginaw
P71	Mycogen 6970	107	Saginaw
P72	NK 4242	100	Saginaw
P73	NKX 423	105	Saginaw
P74	Peyco 614	98	Saginaw
P75	Pioneer 3394	110	Saginaw
P76	Renk 646 PT	105	Saginaw
P77	Rupp XR 1677	106	Saginaw

[a] kindly supplied by Dr. Patrick L. Hart (105 Pesticide Research Center, Michigan State University, MI, 48824.

APPENDIX C

IMMUNOLOGICAL ASSAYS FOR MYCOTOXIN DETECTION

Immunological Assays for Mycotoxin Detection

Enzyme-linked immunosorbent assays have been successfully applied to the screening of mycotoxins in a diverse array of foods

James J. Pestka, Mohamed N. Abouzied, and Sutikno

□ MYCOTOXINS ARE TOXIC SECondary metabolites produced by molds that often contaminate agricultural staples such as corn, wheat, and peanuts prior to harvest and during storage. These compounds have a wide array of chemical structures and are produced by common field and storage fungi, including many species of *Aspergillus*, *Penicillium*, and *Fusarium*.

Mycotoxins can elicit a variety of toxic symptoms in humans and animals, ranging from gastroenteritis to cancer (Table 1). For example, the aflatoxins were identified in the early 1960s as etiologic agents of hepatotoxicity and hepatic cancer in turkey poults and rainbow trout, respectively (Pestka and Casale, 1990). Strict regulations were subsequently established for aflatoxins in food in many countries because of the potential for similar effects in humans.

Besides direct concerns over human health, aflatoxins and other mycotoxins have major economic impact on livestock productivity as a result of lower quantity and quality of animal products, smaller litters, infertility, reduced feed efficiency, impaired resistance to disease, and loss of vaccination efficiency (CAST, 1989). Other economic effects can be more subtle but have major implications worldwide with regard to food production and processing (Table 2). It has been estimated by the Food and Agriculture Organization that 25% of the world's crops are affected by mycotoxins (Mannon and Johnson, 1985), although the absolute quantification of these losses remains enigmatic (Hesseltine, 1986).

Whether a mycotoxin is present in a food is largely dictated by environmental and biological factors (Fig. 1), particularly the regional weather during a growing season and harvest. A major means of eliminating mycotoxins from human and animal food is to detect and divert contaminated raw materials from feed and finished food use. Analytical surveillance can also assist in identifying those geographical regions where mycotoxins are a recurrent problem and provide a database for human exposure in epidemiological studies.

Methods for mycotoxin analysis include thin-layer, liquid, and gas chromatography, as well as mass spectrocopy. A major impediment to the implementation of these approaches is interferences found in extracts of corn, peanuts, wheat, cottonseed, and other foods as well as clinical samples from humans and animals. A series of extensive cleanup steps involving liquid–liquid partition, column cleanup, and evaporation are therefore required to overcome these interferences. The procedures are time-consuming and costly and often involve use of harmful solvents.

Table 1—Major Mycotoxins and their Toxic Effects *in experimental animal models*

Mycotoxin	Toxic effect	Commodity
Aflatoxins B_1, B_2, G_1, G_2	Liver toxicity and cancer	Corn, cottonseed, nuts, peanuts
Aflatoxin M_1	Liver toxicity and cancer	Milk
Cyclopiazonic acid	Muscle necrosis, oral lesions	Corn, peanuts
Fumonisin	Leukoencapholomacia, pulmonary edema	Corn
Ochratoxin	Kidney toxicity, cancer	Wheat, barley, corn, oats
Patulin	Generalized toxicity, neurotoxicity	Apples and apple juice
Trichothecenes, including deoxynivalenol (vomitoxin), nivalenol, T-2 toxin	Feed refusal, diarrhea, oral and GI lesions, immunotoxicity	Corn, wheat, barley
Zearalenone	Estrogenic effects, reproductive problems	Corn, wheat, barley

Research initiated in the late 1970s proposed the application of immunochemical assay, procedures commonly used in clinical laboratories, to the analysis of aflatoxin B_1 (AFB_1) and other mycotoxins (Chu and Ueno, 1977; Langone and Van Vunakis, 1976). These assays typically involve the competition between a free mycotoxin in a sample extract and a labeled mycotoxin for an antibody binding site. Although initially based on radioimmunoassay (RIA), subsequent research has established the feasibility of using enzyme-linked immunosorbent assay (ELISA). These and other assays have been made available commercially for the rapid assay of mycotoxins in food.

Key considerations in the development and application of mycotoxin immunoassays to food safety analysis include method of antibody generation, immunoassay formats, adaptability to food analysis, and evaluation criteria for commercial test kits.

Generation of Mycotoxin Antibodies

Antibodies, or immunoglobulins, are a family of glycoproteins that are produced as a response to foreign molecules in the body (Harlow and Lane, 1988). This response requires the interaction among white blood cells (leukocytes) known as B cells, T cells, and macrophage that culminate in the terminal differentiation of the B cells into antibody-secreting plasma cells.

Immunogenicity refers to the capacity of a macromolecule to induce an immune response and is dependent on its chemical structure and its ability to be recognized as foreign material. The minimum molecular weight of an immunogen is 3,000–5,000. Because mycotoxins are of low molecular weight (300–400), they must be conjugated to a carrier

Author Pestka, a Professional Member of IFT, is Professor, and authors Abouzied and Sutikno are Research Associate and Graduate Student, respectively, Dept. of Food Science and Human Nutrition, Michigan State University, East Lansing, MI 48824. Send reprint requests to author Pestka.

