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dissertation entitled

Recombinant Antibodies and Peptide Mimotopes to Fusarium Mycotoxins

presented by

Qiaoping Yuan

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Botany and Plant Pathology - Environmental Toxicology

L. Patrick Hart
Major professor

Date 8 September 1998

DATE DUE	DATE DUE	DATE DUE

RECOMBINANT ANTIBODIES AND PEPTIDE MIMOTOPES TO *FUSARIUM* MYCOTOXINS

By

Qiaoping Yuan

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology,
and Institute of Environmental Toxicology

1998

ABSTRACT

RECOMBINANT ANTIBODIES AND PEPTIDE MIMOTOPES TO *FUSARIUM* MYCOTOXINS

By

Qiaoping Yuan

Zearalenone, fumonisins and trichothecene mycotoxins are a group of secondary metabolites produced by toxigenic strains of *Fusarium* fungi. These mycotoxins are toxic to both human and animals; Some are also phytotoxic. Modern immunology and molecular biology provide novel approaches to develop low-cost antibodies and alternative antigens with desirable affinity and specificity for mycotoxin detection, neutralization, and detoxification as well as the improvement in plant resistance to mycotoxin associated plant diseases. In this thesis, phage display technology was explored to develop recombinant antibodies and peptide mimotopes for *Fusarium* mycotoxins. Firstly, the heavy and kappa light-chain variable region genes of an anti-zearalenone hybridoma cell line were isolated by polymerase chain reaction and joined by a DNA linker encoding peptide $(Gly_4Ser)_3$ as a single-chain Fv (scFv) DNA fragment. The scFv DNA fragment was cloned and expressed as a fusion protein with an antibody recognition sequence (E-tag) and phage M13 coat protein g3p in *Escherichia coli* TG1. To facilitate the cloning of genes for variable heavy chain, variable kappa light chain, and the assembly of scFv DNA fragment, a new phage display vector, pEY.5, was also constructed. Through affinity panning-elution, high affinity scFv phage antibodies were enriched and selected. Nucleotide sequence analyses revealed that the V_H portion of the cloned scFv antibody contributed more to the specificity than the V_K

portion. The potential of using the soluble scFv antibody for routine screening of zearalenone and its analogues was demonstrated. With a similar approach, attempts were made to generate scFv antibodies from mice immunized with fumonisin B_1-cholera toxin conjugate immunized mice. However, no specific scFv antibody clone was selected. An alternative approach was suggested for the cloning of functional FB_1 specific scFv antibodies. Secondly, the anti-zearalenone scFv DNA fragment was cloned into a plant transformation vector and transferred into *Arabidopsis* immature seeds by vacuum infiltration via *Agrobacterium tumefaciens* mediated plant transformation. Functional anti-zearalenone scFv plantibody was expressed. The plantibody exhibited a high zearalenone-binding affinity comparable to bacteria-produced scFv antibody and the parent monoclonal antibody. Finally, from a phage displayed random 7-mer peptide library, two highly homologous heptapeptide sequences, SWGPFPF and SWGPLPF, were identified that specifically bound a deoxynivalenol-specific monoclonal antibody. In contrast to deoxynivalenol's toxic effects, the peptide did not elicit apoptosis in bone marrow cells, neither did it inhibit protein synthesis in a cell-free translation system. Rather, the peptide mimotope showed an antagonism to deoxynivalenol-induced protein synthesis inhibition. These peptide mimotopes were also evaluated as immunochemical reagents for deoxynivalenol immunoassay.

To my dear parents, my wife, and my lovely twin daughters

ACKNOWLEDGMENTS

I am greatly grateful to my major advisor, Dr. L. Patrick Hart, for his advice, guidance, encouragement, assistance, and understanding throughout the development of this dissertation. I am also indebted to Dr. James J. Pestka, Dr. John E. Linz, and Dr. Dennis W. Fulbright for their invaluable suggestions, advice, and for being members of my graduate guidance committee.

I wish to thank Dr. Hui-Ren Zhou, Dr. James R. Clarke, Ms. Rebecca Uzarski, Mr. Wenqi Hu, and many others from Dr. Hart's and Dr. Pestka's laboratories for their invaluable suggestions, practical help and friendship. Acknowledgment also goes to Dr. Shenyang He and his students for their help with plant transformation and to Mr. Frank E. Klein at the Neogen Company for his help in making deoxynivalenol conjugates.

Most importantly, I would like to thank my wife Youlan Chen, and my daughters, Jing Yuan and Lin Yuan, for their love, support, patience, and understanding during all of these years. I am also very grateful to my father, Junxi Yuan, my mother, Meichun Xiang, my brother Xuewen Yuan and my sister Hongxia Yuan, for their continuous love and support in my life.

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Figure 5.

ABTS
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PBS
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PMS
RAC
scFv
SDS
TBS
TCA
TLC
TMB
V_H: v
V_k: v
V_L: v
ZEN:

LIST OF SYMBOLS AND ABBREVIATIONS

ABTS: 2,2-azinobis(3-3-ethylbenzthiazoline sulfonic acid).
AP: alkaline phosphatase
BCIP: 5-bromo-4-chloro-3-indolyphosphate-*p*-toluidine.
BSA: bovine serum albumin.
CD-ELISA: competitive direct enzyme-linked immunosorbent assay.
CDR: complemetarity-determining region.
CI-ELISA: competitive indirect enzyme-linked immunosorbent assay.
CT: cholera toxin.
DON: deoxynivalenol.
EDTA: ethylenediaminetetraacetate.
ELEM: equine leukoencephalomalacia.
ELISA: enzyme-linked immunosorbent assay.
FB_1 : fumonisin B_1.
FW: framework region.
GC: gas chromatography.
HPLC: high performance liquid chromatography.
HRP : horseradish peroxidase.
IC_{50}: the concentrations required for inhibition of binding by 50%
IPTG: isopropyl-β-D-thiogalactopyranoside.
KLH: keyhole limpet hemocyanin.
mAb: monoclonal antibody.
MBS: *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester.
MS: mass spectroscopy.
NBT: nitroblue tetrazolium.
OVA: ovalbumin.
PAGE: polyacrylamide gel electrophoresis.
PCR: polymerase chain reaction.
PBS: phosphate-buffered saline.
PEP: porcine pulmonary edema.
pNPP: *p*-nitrophenyl phosphate.
PMSF: phenylmethylsulfonyl fluride.
RACE: rapid amplification of cDNA ends.
scFv: single-chain Fv fragment.
SDS: sodium dodecyl sulfate.
TBS: Tris-buffered saline.
TCA: trichloroacetic acid.
TLC: thin layer chromatography.
TMB: 3,3',5,5'-tetramethyl-benzidine.
V_H: variable region of heavy chain.
V_k: variable region of kappa light chain.
V_L: variable region of light chain.
ZEN: zearalenone.

INTRODUCTION

Mycotoxins are a group of secondary metabolites produced by toxigenic strains of fungi. *Fusarium* is one of the major fungal genera that contains species capable of mycotoxin production. Zearalenone, fumonisins and trichothecenes are *Fusarium* mycotoxins that are toxic to both human and animals and are commonly found in grain and grain products infected by *Fusarium* spp. The presence of these toxins in agricultural commodities is primarily governed by environmental and biological factors. 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.

Mycotoxin detection can be carried out using either conventional chemical 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). Immunoassay is commonly preferred to conventional chemical methods because of its high specificity, sensitivity, simplicity, rapidity, and applicability both in the field and the laboratory.

Consequently, immunoassays for zearalenone, fumonisins, and the trichothecene deoxynivalenol (DON) have been developed in our laboratory with both polyclonal and monoclonal antibodies. However, the limits of detection with the existing antibodies to fumonisin and deoxynivalenol are not sufficiently adequate for screening these compounds at <1 ppm in conventional immunoassays, while

detection at ≤0.1 ppm would be desirable in order for the agrifood industry to anticipate problems in a growing season. Therefore, production of higher affinity and low-cost specific antibodies against these *Fusarium* mycotoxins is desirable. Immunoassay for deoxynivalenol encounters further problems because chemical conjugation of DON to a carrier protein or an enzyme has low efficiency and the conjugates are weakly immunogenic. Therefore, alternative reagents are needed to replace deoxynivalenol as immunogens and immunochemical reagents.

With newly developed phage display technology, a specific clone for antibody, or protein, or peptide can be selected from large repertoires because the DNA sequence is linked with its expression product in a single phage particle. This provides a powerful tool to clone mycotoxin antibody genes and to search proteinaceous mimics for mycotoxins. Once the specific antibodies to mycotoxins are cloned, the genes can also be introduced into plants. These plant-produced antibodies (plantibodies) might be very useful to neutralize mycotoxins through passive immunization of animals by feeding and to improve plant resistance against mycotoxin-associated plant diseases.

The research in this dissertation was undertaken to address the above rationales and is presented in five parts. Part I (Literature Review) reviews *Fusarium* mycotoxins especially zearalenone, fumonisins and trichothecenes including their chemical structures, toxicity, natural occurrence, and detection. The development of recombinant antibodies, peptide mimotopes and plantibodies is also reviewed. Part II (Molecular Cloning, Expression and Characterization of A Functional Single-chain Fv Antibody to the Mycotoxin Zearalenone) describes in detail the molecular

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cloning (phage display and expression in bacteria) of a functional single-chain Fv (scFv) antibody to the mycotoxin zearalenone from a hybridoma cell line. The use of the scFv antibody for ELISA development and the application of ELISA for zearalenone detection in spiked corn samples are also demonstrated. Part III (Attempts to Clone Single-Chain Fv Antibodies Against Fumonisin B_1 from Immunized Mice) explains efforts to produce scFv antibodies with phage display from mice immunized with fumonisin B_1-cholera toxin conjugate. Part IV (Expression of A Functional Anti-Zearalenone Single-Chain Fv Antibody in Transgenic *Arabidopsis* Plants) describes in detail the expression of a functional anti-zearalenone scFv antibody in transgenic *Arabidopsis* plants. The construction of a new phage-display cloning vector is also described. Part V (Identification of Mimotope Peptides Which Bind to the Mycotoxin Deoxynivalenol-Specific Monoclonal Antibody) describes the identification of two deoxynivalenol mimotope peptide sequences from a phage-display random 7-mer peptide library. The application of these peptide mimotopes in deoxynivalenol immunoassay and some of their bio-activities are also described.

PART I.

LITERATURE REVIEW

MYCOTOXINS

Mycotoxins are a chemically diverse group of toxic compounds produced as a part of secondary metabolism in a wide variety of filamentous fungi (Pestka and Casale, 1990). They often contaminate agricultural commodities prior to harvest and during storage. These natural toxicants are elaborated in plant tissue infected during the growing season or later in storage environments by common fungi including species of *Aspergillus*, *Penicilium and Fusarium*. Environmental and biological factors, particularly the regional weather during the growing season and at harvest, determine the degree to which a mycotoxin will contaminate a commodity (CAST, 1989). Because mycotoxins are resistant to food processing and do not degrade at high temperatures, they enter the human and animal food supply. In some cases, mycotoxins are passed on through animal feed to meat and poultry foods (Pestka et al., 1995). Human and animal mycotoxicoses include diverse effects such as gastroenteritis, hyperestrogenism, kidney dysfunction, neurotoxicity and cancer (Pestka and Casale, 1990).

Although mycotoxins are defined as fungal secondary metabolites toxic to animals, many of them are also phytotoxic and serve as virulence factors for plant pathogenic fungi (Desjardins and Hohn, 1997). For example, there is a growing body of evidence indicating that trichothecenes, including DON, can enhance the virulence of plant-pathogenic species of *Fusarium* on plant hosts (Adams and Hart, 1989; Desjardins et al., 1989; 1992; Bruins et al., 1993; Wang and Miller, 1988; Scholbrock et al., 1992; Atanassov et al., 1994; Proctor et al., 1995). Fumonisins

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have also been shown to cause necrosis and other disease symptoms at low concentrations and are associated with high level of virulence in pathogenic *Fusarium* (Lamprecht et al., 1994; Desjardins et al., 1995). The mycotoxin zearalenone has also been reported to cause certain phytotoxicities (Vianello and Macri, 1981; Wakulinski, 1989; Nedelnik, 1993).