Table 2—Economic Effects of Mycotoxin contamination in food. Modified from CAST (1989)

Affected group	Economic effect
Producer	Food/feed loss
	Less income
	Possible loss of outlet
	Reduced livestock productivity
Handler/distributor	Less income
	Increased storage costs
	Market loss
	Litigation costs
Processor	Reduced-value products
	Insurance premiums
	Litigation costs
	Lower nutritional quality
	Increased product prices
	Possible chronic health effects
International trade	Unreliable supply, resulting in price fluctuation and market loss
	Problems with establishing production quotas and food security agreements

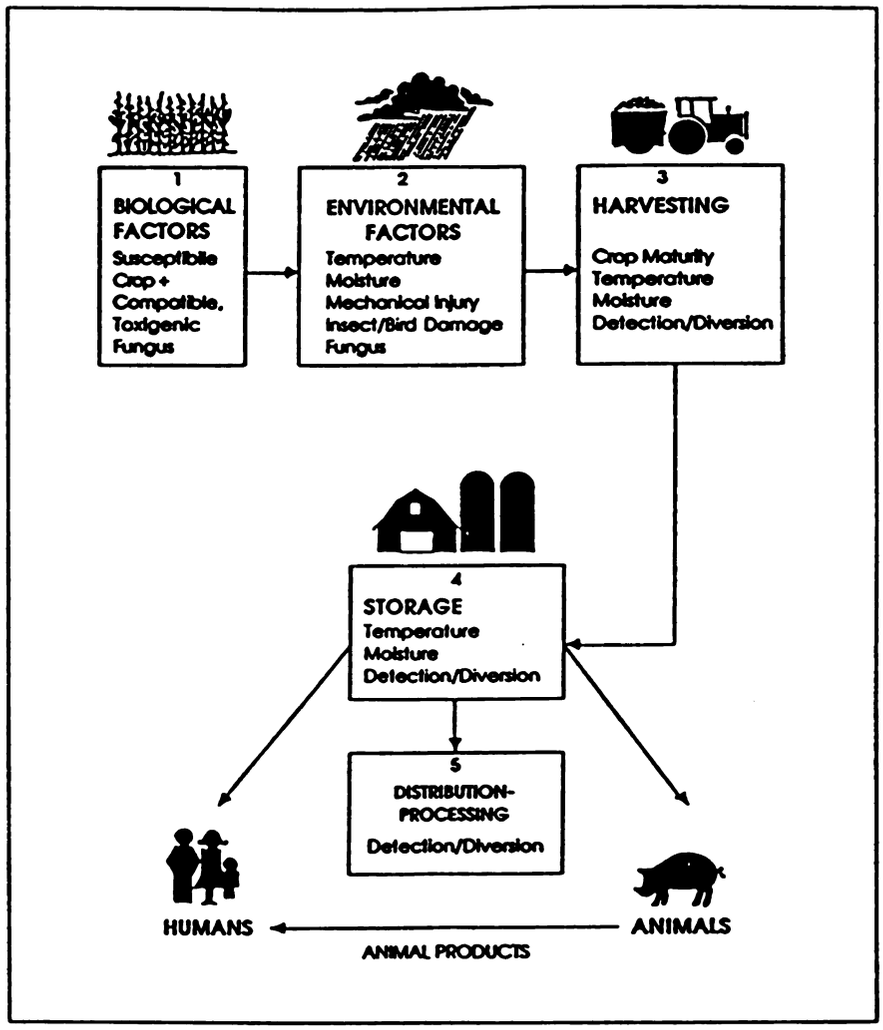


Fig. 1—Factors Affecting Mycotoxin Occurrence in the food chain. From Pestka and Casale (1990)

protein such as bovine serum albumin to be immunogenic.

Often, preparation of a suitable mycotoxin immunogen is the rate-limiting step in the development of an immunoassay. Standard hapten conjugation techniques used for mycotoxins have been reviewed by Chu (1986). If the mycotoxin doesn't have a reactive group for conjugation, it must be derivatized. For example, generation of protein conjugate for the mycotoxin fumonisin, (Azcona-Olivera et al., 1992a, b) simply involves the use of glutaraldehyde linkage via a free amino group (Fig. 2A), whereas conjugation of deoxynivalenol (Casale et al., 1988) is much more difficult because it involves extensive modification and blocking stages (Fig. 2B). Generally, the same conjugation techniques used for immunogen preparation can be applied to link mycotoxins to enzyme markers for the ELISA as long as the reaction conditions do not denature the enzyme.

In some cases, undesirable side reactions can occur during chemical conjugation and can result in antibodies to the by-products (Gendloff et al., 1986). Antibodies may also react with mycotoxin plus bridge groups, bridge group plus carrier protein, or the carrier protein directly. Thus, when characterizing and evaluating mycotoxin antibodies for immunoassay, conjugation reaction protocols must be carefully selected and appropriate controls utilized.

A key aspect in antibody development is the site of chemical conjugation. For example, many approaches have been used to produce antibodies with different specificities for the aflatoxin family (Fig. 3). Those portions of the aflatoxin molecule which project distally from the conjugation site are said to be *immunodominant*, because the resultant antibodies will exhibit the highest degree of recognition for these moieties. Thus, any metabolic precursor or analogues which mimic this immunodominant region will be recognized by an antibody generated against the parent toxin.

Cross-reactivity can be assessed using RIA or ELISA competition curves, where the levels required for 50% inhibition of marker ligand binding are used as the basis of comparison (Fig. 4). Rarely are these competition curves superimposable; thus, an analogue typically cross-reacts to a greater or lesser extent than the parent toxin. Although the presence of such analogues in a sample may render a quantitative assay to the level of semiquantitative, a high level of cross-reactivity can be very useful, as has been observed for the screening of fumonisins (Azcona-Olivera et al., 1992a, b), zearalenones (Dixon et al., 1987; Warner and Pestka, 1986) and the aflatoxins (Dixon et al., 1988).

The most straightforward approach to generation of antibodies is the multiple-site immunization of rabbits with 100–1,000 μg of mycotoxin–protein conjugate. Usable antiserum could be obtained in 3–4 mo. A critical factor in this immunization process is the use of an oil-based adjuvant containing killed *Mycobacterium* such as the "Freund's Complete type" to allow slow release of the immunogen and nonspecifically stimulate the immune response. More recently, we have successfully utilized very low levels of cholera toxin conjugates of fumonisin (Azcona-Olivera et al., 1992a, b) and a trichothecene mycotoxin (Abouzied et al., 1993). Although the mechanism(s) by which choleratoxin (CT) exerts its potent adjuvant effect in the immune system is not fully understood, it has been shown that CT concomitantly stimulates interleukin-1 production and antigen presentation (Bro-

mander et al., 1991).

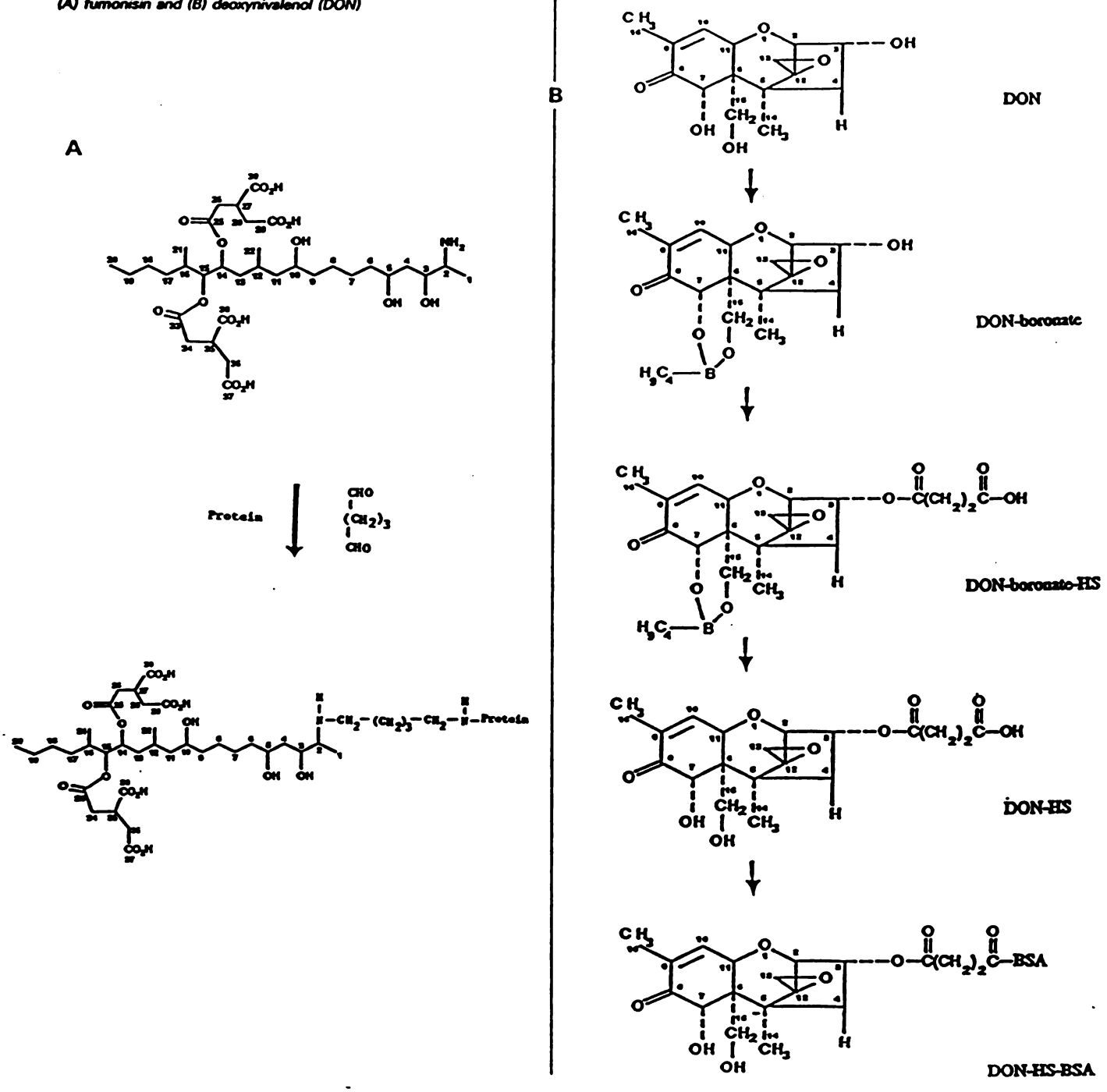
The advantages to this approach are severalfold. First, the procedure is rapid and yields quality antibodies, in comparison to poorer results achieved by standard protocols. Second, since no animal health impairment was observed at the concentrations used in this work, CT might be a humane alternative to Freund's adjuvant, which typically gives rise to abscesses, ulcers, or granulomas at the injection site. And third, the use of CT is also valuable when mycotoxin availability is limited, since relatively low doses of immunogen are required to induce a rapid and strong antibody response.

Rabbit antisera contain antibodies generated by multiple B-cell clones. These vary in specificity and are considered *polyclonal*. A major advantage of polyclonal antisera are that they are of high affinity and inexpensive to produce. However, inherent variability from lot to lot makes it difficult to use them in commercial kits with defined performance characteristics. Hybridomas have therefore been developed by fusions of immunized mouse spleen cells with a myeloma cell line to secrete reagent-quality *monoclonal* antibodies. A major disadvantage to this approach is the requirement for a tissue-culture facility, high cost, and time effort involved.