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

Zearalenone

Production and occurrence. Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid-lactone] (Figure 1.1) is an estrogenic mycotoxin produced by the genus *Fusarium* after infection of corn and small grains (Pestka and Casale, 1990). Species of *Fusarium* are extremely common in nature and worldwide in distribution. They are active pathogens infecting a wide variety of host plants. Since the first finding of zearalenone in *Fusarium*-infected corn in 1960s, zearalenone and its analogs, along with several other mycotoxins (such as deoxynivalenol, nivalenol and fumonisins), have been detected as secondary metabolites of *Fusarium* species in many mold contaminated grains and fruits, including corn, wheat, barley, oats, sorghum, sesame (Mirocha et al., 1977a), soybean (Shotwell et al., 1977), cottonseed, peanut (Price et al., 1993), walnut (Jemmali and Mazerand, 1980), rice, rapeseed (Chakrabarti and Ghosal, 1987), banana (Chakrabarti and Ghosal, 1986), tomato (El-Morshedy and Aziz, 1995),

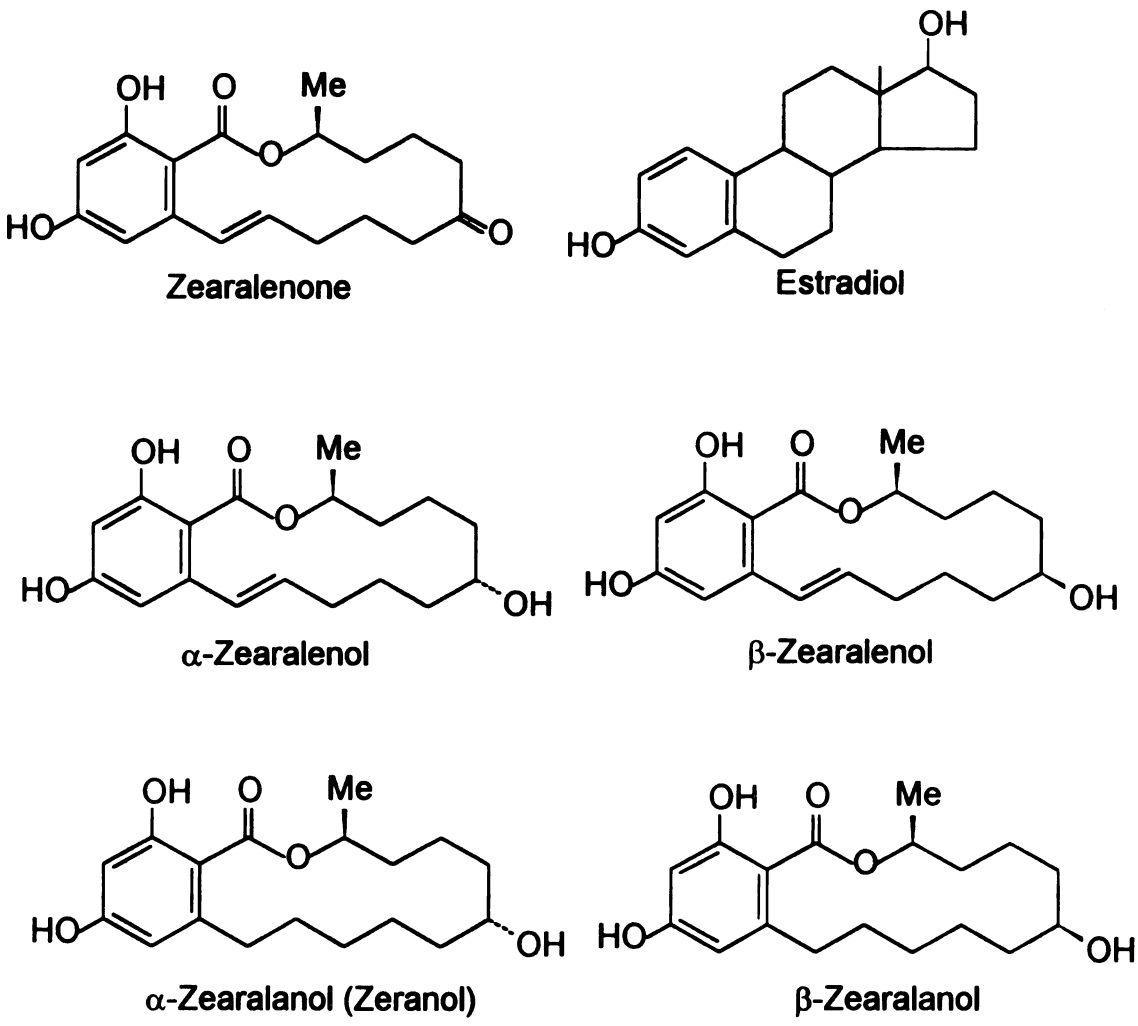

Figure 1.1. Structures of zearalenone and analogues showing structural similarities with estradiol.

beets (Bosch and Mirocha, 1992), and amaranth grain (Bresler et al., 1991). Corn is the most common grain contaminated with zearalenone and its derivatives (Wood, 1992). The occurrence of zearalenone ranged from 0.1 to 8 ppm in corn samples from several surveys in the United States (Stahr et al., 1981; Cote et al., 1984; Abbas et al., 1986; Hart, 1987).

Normally, zearalenone production is promoted by high humidity and low temperatures, as is common in the upper Midwest during autumn harvest (Coulombe, 1993). Zearalenone production is also correlated with plant density, soil fertility, insect damage, mechanical damage during harvest, and improper storage conditions (Mirocha et al., 1977a; Sutton et al., 1980; Montani et al., 1988).

Zearalenone, in spite of its large lactone ring, is surprisingly stable to hydrolytic cleavage (Mirocha et al., 1977a). It is also relatively stable during the processing of feed and foods. Zearalenone and its analogs enter the food chain directly through contaminated grain-based food and animal feed. Once zearalenone and its derivatives are produced in grains, they will remain in the grain-based feed or food. In two separate surveys of processed grain-based products (breakfast cereals, popcorn, snack foods, corn meal, crackers/cookies/muffin mixes) conducted during 1985 and 1989, the same relative concentrations of zearalenone (average 19 to 20 μg/g) were detected (Warner and Pestka, 1987; Abouzied et al., 1991). Additionally, the use of zeranol (Ralgro)(α-zearalanol) (a synthetic derivative of zearalenone) (Figure 1.1) as a growth-promoting agent in beef cattle and sheep provides another route to enter the food chain (Sundlof and Strickland, 1986). Zeranol has been found in the edible tissues (such as muscle, liver, kidney, and fat)

of the animals implanted with this growth-promoting agent (Dixon and Russell, 1986).

Distribution, elimination and metabolism. When ingested orally by animals, zearalenone appears to be fairly rapidly absorbed through the gut into the circulatory system (Dailey et al., 1980; Olsen et al., 1985a). Zearalenone can then be transported to various organs and tissues. The liver appears to be the main organ of deposition upon absorption (Chichila et al., 1988). Zearalenone is also distributed into adipose tissue, ovary, uterus, testicle, and other sites (Ueno et al., 1977; Appelgren et al., 1982).

Zearalenone can be rapidly eliminated from an animal. Following a single oral dose of zearalenone in rats, about 90% of the dose was excreted in feces after 48 hr, while the remaining 10% was excreted in urine (Smith, 1982). Zearalenone and metabolites were excreted mainly in the free form with conjugates being found only in urine. Using different oral doses of zearalenone in rats, Fitzpatrick et al. (1988) found that dose had little effect on metabolites formed, or excretion route. In one study, Pandey et al. (1990) showed that UDP-glucuronyltransferase induction by phenol barbital increased urinary excretion of conjugated α-zearalenol. A more recent study with administration of [^{3}H] zearalenone showed that the biological half-life of zearalenone in plasma was 86 hr for intravenously or orally dosed immature pigs, while only 3.3 hr for intravenously dosed animals after bile removal (Biehl et al., 1993). It provided evidence for extensive biliary secretion and enterohepatic cycling of zearalenone and metabolites in pigs. Besides the route of feces and urine, a small portion of zearalenone can be excreted into milk as

conjugates (Prelusky et al., 1990).

Zearalenone is metabolized mainly in the liver through dehydrogenation and glucuronide conjugation (Kiessling and Pettersen, 1978; Mirocha et al., 1981; Olsen et al., 1981; Bock et al., 1987). Considerable differences between species exist in the ability to reduce zearalenone (Olsen and Kiessling, 1983; Pompa et al., 1986). Among five species examined, swine had one of the greatest abilities to form the more highly estrogenic α-zearalenol (Olsen et al., 1985b). In some cases, sulfo-conjugates of zearalenone and its analogs were also found at low levels (Olsen et al., 1986; Bories et al., 1991).

Toxicity. With their chemical similarity to estradiol (Figure 1.1), zearalenone and its derivatives bind to mammalian estrogen receptors in reproductive and other sensitive tissues (Hurd, 1977). When fed to animals, zearalenone causes hyperestrogenism with symptoms such as enlargement of the uterus and nipples, vulvar swelling, vaginal prolapse, and infertility (Hidy et al., 1977; Mirocha and Christensen, 1974). Swine are the most sensitive of the large domestic animals and most commonly affected with hyperestrogenism on the farm although bovine hyperestrogenism due to zearalenone is also suspected (Mirocha et al., 1977b). Other farm animals affected by zearalenone are dairy cattle, chickens and turkeys. Animals experimentally affected are rats, mice, guinea pigs, monkeys and lambs (Mirocha et al., 1977). Embryotoxicity and teratogenic effects have also been reported in some animals after consumption of zearalenone-contaminated feeds (Miller et al., 1973; Sharma et al., 1974; Shreeve et al., 1978; Chang et al., 1979; Becci et al., 1982). Zearalenone and its derivatives also affect the immune system

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of animals. They are capable of inhibiting mitogen-stimulated lymphocyte proliferation (Forsell and Pestka, 1985). Dietary exposure to 10 ppm of zearalenone for two weeks decreases the resistance of mice to *Listeria monocytogenes* (Pestka et al., 1987). Zeranol could induce acute hepatotoxicity and, subsequently, hepatic carcinogenesis in Armenian hamster (Coe et al., 1992). More recently, DNA adducts were also found in the kidney, liver and ovary of female mice treated with zearalenone (Pfohl et al., 1995; Grosse et al., 1997).

Using the closely related α-zearalenol, the no-hormonal effect level for zearalenone was estimated to be 50-60 μg/kg body weight per day, based on a 90-day oral study in ovariectomized monkeys with vaginal cornification as the endpoint (Griffin et al., 1984; Lindsay, 1985; Kuiper-Goodman et al., 1987). Dividing the non-hormonal effect level by a safety factor of 500, which takes into account inter- and intraspecies differences and uncertainties in the experiment model, Kuiper-Goodman et al. (1987) gave an estimated Tolerable Daily Intake (TDI) of 100 ng/kg body weight per day for humans.

Although zearalenone and its derivatives are toxic at high doses, they act as anabolic hormones enhancing growth when used at the proper time and in the proper amounts. Notably, zeranol (Ralgro) (α-zearalanol), a synthetic derivative of zearalenone, has been approved as a growth-promoting agent in beef cattle and sheep (Umberger, 1975).

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Fumonisins

Historical background. Fumonisins are a group of secondary metabolites produced by *Fusarium moniliforme* (Sheeldon) (Benzuidenhout et al., 1988) and *F. proliferatum* (Ross et al.,1990). These fungi are commonly found on corn, sorghum, and other grain commodities throughout the world (Marasas et al., 1984). *F. moniliforme*, which was firstly described by Sheldon (1904), was implicated as a causative agent of "moldy corn poisoning" in animals in the United States (Peter, 1904). This mold has been associated with an equine poisoning known as equine leukoencephalomalacia (ELEM) (Ross, 1994). This is a neurotoxicosis 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).

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). FB_1 is the most toxic and predominant fumonisin produced by *F. moniliforme* (Gelderblom et al., 1988a), and has been confirmed as an etiologic agent of fatal animal diseases such as ELEM in horses (Kellerman et al., 1990), porcine pulmonary edema (PEP) in pigs (Harrison et al., 1990), and liver cancer in rats (Gelderbolm et al., 1991). In addition, epidemiological studies

indicate that fumonisins are associated with human esophageal cancer in South Africa (Marasas et al., 1988a).

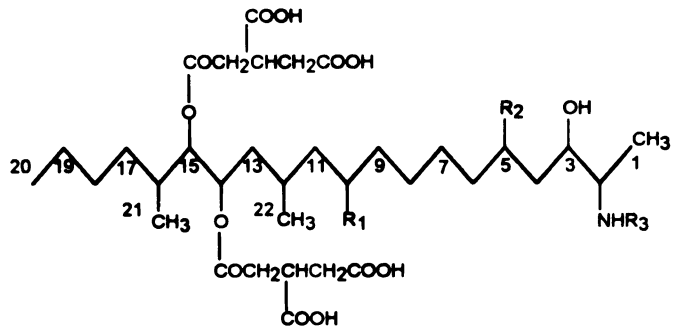
Chemistry. Fumonisins are diesters of 14, 15-propane-1,2,3-tricarboxylic acid and either 2-acetylamino- or 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxy-icosane or its C-10 deoxy analogue (Figure 1.2) (Bezuidenhout et al., 1988). Chemical structures of these toxins are similar to that of *Alternaria alternata* f. sp. *lycopersici* (AAL) toxin and sphingosine (Figure 1.2) (Norred, 1993).

The biosynthetic pathways through which fungi produce fumonisins have only been partially elucidated (Norred, 1993). When Plattner and Shackelford (1992) fed deuterium-labeled methionine to *F. moniliforme* cultures, they observed high levels of deuterium incorporation in FB_1 at the methyl groups on the C-12 and C-16 position of fumonisin backbone. Based on ^{13}C-labeled acetate feeding experiment, Blackwell et al. (1994) concluded that the backbone of fumonisins was synthesized by the fungus through condensation of acetyl coenzyme A and serine. However, more work is still required to fully elaborate the biosynthetic pathway of the fumonisins.

Natural occurrence. *F. moniliforme*, the fumonisin-producing fungus, commonly occurs in the world and it has been isolated in many countries (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 other agricultural products such as beans, peanuts, sugar beats, and bananas (Bacon and Nelson, 1994).

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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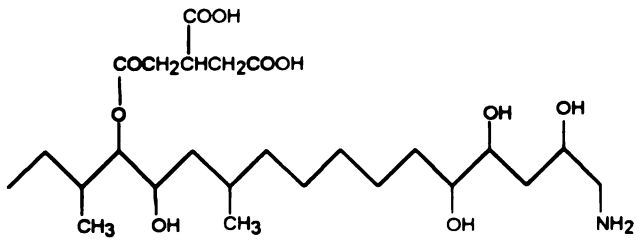

SPHINOSINE

Figure 1.2. Structures of known fumonisins and *Alternaria alternata f. sp. lycopersici* (AAL) toxin, showing structural similarities with sphingosine (Norred, 1993).

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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°C, 32% moisture content of corn media, and aerated atmosphere (cotton-stoppered flasks) were the optimum condition of FB_1 production by *F. moniliforme* in laboratory experiments. *F. moniliforme* can produce FB_1 at level of 300 to 17,000 ppm when cultured on solid corn media at the optimum conditions for 2 to 12 weeks (Bars et al., 1994, Nelson et al., 1991, Alberts et al., 1990).

Toxicity. Equine leukoencephalomalacia (ELEM) is the most studied disease caused by consumption of *F. moniliforme* contaminated corn (Norred, 1993; Marasas et al., 1988b). ELEM develops over a several-week period, during which the horse may refuse feed, becomes lethargic and is disoriented. The progression of the condition includes ataxia, profuse sweating, convulsions, and finally death. ELEM has been reproduced by intravenous administration of 7 doses (0.125mg/kg body weight) of purified FB_1 over 10 days (Marasas et al., 1988b). The horses suffered convulsions and were found to have brain lesions similar to those seen in ELEM cases (Merrill et al., 1995).