Mycotoxin Immunoassay Formats

A number of immunoassay formats have been devised for mycotoxin analysis. Initially, competitive RIAs were used whereby specific antibody is incubated with a constant amount of radiolabeled

Fig. 2—Preparation of immunogens for (A) fumonisin and (B) deoxynivalenol (DON)



toxin in the presence of standard or unknown sample and then various procedures are used to remove the toxin antibody complex from solution. The amount of toxin in a sample is inversely related to the amount of radiolabeled (unbound) toxin in solution. Because of inherent problems with radioactivity, competitive assays based on ELISA (Engvall and Perlman, 1971) were devised. Both direct and indirect assays (Fig. 5) have been applied to mycotoxin detection. Microtiter plates, beads, and Terasaki plates have been used as solid-phase support for ELISA (Pestka et al., 1980; Pestka and Chu, 1984). High-protein-binding polystyrene microtiter plates have been most widely used because they offer an extensive support technology, including removable strips, multiwell pipettes, automated washers, and spectrophotometers.

Membranes are an alternative solid phase that have been employed for yes-no or threshold tests in cups, cards, and dipsticks. We have successfully employed nitrocellulose membranes in a Computer-Assisted-Multianalyte Assay System (CAMAS) for fumonisins, aflatoxins, and zearalenones (Abouzied and Pestka, 1994). Monoclonal antibodies for each of these toxins are immobilized as multiple lines on nitrocellulose membrane strips and sectored into hydrophobic compartments to minimize use of reagents. A modified ELISA is conducted whereby free mycotoxins and horseradish peroxidase-labeled mycotoxins compete for binding to the nitrocellulose-bound antibodies. Color intensity of lines formed by a precipitating substrate is inversely related to myco-

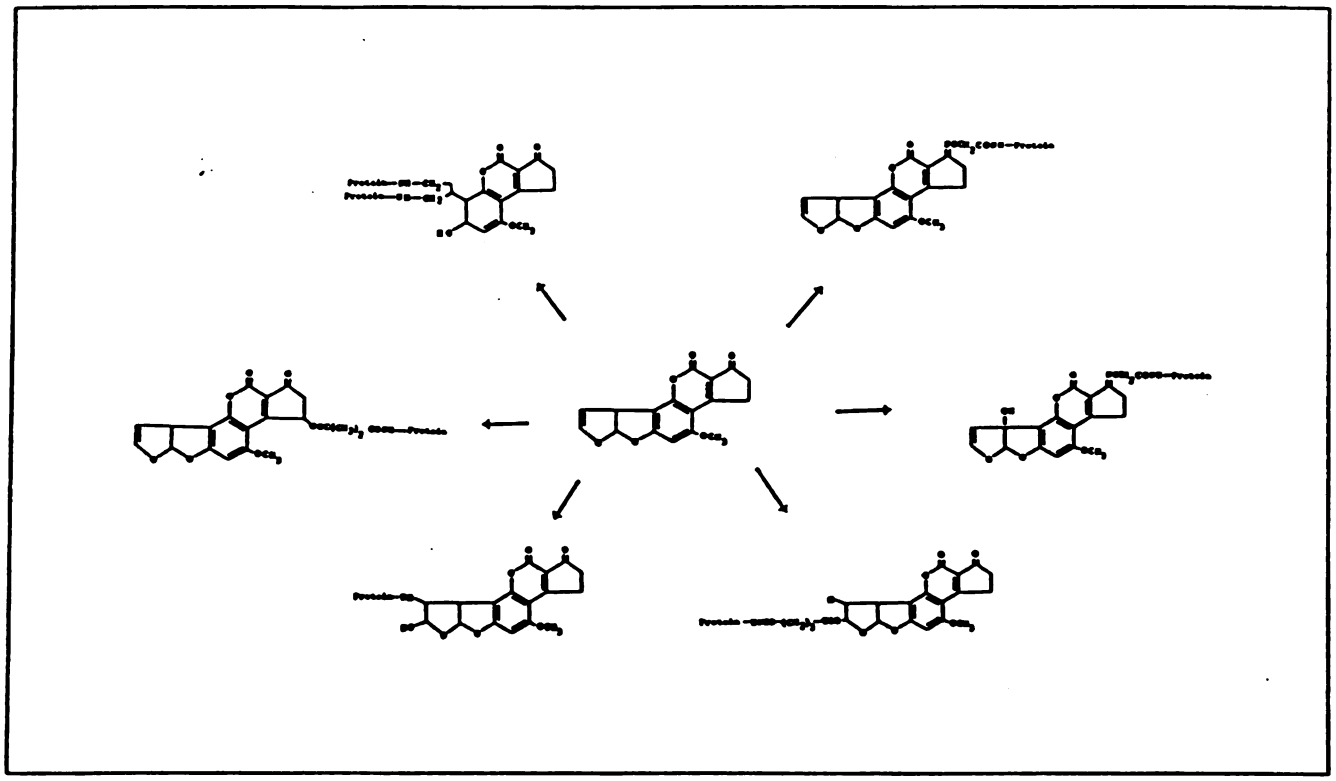


Fig. 3—Approaches for Generation of aflatoxin immunogens

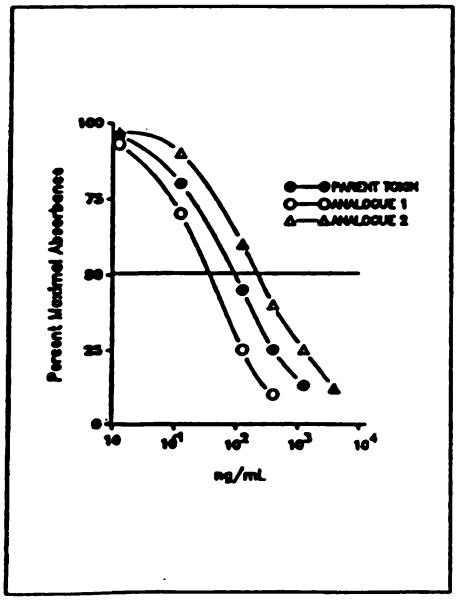
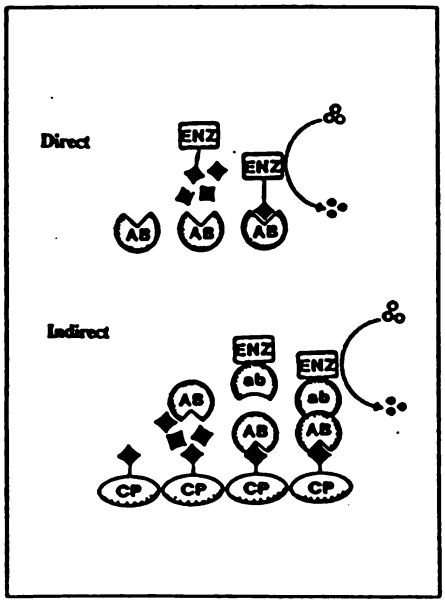


Fig. 4 (left)—Theoretical Binding Inhibition Curves for mycotoxins in a competitive immunoassay. Cross-reactivity of analogue 1 is greater than for parent toxin, while that of analogue 2 is less

Fig. 5 (right)—Competitive ELISAs for Mycotoxins. In direct competitive ELISA, mycotoxin-enzyme conjugate ENZ is simultaneously incubated with unconjugated toxin ♦ over solid-phase-bound antibody AB. Toxin concentration is inversely related to bound enzyme conjugate and thus can be calculated on development of end-product absorbance obtained after addition of enzyme substrate. In indirect competitive ELISA, mycotoxin-specific antibody AB competes with free toxin for binding to solid-phase mycotoxin-carrier protein CP conjugate. Second anti-immunoglobulin enzyme conjugate ab-ENZ is then required to determine total bound antibody. Toxin concentration is inversely related to bound enzyme conjugate



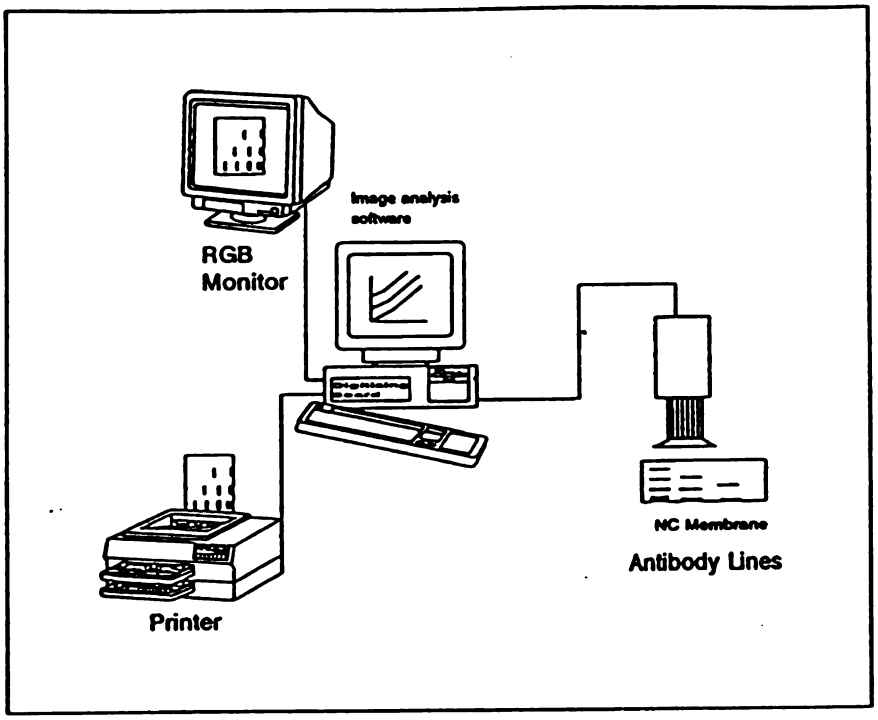


Fig. 6—Image Analysis System used for Computer-Assisted Multianalyte System (CAMAS) for mycotoxins. See Abouzied and Pestka (1993) for details