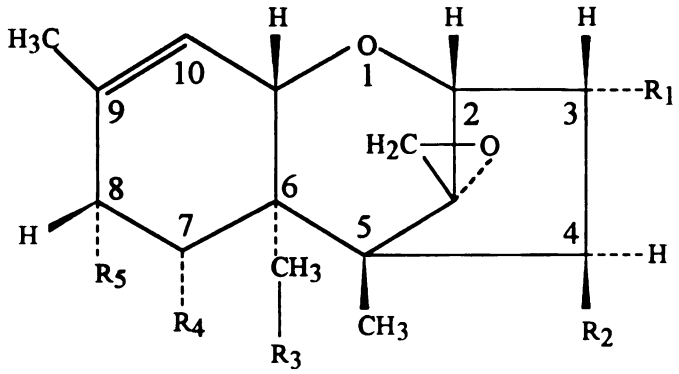
Porcine pulmonary edema (PPE) which develops hydrothorax and lung edema usually resulting in death was particularly prevalent in the United States when pigs were fed contaminated corn (Norred, 1993). Pigs that received pure FB_1 at 0.4 mg/kg body weight were dead on day 5, with typical PPE lesions (Harrison et al., 1990). Haschek et al. (1992) suggested that the disruption of sphingolipid biosynthesis by FB_1 causes membranes damage in hepatocytes and release into

the blood stream. Pumonary intravascular macrophages (PIMs) which phagocytize the membrane fragments are especially rich in the lungs of pigs. This initiates the PIMs to release enzymes and other mediators, which increase capillary permeability in the lung tissue and lead to edema (Haschek et al., 1992; Merril et al., 1995).

In addition, fumonisins can cause nephrotoxicity, hepatotoxicity and hepatocarcinogenicity in some experimental animals (Jaskiewicz et al., 1987; Gelderblom et al., 1991; 1992; Riley et al., 1994; Voss et al., 1995). More importantly, human esophageal cancer in the areas of Southern Africa and China has been correlated with the consumption of corn contaminated by *F. moniliforme* (Marasas et al., 1988a; Marasas, 1995; Cheng et al., 1985; Chu and Li, 1994).

Trichothecenes

Occurrence. Trichothecenes are a group of structurally similar sesquiterpenoid metabolites (Figure 1.3) produced mainly by *Fusarium spp.* (Bamburg and Strong, 1971; Bamburg, 1983; Ueno, 1983; Betina, 1989a). 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 80 trichothecenes have been isolated and characterized (Betina, 1989b). But only a few members of the trichothecenes, such as deoxynivalenol (DON, vomitoxin), nivalenol (NIV), T-2 toxin, HT-2 toxin, and diacetoxyscirpenol (DAS) are detected as major natural contaminants in cereal grains (Betina, 1989b).



Trichothecene	R_1	R_2	R_3	R_4	R_5
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
Diacetoxscirenol	OH	OAC	OAC	H	H
Scirpenetriol	OH	OH	OH	H	H
Deoxynivalenol	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).

Production of trichothecenes in cereal grains is primarily dictated by environmental conditions (Vesonder et al., 1978; Ueno, 1983; Cote et al., 1984). Wet and cool seasons in the spring and early summer favor *Fusarium* infection and trichothecene production in the field (Tuite et al., 1974; Shotwell et al., 1977; 1983; Ueno, 1983; CAST, 1989). In a year when wheat scab is epidemic, the occurrence of deoxynivalenol could be detected up to12 ppm in bulk grain samples (Hart and Schabenberger, 1998).

Toxicity. Symptoms in acute experimental trichothecene poisoning are reduced weight gain, anorexia, dermal irritation, abortion and hematological lesions such as leukopenia, anemia and lack of bone marrow development (Ueno, 1987; Rotter et al., 1996). Major human outbreaks of trichothecene toxicoses have been reported in Russia, Japan, China and India (Beardall and Miller, 1994). Consumption of trichothecene-contaminated grains causes headaches, chills, severe nausea and vomiting, diarrhea, visual disturbances and fatal hemorrhage in humans (Watson et al., 1984). Trichothecenes also modulate immune function and their ingestion over prolonged periods may reduce the immune resistence to infectious diseases, facilitate tumor growth by impairing immune surveillance (Pestka and Bondy, 1994), and cause autoimmune disease, as demonstrated in experimental deoxynivalenol-induced IgA glomerulonephritis (Dong et al., 1991; Dong and Pestka, 1993; Rasooly et al., 1994; Rasooly and Pestka, 1994).

Analytical methods for mycotoxin detection

The presence of mycotoxins in foods and feeds is potentially hazardous to the health of humans and animals. Although it is difficult to remove mycotoxins from human and animal diets, it is possible to decrease the risk of exposure through a rigorous program for monitoring these toxins in foods and feeds and diversion of the contaminated materials (Chu, 1992; Pestka et al., 1995). In general, analysis of mycotoxins in food and feed is a difficult task because only trace amounts of the toxins are present in the sample. The analysis is further complicated by the uneven distribution of mycotoxins in the sample and by sample matrix interference. Normally, the suspected contaminated commodity is first subjected to a rigorous sampling program (Campbell et al., 1986). The toxin is then extracted from the sample and subjected to a very extensive cleanup treatment to remove interfering substances before analysis is conducted. Current analytical methods for mycotoxins include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectroscopy (MS), immunoassay and bioassay.

For mycotoxins that contain a chromophore group with fluorescent properties, such as zearalenone, sample extracts after cleanup are generally subjected either to TLC or to HPLC to separate different structurally related compounds and contaminants (Chu, 1992; Richard et al., 1993). Identification of the toxin is made by comparing the retention factor (Rf) values or retention time in the chromatogram with the standard, whereas quantitative information is obtained from fluorescence intensity or absorbance of the elution peak (or spot). Some

mycotoxins, such as trichothecenes and fumonisins, do not have well-defined absorption maxima or potential for fluorescence under ultraviolet or visible light. Therefore, derivatization reagents are required to provide a fluorescent characteristic for their detection (Chu, 1992; Richard et al., 1993). An effective TLC or HPLC method can detect zearalenone, fumonisins and deoxynivalenol at 50 to 300 ppb (Richard et al., 1993). Higher sensitivity and selectivity of mycotoxin analysis can be achieved by using GC and GC/MS techniques although they require laborious sample cleanups, and expensive instruments (Pestka, 1988, 1994; Chu, 1991).

Immunoassays have been widely used for mycotoxin analysis at the pg and ng levels (Pestka et al., 1995). The most common immunoassay, enzyme-linked immunosorbent assay (ELISA), involves competition between a free mycotoxin in a sample extract with an enzyme-labeled mycotoxin for an antibody binding site. Mycotoxins can be analyzed directly in a liquid system such as milk and solid substrates can be extracted with methanol-water and analyzed directly or after dilution. Because mycotoxins alone are not antigenic, early studies focused on the development of methods for conjugation of mycotoxins to a protein or polypeptide carrier and optimization of conditions for antibody production in rabbits and in other animals (polyclonal antibody). With the advances in hybridoma technology, monoclonal antibodies against many important mycotoxins, including zearalenone, fumonisin B_1, and deoxynivalenol, were also generated (Pestka et al., 1995). Compared with other methods, mycotoxin immunoassays have several advantages

for rapid field tests, including high specificity, sensitivity, facile sample preparation, and ease of use (Pestka et al., 1995).

RECOMBINANT ANTIBODIES AND PEPTIDE MIMOTOPES

The wide application of mycotoxin immunoassay has led to a great demand for immunochemical reagents including low-cost antibodies with high affinity and defined specificity. With the advances in the field of recombinant antibody technology, it is now theoretically feasible to engineer low cost antibodies for mycotoxins with desirable affinity and specificity characteristics by using recombinant DNA technology to manipulate the basic domain structure of the immunoglobulin molecule and to express it in bacteria (Hoogenboom et al., 1992; Lerner et al., 1992). Peptide mimotopes to mycotoxins may also be selected from a random peptide library and used as alternative immunochemical reagents.

Native antibody

An immunoglobulin (Ig) molecule is composed of two heavy (H) and two light (L) protein chains (Figure 1.4). These protein chains contain defined Variable (V), Diversity (D, heavy chain only), Joining (J) and Constant (C) regions. The variable region is positioned at the amino-terminus and is composed of alternating framework (Fw) and hypervariable or complemetarity-determining region (CDRs). The V_L and V_H regions constitute the basic antibody binding site (paratope) which is dictated by the CDRs and, to a lesser extent, the Fw region. This domain structure encoded by variability (V) genes facilitates the extensive gene rearrangement that occur in B cells during *in vivo* immune selection. When newly generated B cells bind antigens via the Ig receptor, they differentiate (with T cell

help) to either short-lived Ig secreting plasma cells or long-lived memory cells that are involved in secondary/tertiary responses. During the selection process, highly frequent somatic mutations in the rearranged Ig genes of the memory B cells greatly improve antibody affinity.

Recombinant antibody and phage display

Recombinant antibody technology involves rearrangement or assembly of antibody V-genes, surface display of antibody, affinity selection, affinity maturation and soluble antibody production (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). But screening specific antibody from large combinatorial repertoires was a difficult task.

In 1985, a report was published describing the in-frame insertion of a DNA fragment encoding a section of the restriction endonuclease *Eco*RI into the gene of a minor capsid protein (g3p) of the filamentous bacteriophage fd (Smith, 1985). The filamentous fd bacteriophage is male specific, containing circular single-strand DNA (Model and Russel, 1988) . The phage genome (approximately 6500 nucleotides in length) encodes 10 proteins (Table 1.1). An important feature of filamentous fd phage is their ability to package longer genomes than the wild-type DNA, simply by further addition of g8p subunits. Fusion phages have been generated with insertions into either the minor capsid protein g3p for large insertions, or the major capsid

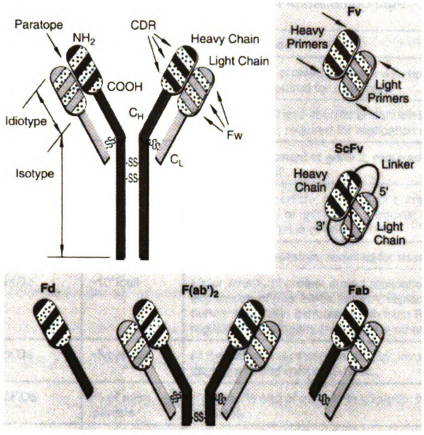


Figure 1.4. Antibody model structures showing subunit composition and domain distribution along the polypeptide chains. Fragments generated by proteolytic cleavage and /or recombinant technology appear in the shaded area (Pharmacia Biotech).

Table 1.1. The genes and proteins of fd bacteriophage

Gene	Protein size	Copy number	Protein function
1	35-40 KDa	few/cell	NS membrane protein; interacts with host thioredoxin; required for assembly.
2	46KDa	~10^3/cell	Site- and (+) strand-specific endonuclease/ topoisomerase; required for replication of RF.
X	12 KDa	~500/cell	N-terminal fragment of gene 2; required for replication of RF.
3	42 KDa	5/particle	At one end of particle; required for correct morphogenesis of unit-length particles; N-terminal part binds to F pilus of host cell.
4	49KDa	few/cell	NS membrane protein; required for assembly.
5	10KDa	~10^5/cell	Major structural protein during replication; controls expression of g2p; binds to DNA; replaced by g8p during assembly; controls switch from RF replication to progeny (+) strand synthesis.
6	12KDa	~5/particle	At the same end of particle as g3p; involved in attachment and morphogenesis.
7/9	3.5KDa	~5 of each /particle	At opposite end of particle to g3p/g6p; involved in assembly;
8	5KDa	2,700-3,000/ particle	Major coat protein.

protein g8p for short peptide insertions (Smith, 1993). In these 'fusion phages', the DNA encoding the insert protein is packaged as an integral part of the phage genome, while the chimeric protein product is expressed on the surface of the bacteriophage particle, allowing the simultaneous selection of a gene and its protein. Since 1985, this ingenious system linking both the DNA sequence and its expression product in a single phage particle has resulted in a rapidly expanding technology with diverse applications (O'Neil and Hoess, 1995; Hill and Stockley, 1996).

A rapidly expanding application of phage display technology has stemmed from the discovery that antibody fragments can be presented on the surface of bacteriophage in a biologically active form (McCafferty et al., 1990) and the repertoires of heavy- and light-chain variable region genes can be built by using polymerase chain reaction (PCR) (Orlandi et al., 1989). In the last decade, many antibody or antibody fragment genes have been cloned with phage display. Antibody phage display is accomplished by fusing the coding sequence of the antibody variable (V) regions to the amino terminus of the phage minor coat protein g3p (McCafferty et al., 1990). Expression of the fusion product and its subsequent incorporation into the mature phage coat results in the antibody being presented on the phage surface, while its genetic material resides within the phage particle. This linkage between antibody genotype and phenotype allows the enrichment of antigen-specific phage antibodies, using immobilized or labeled antigen. In a screening procedure, called "biopanning" (Marks et al., 1991), phage that display a relevant antibody will be retained on the surface coated with antigen, while non-

adherent phage will be washed away. Bound phages can be recovered from the surface, re-infected into bacteria and re-grown for further enrichment and, eventually, for binding analysis (Marks et al., 1991; Hawkins et al., 1992; Yuan et al., 1997).

Affinity and specificity in phage-displayed antibodies can be further enhanced by mimicking B cell somatic mutation via *in vitro* mutagenesis or by shuffling a repertoire of *in vivo* somatically mutated V-genes (Hoogenboom et al., 1992). The CDR regions are often the targets for antibody *in vitro* mutation and maturation. Using "CDR-walking", Yang et al. (1995) created independent libraries with mutations in one of four CDR regions, combined the selected variants in one clone, and obtained a 420-fold increase in affinity. The CDR3 of the heavy chain was demonstrated to be most important for *in vitro* affinity maturation (Thompson et al., 1996; Schier et al., 1996; Yang et al., 1995).

Chain shuffling is the sequential replacement of V_H and V_L region genes with repertoires of V-genes (Marks et al., 1992a). Chain shuffling of V-genes has been used successfully to obtain a 300-fold increase in the affinity of an anti-hapten antibody, originally isolated from a naive human scFv library (Marks et al., 1992b). The source of the V-genes for chain shuffling can be a library of light or heavy chain V-genes from non-immunized animals (naive library) (Marks et al., 1992a), from animals immunized with an antigen of interest (Kang et al., 1991) , or from other positive clones in the initial library (Clackson et al., 1991).