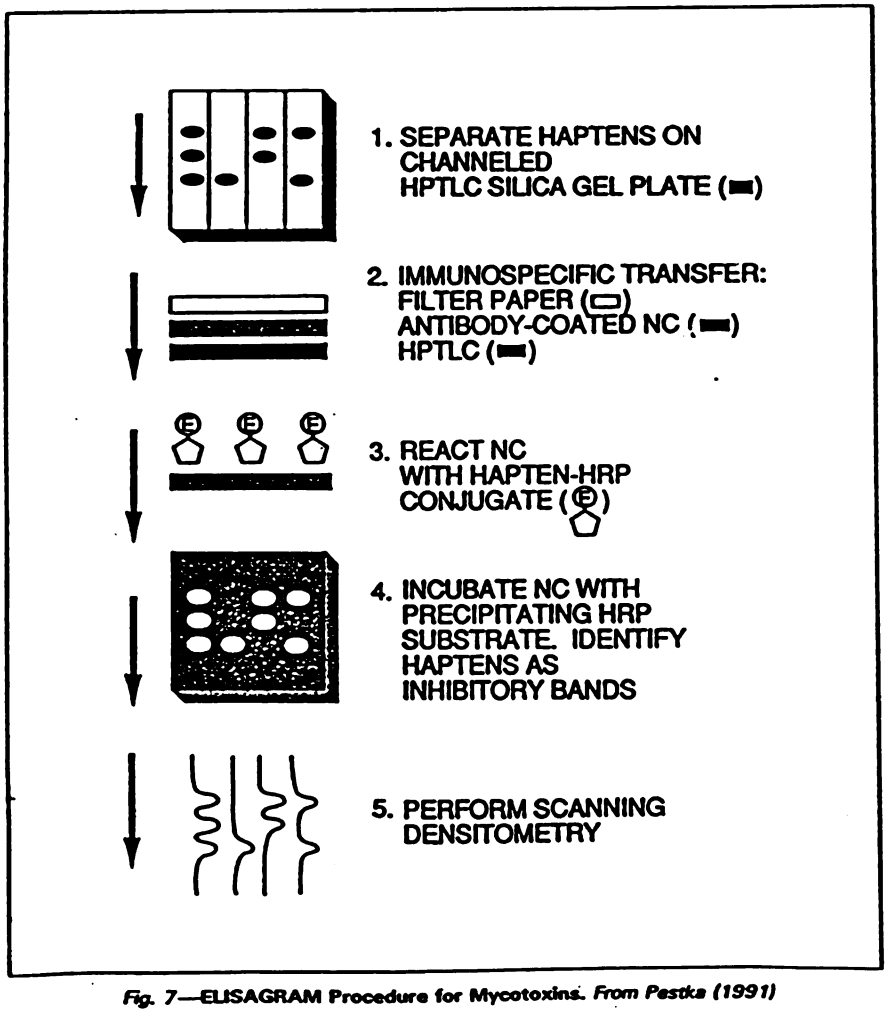


Fig. 7—ELISAGRAM Procedure for Mycotoxins. From Pestka (1991)

toxin concentration. Line density can be quantitatively assessed using a camera, video monitor, and microcomputer equipped with a video digitizing board (Fig. 6). The assay can be used to determine range values for the various mycotoxins in extracts of spiked corn in less than 30 min and record them on the microcomputer hard disk.

Another immunoblot approach, called ELISAGRAM, has been devised that combines the sensitivity and selectivity of competitive ELISA with the capacity of high-performance thin-layer chromatography (HPTLC) to separate structurally related mycotoxins (Pestka, 1991). The procedure (Fig. 7) involves separation of mycotoxin by HPTLC, blotting of the HPTLC plate with nitrocellulose (NC) coated with mycotoxin-specific monoclonal antibody, incubation of NC with mycotoxin-enzyme conjugate to identify unreacted antibody binding sites, detection of bound enzyme conjugate with a precipitating substrate, and visual or densitometric assessment of inhibition bands indicative of a cross-reacting mycotoxin. The technique has been applied to two major mycotoxin families, the zearalenones and aflatoxins. Multiple standard curves for the zearalenones and the aflatoxins can also be constructed using scanning densitometry. Cross-reactivity in ELISAGRAM curves are analogous to that found in competitive ELISA. This procedure could be widely applicable to the simultaneous quantitation and confirmation of multiple haptens with a single cross-reactive antibody.

Mycotoxin antibodies have also been attached to affinity columns and used for analytical purposes. For example, aflatoxins can be bound to an affinity column and then desorbed for subsequent derivatization and fluorescence measurement (Trucksess et al., 1991). This approach requires fluorescent mycotoxin derivatives and additional instrumentation (fluorometer). Another approach is to quantitate mycotoxin in the affinity column eluates by liquid chromatography (reviewed by Chu, 1992).

Immunoassay of Mycotoxins in Foods

Antibodies (polyclonal and monoclonal) have been made to the major known mycotoxins, and both ELISAs and RIAs have been successfully applied to the screening of mycotoxins in a diverse array of foods. Table 3 gives selected examples of reported immunoassays for the aflatoxins, ochratoxins, trichothecenes, fumonisins, and other mycotoxins, many of which can detect picogram or nanogram levels of toxin.

To maintain the native protein structure of the antibody and enzyme conjugate, immunoassays have to be carried out in an aqueous system. In a liquid system such as milk, mycotoxins can be analyzed directly (Pestka et al., 1981a), although the limit of detection can be increased by cleanup and concentration

—Text continued on page 127

Table 3—Selected Immunological Assays for Mycotoxins in Foods

Toxin	Format	Food analyzed	Limit of detection	Reference
Aflatoxin B_1	RIA	Corn, wheat, peanut butter	6 ng/g	El-Nakib et al. (1981)
	ELISA	Corn, wheat, peanut butter	3 ng/g	El-Nakib et al. (1981)
		Peanut butter	2.5 ng/g	Mortimer et al. (1988)
		Corn, cotton-seed	2.5 ng/g	Dixon et al. (1988)
		Barley	0.1 ng/mL	Ramakrishna et al. (1990)
Aflatoxins B_1 and G_1	ELISA	Peanut butter	0.3 ng/g	Morgan et al. (1986b)
Aflatoxin M_1	RIA	Milk	0.5 ng/g	Pestka et al. (1981b)
	ELISA	Milk	0.3 ng/g	Pestka et al. (1981b)
			0.3 ng/g	Fan et al. (1984)
			12 pg/g	Nieuwenhof et al. (1990)
	AC	Milk	50 pg/g	Hansen (1990)
Cyclopiazonic acid	ELISA	Buffer	30 pg/assay	Hainau and Weiler (1991)
Ergot alkaloid	ELISA	Wheat	10 ng/mL	Shelby and Kelley (1992)
Fumonisins	ELISA	Feed	250 ng/g	Azcona-Olivera et al. (1992a, b)
Fusarochromanone	LC-ELISA	Wheat, barley	5 ng/g	Yu and Chu (1991)
Ochratoxin	ELISA	Barley	1.0 ng/mL	Ramakrishna et al. (1990)
			5.0 ng/g	Candlish et al. (1988)
			0.1 ng/g	Morgan et al. (1983)
		Pig kidney	0.5 ng/g	Morgan et al. (1986a)
		Wheat, meat, plasma	1.0 ng/g	Sato et al. (1987)
		Chicken meat, wheat flour, porcine plasma, bovine sera	0.1 ng/mL	Kawamura et al. (1989, 1990)
PR Toxin	RIA	Cheese	50 μg/g	Wei and Chu (1988)
Rubratoxin	RIA	Cultures	0.1 μg	Davis and Stone (1979)
Sterigmatocystin	ELISA	Barley	0.01 pg	Morgan et al. (1986c)
Trichothecenes:				
Acetyldeoxynivalenol	ELISA	Rice	1 ng/g	Kemp et al. (1986)
Diacetoxyscirpenol	ELISA	Culture	16 ng/mL	Hack et al. (1989)
		Wheat	0.3 μg/mL	Mills et al. (1988)
Deoxynivalenol	RIA	Corn, wheat	20 ng/g	Xu et al. (1986)
	ELISA	Corn	200 ng/mL	Casale et al. (1988)
		Wheat	0.1 ng/assay	Mills et al. (1990)
		Grain-based food	1 μg/g	Abouzied et al. (1991)
Nivalenol	ELISA	Barley	0.1 ng/assay	Ikebuchi et al. (1990)
Roridin A	ELISA	Feed	5 ng/mL	Martlbauer et al. (1988)
T-2 toxin	RIA	Corn, wheat	0.1 ng/g	Lee and Chu (1981a)
		Milk	2.5 ng/g	Lee and Chu (1981b)
	ELISA	Corn	50 ng/g	Gendloff et al. (1984)
		Corn, wheat	2.5 ng/g	Pestka et al. (1981a)
		Milk	0.2 ng/g	Fan et al. (1984)
		Wheat	0.5 ng/g	Chiba et al. (1988)
Zearalenone	ELISA	Corn, wheat, feed	1 ng/mL	Liu et al. (1985)
		Corn	1 ng/g	Warner and Pestka (1986)
		Grain-based foods	2.5 ng/g	Warner and Pestka (1987)

Table 4—Commercial Immunoassay Kits for Mycotoxins available as of June 1993

Test kit	Analyte(s)	Format	Detection limit (ppb)	Analysis time[a]	Cost/assay ($)[b]	Application	Comments
EZ-Screen[c]	Aflatoxins	ELISA: Multi-site card	5-20	10	5.00-7.50	Corn, peanuts	Visual, pass/fail
	Aflatoxin M_1	ELISA: Multi-site card	0.5	10	5.00-7.50	Milk	Visual, pass/fail
	Ochratoxin	ELISA: Multi-site card	5	10	5.00-7.50	Corn	Visual, pass/fail
	T-2 toxin	ELISA: Multi-site card	12.5	10	5.00-7.50	Corn	Visual, pass/fail
	Zearalenone	ELISA: Multi-site card	50	10	5.00-7.50	Corn	Visual, pass/fail
Cite Probe[d]	Aflatoxin B_2	ELISA: Tray	5, 20	5	10.00	Corn, cottonseed	Visual, pass/fail, USDA-FGIS certified
Afla 5, 10, 20 Cup[e]	Aflatoxins	ELISA: Cup	5, 10, 20	5	4.00	Corn, peanuts, peanut butter, cottonseed, and feeds	Visual, pass/fail, USDA-FGIS certified, AOAC First Action Approval
One-Step ELISA[e]	Aflatoxin B_1	ELISA: Microwell	5	40	1.00	Corn, peanuts, peanut butter, cottonseed, and feeds	Quantitative; with ELISA reader
	Aflatoxin M_1	ELISA: Microwell	0.5	40	1.00	Milk	Quantitative; with ELISA reader
	Zearalenone	ELISA: Microwell	200	40	1.00	Corn, wheat	Quantitative; with ELISA reader
Agri-screen[f]	Aflatoxins	ELISA: Microwell	5	6-10	3.50	Corn, peanuts, cottonseed, feed	Visual or ELISA reader, pass/fail, USDA-FGIS certified, AOAC First Action Approval
	Deoxynivalenol (vomitoxin)	ELISA: Microwell	1,000	12-20	5.50	Corn, wheat, feed	Visual or ELISA reader, pass/fail, USDA-FGIS certified
	Fumonisin	ELISA: Microwell	500	12-20	5.50	Corn, wheat, feed	Visual or ELISA reader, pass/fail, USDA-FGIS certified
	Ochratoxin	ELISA: Microwell	20	12-20	5.75	Corn, wheat, feed	Visual or ELISA reader, pass/fail,
	T-2 toxin	ELISA: Microwell	500	18-30	5.50	Corn, wheat, feed	Visual or ELISA reader, pass/fail
	Zearalenone	ELISA: Microwell	250	18-30	5.50	Corn, wheat, feed	Visual or ELISA reader, pass/fail,
Veratox[f]	Aflatoxins	ELISA: Microwell	5	15	4.00	Corn, peanuts, cottonseed, feed	Quantitative with ELISA reader, USDA-FGIS certified
	Aflatoxin M_1	ELISA: Microwell	0.25	40	5.50	Milk	Quantitative with ELISA reader,
	Deoxynivalenol	ELISA: Microwell	300	20	5.50	Corn, wheat, feed	Quantitative with ELISA reader,
	T-2	ELISA: Microwell	50	30	6.00	Corn, wheat, feed	Quantitative with ELISA reader,
	Zearalenone	ELISA: Microwell	250	30	6.00	Corn, wheat, feed	Quantitative with ELISA reader,
Dosage[g]	Aflatoxin B_1	ELISA: Microwell	0.5	45	4.00	Corn, peanuts, feed	Quantitative with ELISA reader
Detection[g]	Aflatoxin B_1	ELISA: Tube	1, 5	45	9.00	Corn, peanuts, feed	Visual or reader, pass/fail
Aflatest[h]	Aflatoxins	Affinity column	1.0	10	7.00	Corn, peanuts, feed	Quantitative with fluorometer, USDA-FGIS certified, AOAC First Action Approval, IUPAC approval
	Aflatoxin M_1	Affinity column	0.1	10	10.00	Milk	Quantitative with fluorometer
Fumonitest[h]	Fumonisin B_1, B_2	Affinity column	2	15	10.00	Corn	Quantitative with fluorometer
Ochratest[h]	Ochratoxin	Affinity column	5	10	10.00	Corn	Quantitative with fluorometer
Zearalotest[h]	Zearalenone	Affinity column	500	10	10.00	Corn, wheat	Quantitative with fluorometer