Several types of antibody construct have been displayed including the variable region of single heavy chains, and single-chain Fv fragments (scFv) (Hill

and Stockley, 1996). Fab fragments have also been displayed in a form in which either the light or heavy chain is expressed as a fusion on the bacteriophage surface with the complementary heavy or light chain associated via its secretion into the periplasm of phage-infected cells (Barbas et al., 1991; Hoogenboom et al., 1991).

Compared to Fab and Fv fragments, single-chain Fv fragments fold readily in the correct conformation and have improved stability because the variable heavy (V_H) and variable light chain (V_L) domains are covalently joined by a polypeptide linker (Raag and Whitlow, 1995). Generally, peptide sequences from known protein structures are used as linkers, whose lengths and conformations are compatible with bridging the variable domains of an scFv without serious steric interference (Bird et al., 1988; Takkinen et al., 1991; Mallender and Voss, 1994). The most widely used linker designs have sequences consisting primarily of stretches of Gly and Ser residues for flexibility, sometimes with charged residues such as Glu and Lys interspersed for solubility (Bird et al., 1988; Huston et al., 1988; Whitlow et al., 1993).

The stability of a particular scFv form depends on a number of factors, in addition to the nature of the V_H/V_L interface. These include the presence of antigen, the length of the linker, the solvent system, and temperature. Longer linkers were found to stabilize scFv antibodies (Whitlow et al., 1993; Holliger et al., 1993), while some organic solvents, such as ethylene glycol (Kortt et al., 1994), ethanol (Essig et al., 1993), and methanol (Yuan et al., 1997) were showed to destabilize scFvs. Some strategies generally applicable to protein stabilization may be used to

stabilize the scFv (Raag and Whitlow, 1995). These include the engineering of stronger hydrogen bonding interaction (Pantoliano et al., 1989), disulfide bonds (Glockshuber et al., 1990; Brinkmann et al., 1993; Luo et al., 1995; McCartney et al., 1995), metal binding sites (Wade et al., 1993), increasing buried hydrophobic areas, and adding or improving electrostatic interactions (Jones et al., 1992).

The majority of scFv applications involve a bifunctional task in which scFv binding to a target antigen allows high specific delivery of a second effector function (Raag and Whitlow, 1995). They might also be used as diagnostic reagents. Antibody fragments, specific for an important pollutant or a toxicant, could have considerable potential for the detection and removal of trace levels of contaminants. For example, a cloned antibody fragment to the herbicide paraquat has been shown to be capable of removing paraquat from aqueous environments (Graham et al., 1995). Antibody fragments with catalytic activities have also been developed using phage display (McCafferty et al., 1994; Janda et al., 1994; Baca et al., 1997).

Peptide mimotopes

The physical linkage between a vast library of variants with various binding activities to genes encoding each variant from phage display also permits the identification of peptides with certain binding functions. In 1990, three groups simultaneously reported the construction and screening of highly diverse libraries of unstructured random peptides (6-12 residues) displayed on fd phage or the related phage M13 as g3p fusions (Scott and Smith, 1990; Cwirla et al., 1990; Devlin et al., 1990). In a complementary strategy, peptide libraries fused to the

major coat protein, g8p, have also been used for epitope mapping (Felici et al., 1991). The increased valency of g8p fusions permits selection of lower-affinity ligands, while g3p library generally produce higher affinity ligands.

Peptide libraries can be structurally constrained by flanking the randomized segment with a pair of cysteines which form a disulfide bond (McLafferty et al., 1993; McConnell et al., 1994; Giebel et al., 1995). Cyclization can mimic the native structural context of epitope sequences by "freezing out" unproductive conformations. Structurally-constrained peptide libraries are valuable in identifying structural epitopes (Hoess et al., 1994).

In the last 8 years, random peptide libraries displayed on phage have been extensively used to select for peptide ligands specific for a variety of targets (antibodies, receptors, enzymes, etc.) by using multiple cycles of *in vitro* selection (biopanning) and *in vivo* amplification (Parmley and Smith, 1988; Smith and Scott, 1993). Phage-display libraries have also been used to identify peptide mimics of non-peptide ligands (Devlin et al., 1990; Scott et al., 1992; Oldenburg et al., 1992; Hoess et al., 1993; Adey et al., 1995).

PLANTIBODIES

Antibodies historically have been considered as part of mammalian defense systems. With the advances in antibody gene cloning and plant genetic transformation, however, the ability to produce antibodies and antibody fragments in plants presents some very interesting possibilities. These so-called "plantibodies" can be produced on an agricultural scale for therapeutic or diagnostic use (Smith 1996). They also provide the opportunity to manipulate plant traits or reduce pathogenic infections in plants through antibody-mediated modifications of antigen activity *in planta* (Hiatt, 1990; Schots et al., 1992; Tavladoraki et al., 1993; Voss et al., 1995; Whitelam, 1995). The binding and retention capacity of antibodies could also be used for the isolation and processing of environmental contaminants, a process known as biofiltration (Hiatt, 1990).

For expression of complete antibodies or Fab fragments, in one approach, light- and heavy-chain genes were separately introduced into plants. Plants expressing light and heavy chains were then crossed and assembled antibodies were detected in the F1 generation (Hiatt et al., 1989; Ma et al., 1994; Ma et al., 1995). Alternatively, double transformations were performed or the genes for expressing light and heavy chains were cloned into one expression cassette (De Neve et al., 1993; Düring et al., 1990; Voss et al., 1995). The expression of a functional scFv antibody in plants is relatively simple because it requires only correct folding and it does not have to be assembled like complete antibodies.

Antibodies have been successfully expressed in plants both intracellularly and intercellularly. Full length antibodies in algal cytoplasm can form correctly folded antigenic binding sites even though the relatively high reducing conditions in the cytoplasm do not allow formation of disulfide bonds (Stieger et al., 1991). However, functional full length antibodies in the cytoplasm have not yet been reported in higher plants. It is possible that the cytoplasm of algae is less reducing and contains the chaperonins necessary for folding of full-size immunoglobulins (Smith, 1996). In contrast, the expression of scFv antibodies in the plant cytoplasm has been more successful. Some groups have reported successful expression of scFv antibodies in the cytoplasm (Owen et al., 1992; Tavladoraki et al., 1993), although others failed to get any antibody accumulation unless an endoplasmic reticulum (ER) retention signal (a tetrapeptide KDEL) was included (Schouten et al., 1996).

The production of secreted antibodies in plants normally requires a signal sequence, although the origin of the signal sequence does not appear to be critical, since plant, mouse, and yeast sequences can be used (Düring et al., 1990; Hein et al., 1991). The signal sequences not only direct the antibody fragments to plant secretory pathways but also stabilize the antibody and increase the yield (Hiatt et al., 1989; Firek et al., 1993). Although plant cell walls are believed to have exclusion limits of between 17 and 60 KDa (Carpita et al., 1979; Tepfer and Taylor, 1981; De Wilde et al., 1996), the results of De Wilde et al. (1996) suggest that the mesophyll cell walls of *Arabidopsis* are permeable to intact IgG molecules, which have a molecular weight of 146 KDa. These findings are of particular interest to any application where extracellular antigen binding is desired. When a suitable signal

sequence was used, antibody was also targeted into plant nuclei (De Neve et al., 1993).

Antibody can also be targeted to specific plant organs. Fiedler and Conrad (1995) directed active scFv molecules into tobacco seeds and stored them stably for one year at room temperature. Such a system would be valuable for long-term storage and easy delivery of large quantities of antibodies for passive immunization.

As in mammals, γ-heavy chain expressed in plant has N-linked glycosylation, in which a high-mannose core oligosaccharide is attached to the asparagines contained within the canonical Asn-X-Ser/Thr sequence (Hein et al., 1991). However, the composition of terminal residues on the plant glycan appears to be distinct from that in mammals. The differences in glycosylation patterns of plant antibodies had no effect on antigen binding or specificity (Hein et al., 1991). However, for human therapy, the presence of plant-specific glycan might increase the immunogenicity of the recombinant antibody.

Since the first plantibody was expressed in tobacco (Hiatt et al., 1989), a number of antibodies or antibody fragments have been successfully produced in plants for various purposes (Table 1.2). Full length antibodies have been expressed in plants with the aim of producing large quantities for use in immunotherapy. Indeed, the production of multimeric secretory antibodies (Ma et al., 1995) and the ability to store antibodies long-term in seeds or other organs (Fiedler and Conrad, 1995) showed great promise in immunotherapy and passive immunization by using plantibody. In an effort to confer viral resistance to plants, Tavladoraki et al. (1993) expressed an scFv antibody against the artichoke mottled crinkle virus in the

cytoplasm of tobacco. These transgenic plants were specifically protected from viral attack, in that they had a reduced incidence of infection and showed delayed symptom development (Tavladoraki et al., 1993). The possibility of using full length antibodies to interfere with fungal pathogenesis in the apoplast has also been examined (van Engelen et al., 1994). With the intracellular expression of specific antibody fragments in plants, plant physiological traits such as phytochrome-mediated seed germination (Owen et al., 1992) and abscisic acid-controlled physiology (Artsaenko et al., 1995; Phillips et al., 1997), have successfully been altered.

The feasibility of producing specific antibody or antibody fragments in plants allows us to speculate that the expression of mycotoxin specific antibody in plants may be useful to reduce plant and animal diseases associated with the mycotoxin by antibody-mediated neutralization and degradation.

Table 1.2. Antibody-derived molecules produced in transgenic plants

Antibody form	Antigen	Plant species	Reference
Single domain (sAb)	Substance P (neuropeptide)	*Nicotiana*	Benvenuto et al., 1991
Single chain Fv (scFv)	Phytochrome	*Nicotiana*	Firek et al., 1993; Owen et al., 1992
Single chain Fv (scFv)	Artichoke mottled crinkle virus coat protein	*Nicotiana*	Tavladoraki et al., 1993
Single chain Fv (scFv)	Beet necrotic yellow vein virus coat protein	*Nicotiana*	Fecker et al., 1996
Single chain Fv (scFv)	Abscisic acid	*Nicotiana*	Artsaenko et al., 1995 Phillips et al., 1997
Fab; IgG (κ)	Human creatine kinase	*Nicotiana* *Arabidopsis*	De Neve et al., 1993
IgG (κ)	Transition-state analogue	*Nicotiana*	Hiatt et al., 1989; Hein et al., 1991
IgG (κ)	Fungal cutinase	*Nicotiana*	van Engelen et al., 1994
IgG (κ/λ)	TMV	*Nicotiana*	Voss et al., 1995
IgG (κ) and IgG/A hybrids	Streptococcus mutants adhesin	*Nicotiana*	Ma et al., 1994
Secretory IgA/G	S. mutants adhesin	*Nicotiana*	Ma et al., 1995
IgM (λ)	NP (4-hydroxy-3-nitrophenyl) acetyl hapten	*Nicotiana*	Düring et al., 1990
IgG-like IgM	Nematode stylet secretions	*Nicotiana*	Baum et al., 1996 Rosso et al., 1996

PART II

MOLECULAR CLONING, EXPRESSION AND CHARACTERIZATION OF A FUNCTIONAL SINGLE-CHAIN Fv (scFv) ANTIBODY TO THE MYCOTOXIN ZEARALENONE

ABSTRACT

MOLECULAR CLONING, EXPRESSION AND CHARACTERIZATION OF A FUNCTIONAL SINGLE-CHAIN Fv (scFv) ANTIBODY TO THE MYCOTOXIN ZEARALENONE

The heavy and kappa light-chain variable region genes of an anti-zearalenone hybridoma cell line (2G3-6E3-2E2) were isolated by polymerase chain reaction (PCR) and joined by a DNA fragment encoding the linker peptide $(Gly_4Ser)_3$ to generate a single-chain Fv (scFv) DNA fragment. The scFv DNA fragment was cloned into a phagemid (pCANTAB5E) and expressed as a fusion protein with an antibody recognition sequence (E-tag) and phage M13 coat protein g3p in *Escherichia coli* TG1. In the presence of helper phage M13K07, the scFv fusion protein was displayed on the surface of recombinant phage. High affinity scFv phage antibodies were enriched through panning-elution selection in microtiter wells coated with zearalenone-ovalbumin conjugate. The selected recombinant phages were used to infect *E. coli* HB2151 for the production of soluble scFv antibodies. One selected clone (pQY1.5) in HB2151 secreted a soluble scFv antibody (QY1.5) with high zearalenone binding affinity (IC_{50}=14 ng/ml), similar to that of the parent monoclonal antibody in a competitive indirect ELISA. However, scFv QY1.5 exhibited higher cross-reactivity with zearalenone analogues and had increased sensitivity to methanol destabilization when compared with the parent monoclonal antibody. Nucleotide sequence analyses revealed that the light chain portion of scFv QY1.5 had a nucleotide sequence identity of 97% to a mouse germline gene

V_K23.32 in mouse kappa variable light chain subgroup V, whereas the heavy chain nucleotide sequence was classified as mouse heavy chain subgroup III (D) but without any closely related members having highly homologous complementary-determining regions (CDRs) sequences. The potential of soluble scFv QY1.5 for routine screening of zearalenone and its analogues was demonstrated with zearalenone spiked corn extracts. A portion of the research presented here has been published (Yuan, Q., Clarke, J. R., Zhou, H. R., Linz, J. E., Pestka., J. J., and Hart, L. P. 1997. Molecular cloning, expression and characterization of a functional single-chain Fv antibody to the mycotoxin zearalenone. Appl. Environ. Microbiol. 63:263-269).

INTRODUCTION

Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone] (Figure 1.1) is a mycotoxin produced by members of the genus *Fusarium* after infection of corn and small grains (Mirocha et al., 1971; 1977; Hart et al., 1982). When fed to animals, the compound causes hyperestrogenism with symptoms such as enlargement of the uterus and nipples, vulvar swelling, vaginal prolapse, and infertility (Mirocha and Christensen, 1974; Hidy et al., 1977). As a consequence of these estrogenic effects, there is a need for routine screening of agricultural commodities used for human and animal consumption.

Methods for the analysis of zearalenone in food and feeds include thin-layer chromatography (TLC) (Gimeno, 1983; Scott et al., 1978; Swanson et al., 1984), gas-liquid chromatography (Scott et al., 1978; Thouvenot and Morfin, 1979), high-pressure liquid chromatography (Scott et al., 1978; James et al., 1982; Turner et al., 1983) and more recently immunoassays (Thouvenot and Morfin, 1983; Liu et al., 1985; Warner et al., 1986; Dixon et al., 1987; Teshima et al., 1990; Bennett et al., 1994). Compared with other methods, immunoassays have several advantages for rapid field tests including high specificity, sensitivity, facile sample preparation and ease of use (Pestka et al., 1982). However, the development of antibodies requires the use of animals, specialized cell culturing facilities, and an extensive commitment of time and labor. Production of monoclonal antibodies from hybridoma cell lines also requires specialized cell culture facilities and usually is time consuming.

Advances in the field of recombinant antibody technology provide an alternative means to engineer low cost antibodies with desirable affinity and specificity by enabling one to manipulate the basic domain structure of the immunoglobulin (Ig) molecule. One of the most successful approaches is to display scFv antibodies on filamentous phage (McCafferty et al., 1990; Barbas et al., 1991; Clackson et al., 1991; Marks et al., 1991; 1992; Griffiths et al., 1993). ScFv is an antigen-binding protein, composed of an immunoglobulin heavy chain variable domain (V_H) and a light chain variable domain (V_k or V_λ) joined together by a flexible peptide linker (Figure 1.4). When expressed with phage protein g3p (fd g3 protein of phage M13K07) as a fusion protein, a high affinity scFv producing phage clone can be enriched by a procedure called "panning" (Marks et al., 1991; Hawkins et al., 1992). In this part, we describe the molecular cloning, expression and characterization of a functional scFv with high specificity and sensitivity to zearalenone and its analogues, and the application of this scFv to the analysis of zearalenone in spiked corn samples.

MATERIALS AND METHODS

Materials, strains and general methods

All chemicals and organic solvents were reagent grade or better. Zearalenone was generously supplied by International Minerals and Chemicals Corp. (Terre Haute, IN). Zearalenone-ovalbumin and -keyhole limpet hemocyanin (KLH) conjugates were prepared as described by Dixon et al. (1987) (Figure 2.1). Anti-zearalenone hybridoma cell line 2G3-6E3-2EZ (Dixon et al., 1987) was kindly supplied by M. A. Abouzied (Neogen Company, Lansing, MI). Plasmid pCANTAB5E, *E. coli* TG1 and HB2151, M13K07 helper phage, horseradish peroxidase conjugated sheep anti-M13 antibody and mouse anti-E tag antibody were obtained from Pharmacia Biotech (Piscataway, NJ). Cell culture components and reagents, goat anti-mouse IgG peroxidase and rabbit anti-mouse IgG alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum, oligo(dT) cellulose columns and *Not* I restriction enzyme were supplied by Gibco BRL (Gaithersburg, MD). PCR amplification primers were synthesized by Gibco BRL(Gaithersburg, MD). The restriction enzyme *Sfi* I was purchased from New England BioLabs (Beverly, MA). All DNA manipulations, if not described, were carried out by standard procedures (Sambrook et al., 1989).

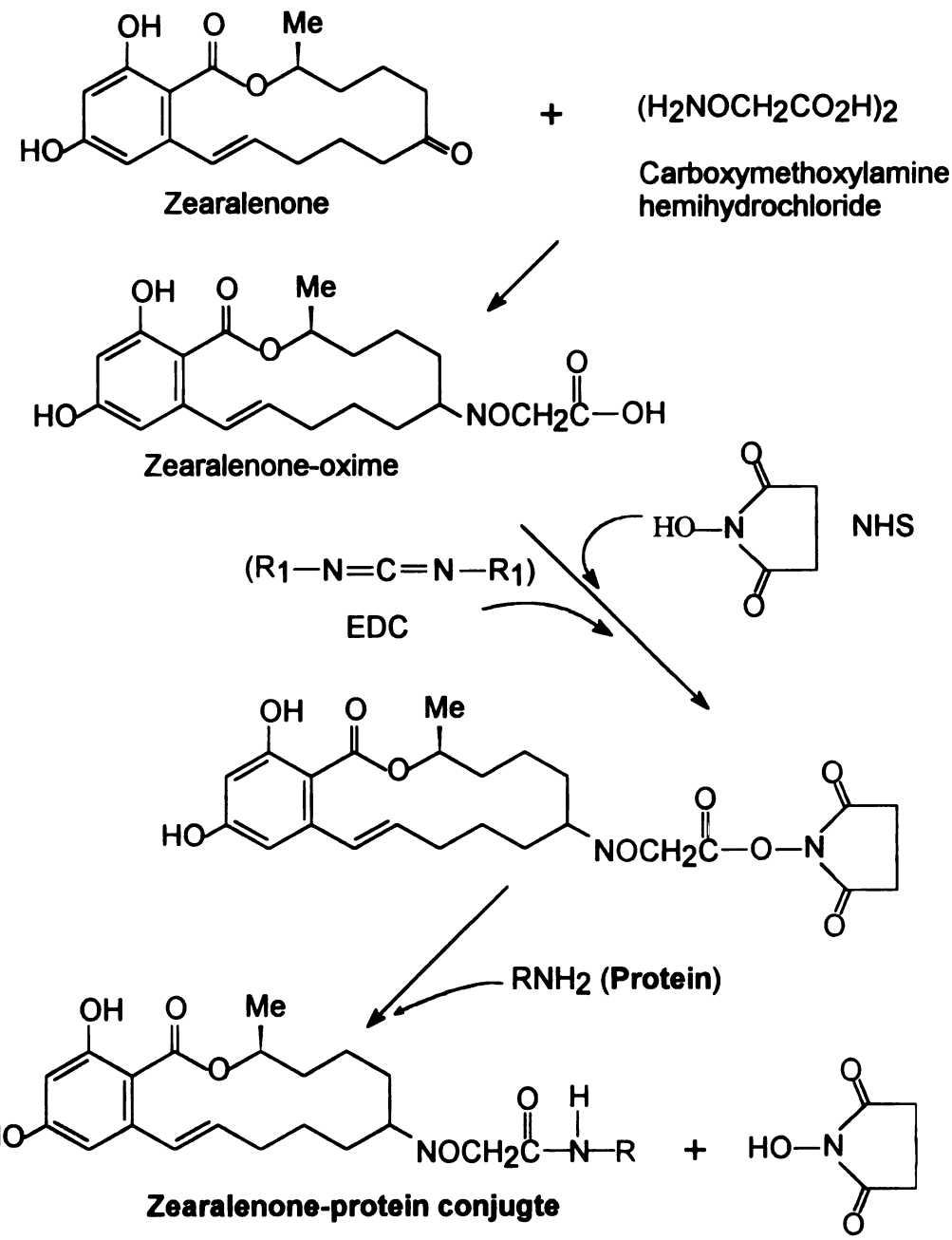


Figure 2.1. Preparation of zearalenone immunogens through oxime chemistry (based on Thouvenot and Morfin, 1983).

mRNA isolation

Hybridoma cell line 2G3-6E3-2EZ producing anti-zearalenone antibody (Dixon et al., 1987) was cultured in macrophage conditioned DMEM medium containing 20% (v/v) fetal bovine serum supplemented with 100 U/ml penicillin/streptomycin in a 5% CO_2 humidified chamber. After the cells were grown to a density of 10^6 cells/ml, they were pelleted by centrifugation at 1000 x *g* for 5 min. For total RNA isolation, the pelleted cells were homogenized in RNA STAT-60 (TEL-TEST "B", Inc., Friendswood, TX) at $5x10^6$ cells/ml, followed by chloroform extraction and isopropanol precipitation. mRNA was purified by affinity chromatography with an oligo (dT)-cellulose column according to manufacturer instructions.

cDNA synthesis, PCR amplification and scFv cloning

First strand cDNA was synthesized from mRNA template with M-Mulv reverse transcriptase and random hexadeoxyribonucleotide [pd$(N)_6$] primers (Pharmacia Biotech, Piscataway, NJ). The variable regions of heavy chain (V_H) and kappa light chain (V_K) were amplified from first strand cDNA using *Taq* DNA polymerase with 30 cycles of PCR (94°C, 1 min; 55°C, 1 min; 72°C, 2 min). The primers used in the PCR amplification were based on Orlandi et al. (1989) and Clackson et al. (1991), and modified for facilitating ligase-free UDG-ligation (Nisson et al., 1991) (Table 2.1). VHBACK and du-VHFOR were used for amplification of V_H; dU-VKBACK and VKFOR were used for amplification of V_K. A 93-bp DNA linker (Pharmacia Biotech, Piscataway, NJ), containing a sequence encoding a short

flexible peptide, $(Gly_4Ser)_3$, was also amplified with LINKBACK and du-LINKFOR. After gel purification with a QIAEX gel extraction kit (QIAGEN Inc., Chatsworth, CA), 25ng linker DNA and 75ng V_K DNA were mixed and treated with 1 unit uracil DNA glycosylase (UDG) (USB, Cleveland, OH) at 37°C in a 20µl reaction with 1x PCR buffer for 1.5 hr. The UDG treated fragments were then scaled up to a 40µl PCR reaction without primers and cycled 15 times (94°C, 1 min; 60°C, 1 min; 72°C, 2 min) for joining the linker DNA with V_K DNA. The joined linker-V_K DNA was amplified in a 100µl PCR reaction (94°C, 1 min; 60°C, 1 min; 72°C, 2 min) for 30 cycles with 75 pmol each VKFOR and du-LINKBACK. Gel-purified linker-V_K DNA fragment (40 ng) was joined with V_H DNA (40 ng) into scFv (V_H-linker-V_K) DNA fragment in a 20 µl PCR fill-in reaction after UDG treatment. The assembled scFv DNA was amplified with 75 pmol each VHBACK and VKFOR in a 100 µl PCR amplification reaction for 30 cycles as described above. Finally, the assembled scFv products were gel-purified and reamplified with restriction site tagged primers (RS Primers Mix) (Pharmacia Biotech, Piscataway, NJ) to append an *Sfi*I site on the 5' end and a *Not*I site on the 3' end of the scFv DNA. The scFv DNA products were digested with *Sfi*I and *Not*I restriction enzymes, gel purified and then ligated into the phagemid pCANTAB5E (digested with these same enzymes).

Table 2.1: Sequences of oligonucleotide PCR primers for PCR amplification of V_H, V_K, and linker DNA*

VHBACK:	5' AGGTSMARCTGCAGSAGTCWGG-3'
dU-VHFOR:	5' UGAGGAUACGGUGACCGUAGUACCUUGGCCCC-3'
dU-VKBACK:	5' GACAUCGAGCUCACUCAGUCUCCA-3'
VKFOR:	5' CCGTTTBAKYTCCARCTTKGTSCC-3'
LINKBACK:	5' GGTACTACGGTCACCGTATCCTCAGGTG-3'
dU-LINKBACK:	5' GGUACUACGGUCACCGUAUCCUCAGGUG-3'
dU-LINKFOR:	5' AGACUGAGUGAGCUCGAUGUCCGAUCC-3'

*: U=deoxyuracil; R=A or G; Y=C or T; M=A or C; K=T or G; S=C or G; W=A or T; B=T or C or G;

ScFv antibody expression and panning-elution selection

To display scFv as a fusion protein with E-tag and M13 g3p (there is an amber stop codon between E-tag and fd g3 in pCANTAB5E), the ligated products were used to transform *E. coli* TG1 competent cells which is an amber suppressor strain (*sup*E). Transformed cells were plated on SOB medium (Sambrook et al., 1989) containing 100 μg/ml ampicillin and 2% glucose and incubated at 30°C overnight. Colonies were pooled and infected with M13K07 helper phage in 2x YT medium (Sambrook et al., 1989) containing 100 μg/ml ampicillin and 50 μg/ml kanamycin to rescue the phagemid with its scFv gene inserts and to display scFv fusion protein on the surface of the recombinant phages. The recombinant phages from the supernatant were filtered through a 0.45 μm-pore-size filter.

For panning-elution selection (Figure 2.2), wells of sterile polystyrene Immulon-4 microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight (4 °C) with 100 μl of zearalenone-ovalbumin conjugate (10 μg/ml) in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Plates were washed four times by filling each well with 300 μl of 0.05% (v/v) Tween-20 in 0.01 M PBS (PBS-T) and aspirating the contents. Non-specific binding was blocked by addition of 300 μl "blocking buffer" [1% ovalbumin (w/v) dissolved in PBS (PBS-OVA)] to each well for 1 hr at 37 °C and then washing 6 times with PBS-T. Recombinant phages (about 5×10^{10} pfu/ml) were then added to the wells (100 μl/well) of a zearalenone-ovalbumin coated plate. The plates were incubated for 2 hr at 37 °C followed by ten washes with PBS and ten washes with PBS-T. One hundred microliters of free

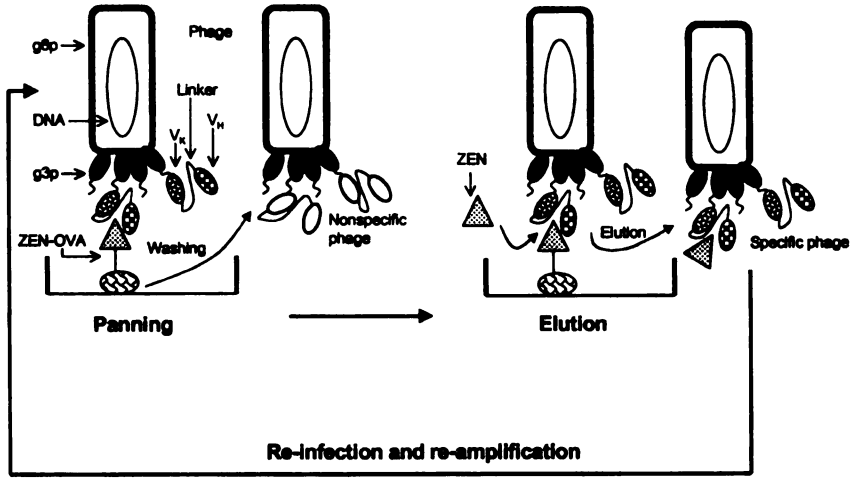


Figure 2.2. Panning-elution selection procedure for anti-zearalenone scFv phage in zearalenone-ovalbumin (ZEN-OVA) coated Immunlon-4 microtiter wells.