[a]Analysis time does not include sample extraction
[b]Based on purchase of smallest available unit
[c]DIAGnostix, Inc., Burlington, NC 27215 (phone 800-334-1146)
[d]Idexx Labs, Westbrook, ME 04092 (800-548-6733)
[e]International Diagnostics, St. Joseph, MI 49085 (616-983-0972)
[f]Neogen Corp., Lansing, MI 48912 (800-234-5333)
[g]Transia, Lyon, France 69007 (33-72730381)
[h]Vicam, Somerville, MA 02146 (800-338-4381)

on a Sep-Pak or affinity column. Food samples were originally extracted with a solvent by standard protocols, evaporated, and reconstituted in an aqueous buffer for assay. However, based on our initial observation that mycotoxin–horseradish peroxidase and solid-phase antibodies retain sufficient stability for ELISA when incubated with as much as 35% (w/vol) methanol (Ram et al., 1986a), a direct approach was developed whereby solid substrates are blended with methanol–water extraction solvent and the extract analyzed directly or after dilution.

Immunoassay and various chromatographic methods for mycotoxin detection in foods are usually comparable when they are performed in the research laboratory (Ram et al., 1986a, b; Chu et al., 1987). However, sometimes toxin-free food extracts can interfere with mycotoxin–enzyme binding to the solid-phase antibody and therefore yield a low false-positive response when compared to a standard curve prepared in extraction solvent with buffer. Samples can be diluted more extensively to eliminate this interference, but this will decrease sensitivity. Alternatively, interference can be minimized by incorporating toxin-free sample extracts during standard-curve preparation. Another factor that must be considered is sample pH, which must sometimes be adjusted prior to immunoassay, since antibody–antigen binding occurs optimally at neutral pH.

Commercial Mycotoxin Immunoassays

The above research has led to the development of a number of commercial kits that have been marketed in the United States for food safety verification (Table 4). Commercial immunoassay kits have generally performed well in routine analyses performed in the laboratory and the field (Koeltzow and Tanner, 1990; Azer and Cooper, 1991; Dorner et al., 1993). However, when Horwitz et al. (1993) recalculated the precision performance parameters of collaborative studies for mycotoxins through 1991, they found that ELISA had somewhat poorer precision than thin-layer chromatography and liquid chromatography. Thus, when adopting a commercial immunoassay for rapid testing of mycotoxins, food analysts must critically evaluate the system in light of their specific needs and the limitations of the assay (Table 5).

Several organizations have provided leadership in evaluating these tests. Official First Action approval has been given for detection of aflatoxins by AOAC International (formerly Association of Official Analytical Chemists) in various commodities, using microtiter-well (Park et al., 1989a, b; Patey et al., 1992), cup (Trucksess et al., 1989), and affinity-column (Trucksess et al., 1991) tests. The U.S. Dept. of Agriculture's Federal Grain Inspection Service (FGIS) has tested and approved a number of kits for qualitative screening and quantitation of aflatoxins (Table 4).

Table 5—Suggested Criteria for Adoption of a Mycotoxin Immunoasssay. Summarized from Pestka (1988)

Limits of detection and sensitivity range
Requirements for rapid screening and/or quantitation
Specificity
Necessity for adaptability to wide range of sample types
Effectiveness of recommended extraction procedure
Sample throughput
Field stability
Inter- and intra-assay reproducibility

To further facilitate kit evaluation, AOAC International has created the AOAC Research Institute with the charge of evaluating and certifying rapid test kits used for safety screening of foods. During summer 1993, this Institute evaluated aflatoxin test kits in conjunction with a Memorandum of Understanding signed in October 1992 with FGIS which recognizes the Test Kit Performance Testing Program for test kits that detect aflatoxins in grain. Upon successful evaluation using protocols by FGIS, test kits are certified to claim "Performance Tested in Accordance with Standards Established by FGIS for Test Kits to Detect Aflatoxin Residues in Grain and Grain Products." More recently, two deoxynivalenol ELISAs have been similarly certified by FGIS. It is anticipated that food analysts will be assisted by similar evaluation studies that focus on mycotoxins of potential health and economic significance, such as deoxynivalenol, the fumonisins, ochratoxins, and zearalenone.

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