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zearalenone (0, 0.01, 0.1, 1.0, 5.0, 10.0, 15.0, and 50.0 μg/ml) diluted with PBS containing 1.5% methanol were added to the wells and incubated at 37 °C for 1 hr with rotary shaking (150 rpm) to elute the bound phages. The zearalenone eluted phages were collected and passed through a 0.45 μm-pore-size low-protein-binding syringe filter (Gelman Sciences, Ann Arbor, MI). These zearalenone specific phages were used to reinfect *E. coli* TG1 cells for subsequent rounds of selection. The affinities of recombinant phage antibodies from pooled or individual colonies after each round of selection was tested with a modified CI-ELISA described below.

Recombinant phages from selected clones were used to infect *E. coli* HB2151 for production of soluble scFv antibodies. Briefly, the infected *E. coli* HB2151 cells were cultured in SB medium (35 g/l tryptone, 20 g/l yeast extract, 5 g/l NaCl) supplemented with 100 μg/ml ampicillin at 30°C with 250 rpm shaking until they reached an A_{600} of 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to 1 mM final concentration and the cells were incubated on a shaker at 30 °C overnight. Supernatants containing the extracellular soluble scFv antibodies were separated from the cell pellets by centrifugation at 1500 x *g* for 15 min, and filtered through a 0.45 μm-pore-size filter. The affinity of soluble scFv antibodies was detected with a modified CI-ELISA described below.

Competitive Indirect ELISA (CI-ELISA)

Several CI-ELISAs were developed and used to determine the sensitivities and specificities of the scFv antibodies (phage-associated or soluble forms). Briefly, microtiter plates were coated and blocked as described above. Next, 50 μl of

zearalenone standard (or analogues) at various concentrations dissolved in PBS containing 1% methanol was added to the coated wells and simultaneously incubated with 50 μl of phage-associated (about 5 x 10^{10} pfu/ml in 2x YT medium) or soluble scFv antibody (various dilutions with PBS-OVA) or parent monoclonal antibody (diluted 1:5 with PBS-OVA) from the supernatant of hybridoma cell culture for comparison. The plates were incubated for 1 hr at 37 °C then washed 6 times with PBS-T. The amount of bound phage-associated scFv antibody was determined by the addition of 100 μl anti-M13-HRP conjugate diluted 1:2500 with PBS-OVA. The amount of soluble scFv antibody bound was determined by incubation with 100 μl mouse anti-E tag (diluted 1:2500 with PBS-OVA) at 37°C for 1 hr followed by washing (six times) and a subsequent incubation with goat anti-mouse IgG HRP conjugate (diluted 1:500 with PBS-OVA). The bound monoclonal antibody was also detected by the addition of 100 μl goat anti-mouse IgG HRP conjugate (diluted 1:500 with PBS-OVA). The plates were incubated for 1 hr at 37°C and then washed 6 times with PBS-T. The amounts of anti-M13-HRP and goat anti-mouse IgG HRP conjugates bound were assessed by the addition of 100 μl of 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid) (ABTS) substrate (Pestka et al., 1982). The plates were incubated for 15 min for color development and the absorbances were determined at 405 nm with a microtiter plate reader (Molecular Devices Corporation, Menlo Park, CA).

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SDS-PAGE and Western blot analysis

Supernatants, containing the extracellular soluble scFv antibodies in SB medium from *E. coli* HB2151 cell culture, were precipitated with trichloroacetic acid (TCA) at a final concentration of 10% (w/v) on ice for 20 min. After centrifugation in a microcentrifuge at 14,000 rpm for 10 min, the pellet was resuspended in 15 μl of 0.5 M Tris buffer (pH 8.0), heated for 5 min at 95°C after adding 5 μl loading buffer (4 x), and subjected to SDS-12% polyacrylamide gel electrophoresis with a 0.5 mm thick gel in a Bio-Rad Mini-Protein II Eletrophoresis System (Bio-Rad Laboratories, Hercules, CA). Prestained low range SDS-PAGE standards (Bio-Rad laboratories) were used to calibrate protein mobilities. Two identical gels were run in parallel. After separation, the protein bands in one gel were stained with Coomassie brilliant blue G-250, those in the other gel were transferred to a nitrocellulose membrane (Schleicher & Schuell, Kneene, NH) in a Multiphor II Electrophoresis System (LKB Produkter AB, Bromma, Sweden). The transblotted membrane was probed with anti-E tag antibody (about 8 μg/ml in PBS-OVA containing 0.05% Tween 20) followed by incubation with rabbit anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Company, St. Louis, MO) as described by manufacturer's instructions. Nitroblue tetrazolium and 5-bromo-4-chloro-3- indolyphosphate-*p*-toluidine (NBT/BCIP)(Pierce, Rockford, IL) were used as the substrate for visualization.

Sequence analysis

Plasmid DNA of anti-zearalenone producing clones was isolated from *E. coli* HB2151 by alkaline lysis. V_H and V_K DNA portions in the plasmid were sequenced on both strands with the pCANTAB5 sequence primer set (Pharmacia Biotech, Piscataway, NJ) by using *Taq* cycle sequencing and dye terminator chemistry at the MSU Sequencing Facility. Sequence comparisons were performed by searching the Kabat Database at the Internet Web site (http://immuno.bme.nwu.edu).

CI-ELISA for spiked corn extracts

The procedures for extraction of corn and spiking were similar to those described by Liu *et al.* (1985). Briefly, zearalenone-free homogeneous ground corn was extracted with five volumes (w/v) of methanol-water (70:30) for 5 min, with constant stirring. The mixture was immediately filtered through a Whatman No. 4 filter paper and the filtrate was spiked directly with zearalenone, or the filtrate was diluted 2 times with 1x PBS and then spiked with zearalenone. The spiked extracts were then subjected to CI-ELISA using the soluble scFv antibodies and monoclonal antibody in a similar procedure as described above with the following modifications. (i) Since anti-E-tag antibody, which was used as a second antibody to detect the bound scFv antibodies, exhibited nonspecific binding to methanol treated carrier protein ovalbumin, zearalenone-KLH conjugate was used in the CI-ELISA for methanol extracted corn samples. (ii) Coated wells were blocked by PBS containing 10% nonfat dry milk. (iii) Antibodies were diluted in PBS containing 10%

nonfat dry milk. The spiking experiment was repeated once with the same procedure.

Nucleotide sequence accession numbers

The sequences determined for V_H and V_K from this clone are available under GenBank database accession numbers U74671 and U74672, respectively.

RESULTS

PCR amplification

V_H and V_K cDNA from hybridoma cell line 2G3-6E3-2EZ were amplified by PCR and assembled into an scFv encoding DNA fragment (Figure 2.3). Amplification of V_K generated a major DNA fragment with the expected length (about 324 bp), while V_H generated an expected 340 bp fragment and two other closely migrating fragments. The PCR assembly of linker-V_k produced two major closely migrating fragments (lane 5 in Figure 2.3) with approximately the expected size plus a minor band much larger than the expected product. The two major linker-V_k fragments, which might be generated by alternative priming of the linker DNA with its nearly identical repeats, were collectively isolated, purified, and then PCR assembled with V_H products (the mixture of three major bands in lane 2 of Figure 2.3) to form the scFv fragments in lane 6 of Figure 2.3. For the assembly of scFv, a preliminary experiment with a one step PCR fill-in reaction failed to join V_H, linker, and V_K . The modified two-step procedure described in Materials and Methods greatly improved the joining.

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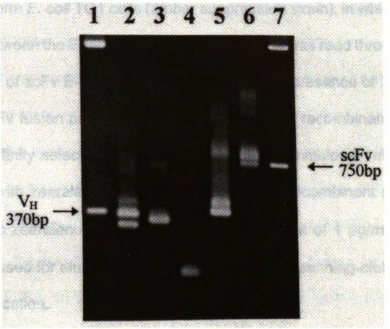

Figure 2.3. V_H and V_K amplification and assembly of scFv. Lanes are: (1): V_H marker (pUC18/V_H, *Eco*R I/*Xba* I digested, Pharmarcia Biotech); (2): V_H PCR product; (3): V_K PCR product; (4): Linker PCR product; (5): Linker-V_K PCR product; (6): V_H-Linker-V_K (scFv) PCR product; (7): scFv marker (pUC18/A10B, *Sfi* I/ *Not* I digested, Pharmacia Biotech).

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Phage display of scFv antibody and panning-elution selection

Two major scFv DNA fragments in lane 6 of Figure 2.3 were collectively isolated, purified, digested, then ligated into pCANTAB5E, and translationally fused with the E-tag DNA fragment (Figure 2.4). The resulting library, named pQY1, was used to transform *E. coli* TG1 cells (amber suppression strain), in which the amber stop codon between the E-tag DNA sequence and fd g3 was read through, allowing the production of scFv E-tag-g3p fusion protein. In the presence of helper phage (M13K07), scFv fusion products were displayed on the recombinant phage tips, allowing for affinity selection with panning-elution. In Immulon-4 microtiter plate wells coated with zearalenone-ovalbumin, the bound recombinant phages were eluted by free zearalenone at a minimum concentration of 1 μg/ml. The eluted phages were used for elution in the sequential round of panning-elution selection after re-amplification.

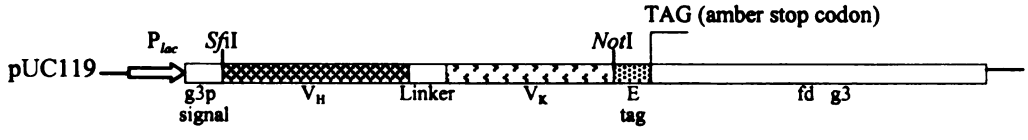

Figure 2.4. pQY1 construct. PCR amplified scFv was cloned into *Sfi* I and *Not* I sites of pCANTAB5E, a pUC119 based vector from Pharmacia Biotech. The linker DNA sequence was 5'GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTG GCGGATCG-3', which encoded a short peptide [$(Gly_4Ser)_3$].

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Prior to affinity selection, the pooled recombinant phage library exhibited very low zearalenone-binding activity (Figure 2.5). After one round of affinity selection, a clear enrichment for scFv clones expressing zearalenone-binding was observed (Figure 2.5). The IC_{50} (concentrations of free zearalenone required for inhibition of binding by 50%) for preselection phage, first round selected phage and second round selected phage were >1000, 31, and 28 ng/ml, respectively. The second-round selection mainly increased yield of functional scFv fusion protein. Following each round of panning-elution selection, individual clones (in TG1) were randomly isolated and the scFv DNA insert in these clones was confirmed by PCR amplification and restriction digestion with *Sfi*I and *Not*I. Three individual clones from each selection group were rescued with helper phage M13K07 for scFv phage display. When the resultant recombinant phages were tested for zearalenone binding in CI-ELISA, all three preselection clones were negative whereas those from first and second round selection were positive.

Soluble scFv antibody production and characterization

In the nonsuppressor strain, *E. coli* HB2151(*sup*E^-), the amber stop codon between E- tag and fd g3 in scFv clones is recognized as a stop codon and a soluble scFv-E tag fusion protein is produced as a consequence. Three individual recombinant phage clones (the same clones tested above for zearalenone binding) from each selection group were used to infect HB2151. The expression of scFv E-tag fusion protein in the resultant HB2151 clones was induced by IPTG, and soluble scFv antibodies were secreted to the culture supernatants.

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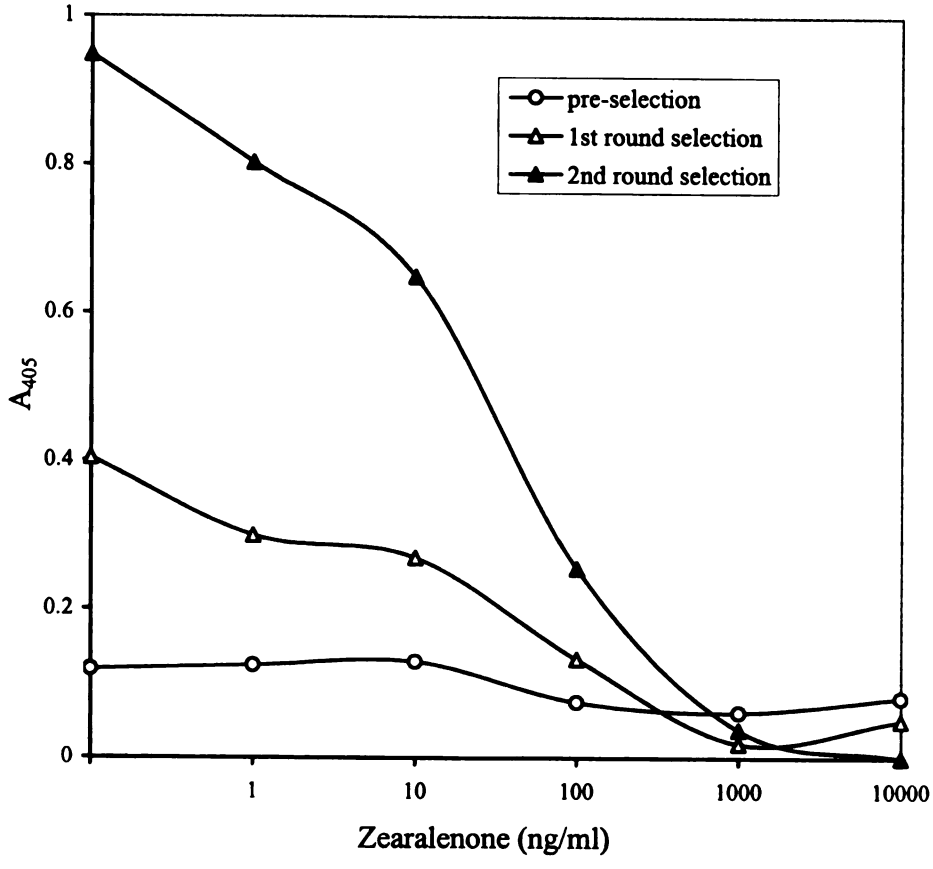

Figure 2.5. Zearalenone-binding activities of recombinant phage following each round of panning-elution selection measured by CI-ELISA in zearalenone-ovalbumin coated Immulon-4 microtiter wells. Bound phages were detected by horseradish peroxidase conjugated sheep anti-M13 antibody.

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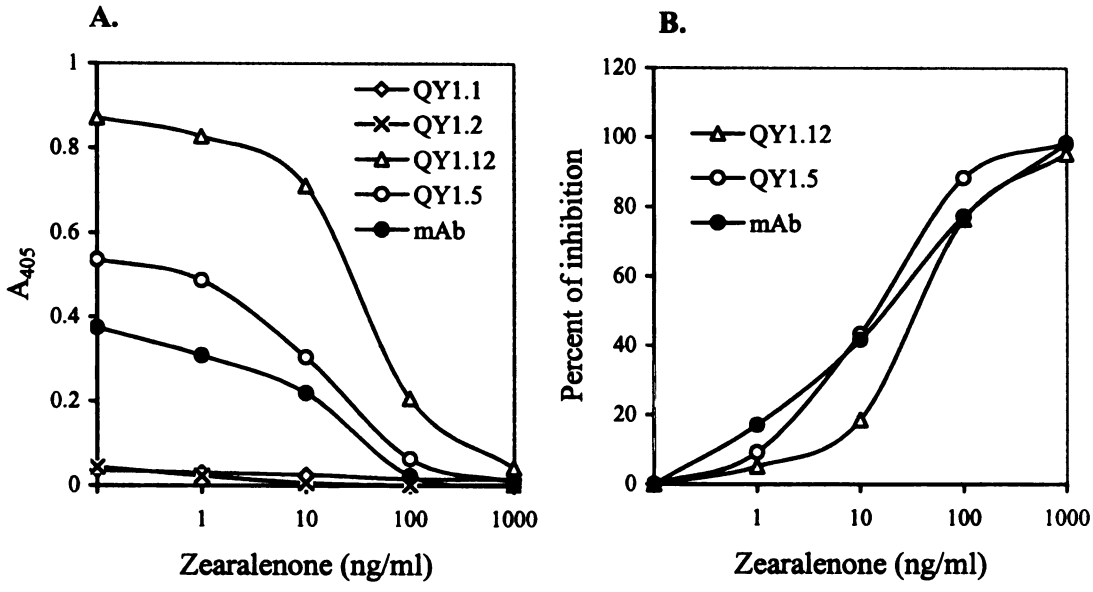

Figure 2.6. Sensitivity of soluble scFv antibodies tested in CI-ELISA in zearalenone-ovalbumin coated Immulon-4 microtiter wells. QY1.1 and QY1.2 (no dilution) were from preselection; QY1.5 (5x dilution) was from first round of panning-elution selection; QY1.12 (10 x dilution) was from second round of panning-elution selection; mAb (supernatant of hybridoma cell culture) was diluted 5 times. Bound soluble scFv antibodies were detected by mouse anti-E tag antibody, followed by horseradish peroxidase conjugated goat anti-mouse IgG. A: Absorbance reading at 405 nm. B: Percent of binding inhibition determined by dividing sample absorbance by control absorbance x 100.

Table 2.2: Comparison of cross-reactivities between scFv antibody and mAb toward zearalenone analogues

Analogue	mAb		scFv QY1.5	
	IC_{50} (ng/ml)	Cross-reactivity* (%)	IC_{50} (ng/ml)	Cross-reactivity (%)
Zearalenone	17	100	14	100
α-zearalenol	66	26	17	82
β-zearalenol	159	11	54	26
α-zearalanol	212	8	23	62
β-zearalanol	175	10	54	26

*: Cross-reactivity defined as $(IC_{50\ zearalenone})/(IC_{50\ analogues}) \times 100\ \%$.

The zearalenone-binding pattern of soluble scFv antibodies from these nine HB2151 clones was the same as their parent phage scFv antibodies. CI-ELISA results for four typical scFv clones are shown in Figure 2.6A. Two of the scFv antibodies (QY1.5 and QY1.12 from first round and second round selection, respectively) exhibited zearalenone-binding affinities similar to those of the parent monoclonal antibody (Figure 2.6B). IC_{50} for scFv QY1.5, QY1.12 and their parent monoclonal antibody were 14, 35, 17 ng/ml, respectively. Compared with the parent monoclonal antibody, however, soluble scFv QY1.5 exhibited similar sensitivity but higher relative cross-reactivities to hydroxylated zearalenone analogues (Table 2.2).

SDS-PAGE and Western blot analyses

An approximately 32 KDa scFv-E tag fusion protein was expressed from HB2151/pQY1.2, HB2151/pQY1.12 and HB2151/pQY1.5, while HB2151/pQY1.1 expressed a smaller protein, which probably lacked an E tag and could not be detected by anti-E tag antibody (Figure 2.7). Among the two positive zearalenone binding clones, HB2151/pQY1.12 expressed higher yields of scFv than HB2151/pQY1.5; however, the affinity of its scFv antibody (QY1.12) for zearalenone appeared slightly lower than that of QY1.5 (Figure 2.5B).

Sequence analysis

Clone pQY1.5, producing scFv with the highest affinity for zearalenone in HB2151, was chosen for DNA sequencing. The deduced amino acid sequences of V_H and V_K in pQY1.5 are shown in Figure 2.8. According to the amino acid

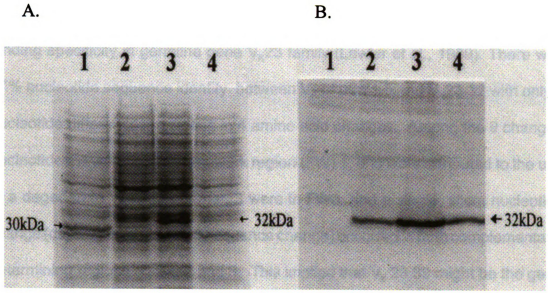

Figure 2.7. SDS-PAGE gel and Western blot analysis of scFv expression. (A): Supernatants of QY1.1 (lane 1; 0.3 ml), QY1.2 (lane 2; 0.3 ml), QY1.12 (lane3; 0.3 ml) and QY1.5 (lane 4; 0.6 ml) concentrated in 10% trichloroacetic acid were subjected to SDS-PAGE and stained with Coomassie brilliant blue G-250. (B): Western blot of duplicate SDS/PAGE gel.

sequences, the V_H was classified as mouse heavy chain subgroup III (D), while V_K fell into mouse kappa light chain subgroup V (12) (Kabat et al., 1991). The sequences of V_H and V_K were screened against the entries of Ig heavy chain and Ig kappa light chain respectively in the Kabat Database. The V_K sequence was closely related to that of a genomic DNA clone V_K23.32, a member with no identified binding specificity in germline gene V_K23 family (Lawler et al., 1989). There was 97% nucleotide sequence identity between V_K of pQY1.5 and V_K23.32 with only 9 nucleotide differences, resulting in 4 amino acid changes. Among the 9 changed nucleotides, 4 were in the framework region (FW) 1, and were attributed to the use of a degenerate VKBACK primer, 3 were in FW3, and a single silent nucleotide change (causing no amino acid sequence change) occurred in both complementary-determining regions (CDR) 2 and 3. This implied that V_k 23.32 might be the germ line gene for V_k of scFv QY1.5. Thus, few somatic mutations in the kappa light chain occurred during the *in vivo* affinity maturation of its parent anti-zearalenone antibody. However, the sequences of CDRs (especially CDR2 and CDR3) in the V_H of pQY1.5 were not related to any known members in the database. The closest V_H sequence was that of L3 10A (Kettleborough et al., 1994), an antibody with specificity to human epidermal growth factor receptor. But there was only 68% and 47% sequence identity between scFv QY1.5 and L3 10A in their V_H CDRs at the nucleotide and amino acid levels, respectively, although they had 96% identity in the FWs at both nucleotide and amino acid levels.

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A. V_H sequence:

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_____FW1_____ CDR1 _____FW2_____ CDR2
MA QVKLQESGGGLVQPGGSLKLSCAASGFTFS NYGMS WVRQTPDKRLEFVA NINGNGGKTYYPGSVKG

_____FW3_____ CDR3 ____FW4____
RFTISRDNAKNTLYLQMSSLKSEDTAMYYCVR VAFDGYYDDF WGQGTTVTVSS
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B. V_K sequence:

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_____FW1_____ CDR1 _____FW2_____ CDR2
DIELTQSPATLSVTPGDRVSLSC RASQSISDYLH WYQQKSHESPRLLIK YASQSIS

_____FW3_____ CDR3 ___FW4___
GIPSRFSGSGSGSDFTLSINSVEPEDVGVYYC QNGHSFPPT FGGGTKLEIK
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Figure 2.8. Deduced amino acid sequences of V_H and V_K in scFv QY1.5. Complementary-determining regions (CDR) and framework regions (FW) are indicated.

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Application of scFv QY1.5 to a spiked corn extract

Using zearalenone-KLH conjugate coated microtiter plates, soluble scFv QY1.5 showed higher sensitivity to zearalenone in corn extracts than its parent monoclonal antibody when the final methanol concentration was 17.5% in the CI-ELISA solution (Figure 2.9). Corn extracts containing as little as 10 ng zearalenone/ml caused a visually distinct inhibition of absorbance with soluble scFv QY1.5 in CI-ELISA. However, for undiluted corn extract (35% methanol in the final CI-ELISA solution), soluble scFv QY1.5 failed to detect any level of zearalenone in CI-ELISA because zearalenone specific binding of QY1.5 was eliminated by methanol at concentrations higher than 25% in the final CI-ELISA solution (Figure 2.10). In contrast, the parent monoclonal antibody exhibited much higher tolerance to methanol destabilization (Figure 2.10). Regardless, scFv QY1.5 did bind zearalenone with high specificity and sensitivity at practical levels of methanol concentration (less than 20%). Thus, the scFv antibody described here should be applicable for routine screening of zearalenone in corn and perhaps other cereal grains.

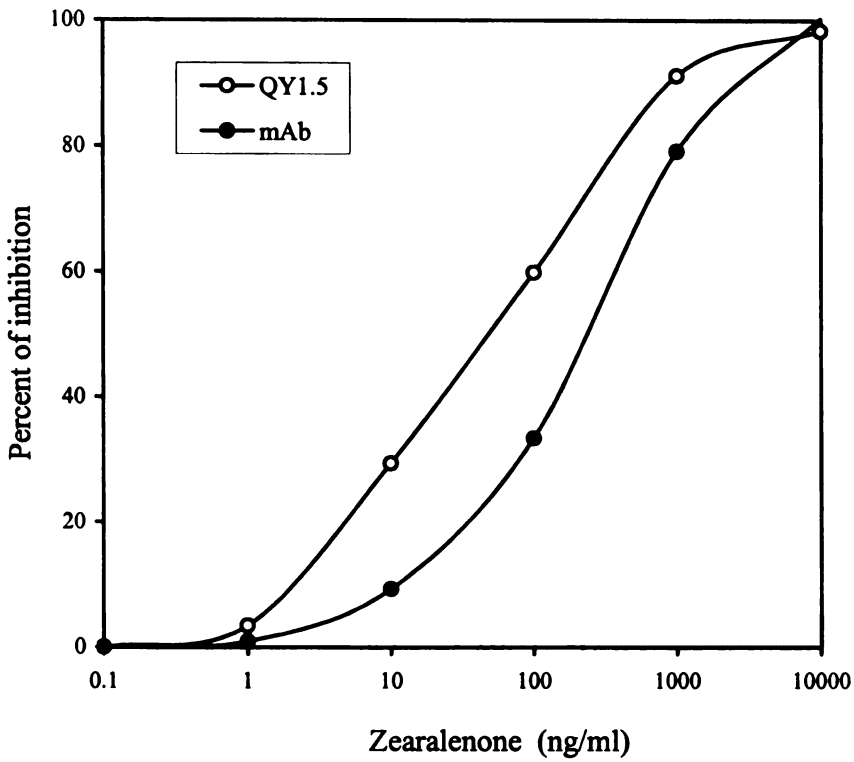

Figure 2.9. Zearalenone sensitivities in spiked corn extracts with monclonal antibody and soluble scFv antibody QY1.5 measured by CI-ELISA in zearalenone-keyhole limpet hemocyanin conjugate coated Immulon-4 microtiter wells. Both scFv QY1.5 and mAb were diluted 1:4 in PBS containing 10% nonfat dry milk. Zearalenone in ground corn was extracted with 5 volumes (wt/vol) of methanol-water (70:30) and the extracts were then diluted 1:1 with PBS.

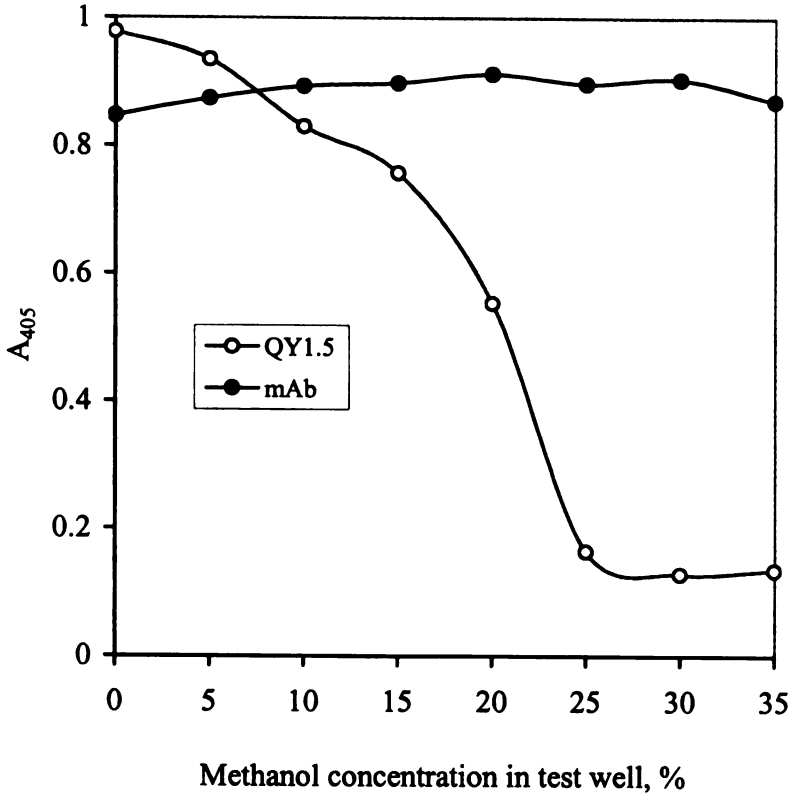

Figure 2.10. Effects of methanol on the binding of mAb and scFv QY1.5 to zearalenone-keyhole limpet hemocyanin conjugate. Immulon-4 microtiter wells were coated with zearalenone-keyhole limpet hemocyanin conjugate. Bound soluble scFv antibody was detected by mouse anti-E tag antibody, followed by horseradish peroxidase conjugated goat anti-mouse IgG. Bound monoclonal antibody was detected by horseradish peroxidase conjugated goat anti-mouse IgG.

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DISCUSSION

This study describes the successful isolation and cloning of V_H and V_K genes from a zearalenone-specific hybridoma, their soluble expression as an scFv protein, and their use in a zearalenone CI-ELISA. To our knowledge, this is the first successful example of the development of a recombinant antibody for mycotoxins, and we think it could serve as a model for development and construction of novel antibodies for mycotoxins and other potentially toxic residues.

When a hybridoma is used as a mRNA source for construction of scFv antibody, it is most desirable to use a cell line that produces high-affinity antibody for the target hapten or antigen. The hybridoma cell line we used in this study produced monoclonal antibody with high affinity (IC_{50}=17 ng/ml) for zearalenone, which made the cloning of high affinity scFv antibody possible. In contrast, only a very low sensitive scFv antibody specific for the mycotoxin fumonisin B_1 was developed in a previous study because, in part, the hybridoma cell line used produced a lower affinity antibody specific to fumonisin B_1 (Zhou et al., 1996).

Although hybridomas producing high affinity antibodies are good sources of mRNA for the facile cloning of desired variable region (V region) genes, additional irrelevant transcripts are possible. Thus, it was notable that amplification of the V_H gene from hybridoma 2G3-6E3-2EZ, which produces anti-zearalenone antibody, gave three different PCR products. One of these products may have been the amplified product from the fusion partner, myeloma cell line NS-1, which produces an aberrant V_H transcript with a 50 nucleotide deletion at FW3-CDR3 boundary

(Thammana, 1994). Other irrelevant V regions might possibly result from non-productive rearrangements. Practically, a hybridoma may contain several different cell populations as a result of recombination during extended culture after limiting dilution. So V regions which are productive but do not recognize the antigen of interest may also be isolated. Additionally, modifications could be generated in the PCR amplification and assembly because of the use of degenerate primers and *Taq* DNA polymerase with low fidelity. Affinity selection was therefore necessary for isolation of the variable regions with the desired specificity. Like the scFv antibodies before panning elution selection in this study, the fumonisin B_1 specific recombinant scFv antibody (Zhou et al., 1996), selected only by panning without affinity elution selection (through hapten elution), were 10 to 100 times less sensitive compared with anti-fumonisin B_1 polyclonal or monoclonal antibodies. Clackson et al. (1991) also showed that affinity elution with free antigen or hapten elution after panning greatly enriched clones producing high affinity scFv antibody.

Since only a few species of mRNA transcripts from a specific hybridoma cell line contain V regions, a few rounds of panning-elution selection should select the desired V regions. In this study, a functional anti-zearalenone scFv protein with high affinity was isolated after only one round of panning-elution selection. Sequential selections mainly appeared to enrich clones with a higher yield of scFv protein, not necessarily improving the affinity.

Theoretically, a scFv will have similar specificity and sensitivity as its parent immunoglobulin molecule. In this study, soluble scFv antibody QY1.5 exhibited an affinity to zearalenone similar to that of its parent monoclonal antibody, but it was

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shown to have higher cross-reactivity to hydroxylated zearalenone analogues. Since these analogues are naturally occurring toxic metabolites of zearalenone (Mirocha et al., 1981; Hagler et al., 1979), the ability of the scFv antibody to detect them is particularly advantageous when detection of these analogues is desirable. In general, a change in specificity and sensitivity could result from sequence changes and conformation differences between scFv and the monoclonal antibody. Sequence changes may occur during the scFv construction procedures, including reverse transcription, PCR amplification, enzyme digestion and ligation. Even if the sequences of V_H and V_K in the scFv are the same as those naturally occuring in an IgG molecule, their conformations may also differ because scFv has V_H and V_K covalently coupled by a flexible peptide linker while natural IgG molecule does not.

ScFv QY1.5 also differed from its parent monoclonal antibody in its sensitivity to the destabilizing influence of methanol, which drastically decreased the binding of scFv QY1.5 to zearalenone. Muller et al. (1994) observed a similar destabilizing effect of glycerol on scFv, which promoted the dissociation of two variable domains of scFv protein but did not affect the domain structure of scFv. In contrast, destabilization was not observed for Fab fragments and whole monoclonal antibody molecules in that study. It was believed that the constant domains in Fab or monoclonal antibody may provide additional stabilization of the molecular structure at the antigen binding site. Some general strategies for protein stabilization may be used to stabilize scFv protein by strengthening the interactions between V_H and V_K domains. These include engineering of stronger hydrogen bonds, disulfide

bonds, metal binding sites, and increasing the hydrophobic area or electrostatic interactions (Raag and Whitlow, 1995).

It was notable that the V_H sequence of scFv QY1.5 exhibited numerous sequence differences from sequenced members in the antibody database. As might be expected, the changes are clustered in the CDRs, especially in CDR2 and CDR3. This implies that CDR2 and CDR3 in the V_H of scFv QY1.5 may play an important role in determining the specificity for zearalenone binding. In contrast, the V_K in scFv QY1.5 may contribute less to the specificity, since there were few differences in its sequence compared with its possible germ line gene. The latter may have a broad range of specificities without somatic mutations.

As immunoassays become a more common method for the routine screening of mycotoxins and other natural toxicants in food and agriculture products, larger supplies of low-cost high affinity antibodies will be needed. The recombinant antibody technique provides a promising alternative for producing low-cost antibodies with the desirable specificity and sensitivity. The recombinant antibody scFv QY1.5 has high sensitivity and specificity to zearalenone and its analogues, and should be readily applicable to the routine screening for these mycotoxins.

PART III

ATTEMPTS TO CLONE SINGLE-CHAIN FV (SCFV) ANTIBODIES AGAINST FUMONISIN B_1 FROM IMMUNIZED MICE

ABSTRACT

ATTEMPTS TO CLONE SINGLE-CHAIN FV (SCFV) ANTIBODIES AGAINST FUMONISIN B_1 FROM IMMUNIZED MICE

High affinity (IC_{50}, 0.27 μg fumonisin B_1 /ml) mouse polyclonal antibodies were readily generated after immunization with fumonisin B_1 -cholera toxin (FB_1-CT) conjugate but not produced with fumonisin B_1-keyhole limpet hemocyanin (FB_1-KLH) conjugate immunization. From the splenic cDNA of FB_1-CT immunized mice, variable region genes of heavy- (V_H) and kappa light-chain (V_K) were successfully amplified by PCR with previously designed primers (Yuan et al., 1997). The V_H and V_K genes were assembled with linker DNA to form scFv DNA fragments by PCR and cloned in a phage-display vector. However, panning-elution selection from this scFv phage-display library did not result in enrichment of FB_1 specific phage clones. Different FB_1 conjugates, phage incubation times and FB_1 concentrations in the eluting solution were investigated for their effects on specific binding and elution. No conditions were found suitable for FB_1 specific scFv phage selection in the panning-elution selection procedure. The soluble form of scFv proteins from approximately 300 randomly selected individual clones did not show any FB_1 specific binding activity either. An alternative method is suggested to clone functional FB_1 specific scFv antibodies.

INTRODUCTION

Fumonisins are toxic secondary metabolites produced by certain species of Fusarium during pathogenesis on corn and sorghum (Sydenham et al., 1990). Interest on fumonisins has intensified recently because of its cytotoxic properties and carcinogenic potential (Thiel et al., 1991; Gelderblom et al., 1993). 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 animal species, including hepatic cancer in rats, brain lesions in horses (equine leukoencephalomalacia, ELEM), and pulmonary lesions in pigs (porcine pulmonary edema, PPE).

Elimination of fumonisins from human and animal food requires detection and diversion of contaminated raw materials from feed and finished food use. Commonly used chemical analytical methods for detecting 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 (Geldeblom et al., 1988a; Shephard et al., 1990; Cawood et al., 1991; Rottinghaus et al., 1992; Scott and Lawrence, 1992). Regardless of sensitivity and selectivity, these chemical analytical methods are generally expensive, complex, and time consuming (Pestka et al., 1995). More

recently, immunoassays have proven to be useful in screening for mycotoxins. Compared to the chemical analytical methods, immunoassays have several advantages for rapid field tests, including high specificity, sensitivity, facile sample preparation, and ease of use (Pestka et al., 1995). As a consequence, both polyclonal (Azcona-Olivera et al., 1992a) and monoclonal (Azcona-Olivera et al., 1992b) antibodies to FB_1 have been developed and applied to the enzyme-linked immunosorbent assays (ELISAs) (Elissalde et al., 1995; Fukuda et al., 1994; Pestka et al., 1994a; Tejada-Simon et al., 1995).

Such developments have lead to a great demand for antibodies in the fumonisin immunoassays. However, the limit of detection with most current antibodies is not sufficiently adequate for screening fumonisins at <1 ppm in conventional ELISA or multianalyte assays. Furthermore, the development of these natural antibodies requires the use of animals, specialized cell culturing facilities, and an extensive commitment of time and labor.

It is now theoretically feasible to engineer low cost antibodies with desirable affinity and specificity characteristics by using recombinant DNA technology to manipulate the basic domain structure of immunoglobulin molecule (Hoogenboom et al., 1992; Lerner et al., 1992). Using filamentous phage to display antibodies, antibody fragments can now be engineered from antibody V-gene repertoires (McCafferty et al., 1990; Clackson et al., 1991; Marks et al., 1991) and high affinity clones can be selected by a procedure called "panning" (Marks et al., 1991; Hawkins et al., 1992).

Previously, several single-chain Fv clones were developed in our laboratory using mRNA from either spleen cells of mice immunized with FB_1-bovine serum albumin (FB_1-BSA) conjugate or from an existing hybridoma cell line that produces anti-FB_1 antibody (Zhou et al., 1996). However, these scFv antibodies had very low affinities to fumonisins and were even 10 to 100 times less sensitive than the parent natural antibodies. The failure to develop high affinity scFv antibodies to fumonisins in the previous study might be due to (i) the use of a hybridoma cell line or immunized mice which produced low affinity antibodies; (ii) only panning was used in the selection, without affinity elution. Based on the successful development of anti-zearalenone scFv antibodies (Yuan et al., 1997), it is desirable to use a cell line or immunized mice that produce high-affinity antibodies to start scFv fragments cloning. For the selection of specific scFv antibodies, it is also critical to conduct an affinity elution by free hapten after the regular panning. In this study, the possibility of developing high affinity scFv antibodies to fumonisin B_1 was investigated by using (i) spleen mRNA from immunized mice that produce high affinity antibodies to fumonisin B_1; (ii) affinity elution with free fumonisin B_1 after the regular panning.

MATERIALS AND METHODS

General procedures

All chemicals and organic solvents were reagent grade or better. Fumonisin B_1, cholera toxin (CT), ovalbumin (OVA), bovine albumin (BSA), glutaraldehyde, goat anti-mouse IgG peroxidase and rabbit anti-mouse IgG alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO). Inject® keyhole limpet hemocyanin (KLH) was purchased from Pierce Chemical Company (Rockford, IL). Plasmid pCANTAB5E, *E. coli* TG1 and HB2151, M13K07 helper phage, horseradish peroxidase conjugated sheep anti-M13 antibody and mouse anti-E tag antibody were obtained from Pharmacia Biotech (Piscataway, NJ). Oligo(dT) cellulose columns and *Not* I restriction enzyme were supplied by Gibco BRL (Gaithersburg, MD). PCR amplification primers were synthesized by Gibco BRL(Gaithersburg, MD). Restriction enzyme *Sfi* I was purchased from New England BioLabs (Beverly, MA). All DNA manipulations, if not described, were carried out by standard procedures (Sambrook et al., 1989).

Preparation of immunogens

Fumonisin B_1 was conjugated to KLH (FB_1-KLH), cholera toxin (FB_1-CT), ovalbumin (FB_1-OVA), and bovine serum albumin (FB_1-BSA) with glutaraldehyde linkage via a free amino group (Figure 3.1) as described by Azcona-Olivera et al. (1992a). FB_1-KLH and FB_1-CT conjugates were used as immunogens, and FB_1-OVA and FB_1-BSA conjugates were used as solid-phase antigens for affinity

selection. Briefly, the coupling reaction was carried out at 4°C in 0.01 M phosphate-buffered saline (pH7.4) (PBS). FB_1 was added to a 1-mg/ml suspension of protein at a molar ratio of 50:1 (toxin/protein), and then an equal volume of glutaraldehyde (2% vol/vol) was added dropwise with constant stirring. After 1 hr, the reaction was stopped and the bond stabilized by reduction via the addition of sodium borohydride to a final concentration of 10 mg/ml. One hour later, the mixture was dialyzed for 72 hr (three changes) against 0.01 M PBS (pH7.4). The conjugates then were aliquoted in fractions of 1 mg (total protein) and stored at -20°C until required.

Animal immunization

Female BALB/c mice (7 to 8 weeks of age) were immunized by intraperitoneal injections. Mice immunized with FB_1-KLH conjugate received three 100-μg doses of immunogen at 2-week intervals. The first injection consisted of 0.1 ml of conjugate in saline mixed with (1:1 volume ratio) Freund's complete adjuvant. The second and third injections consisted of 0.1 ml of conjugate in saline mixed with (1:1 volume ratio) Freund's incomplete adjuvant. Mice immunized with FB_1-CT were injected three times (10-day interval) with 10 μg of immunogen dissolved in 0.2 ml of PBS. Ether-anesthetized mice were bled from the eye retrobulbar plexus, and serum was obtained after overnight incubation of blood at 4°C and centrifugation at 1,000 x *g* for 10 min.

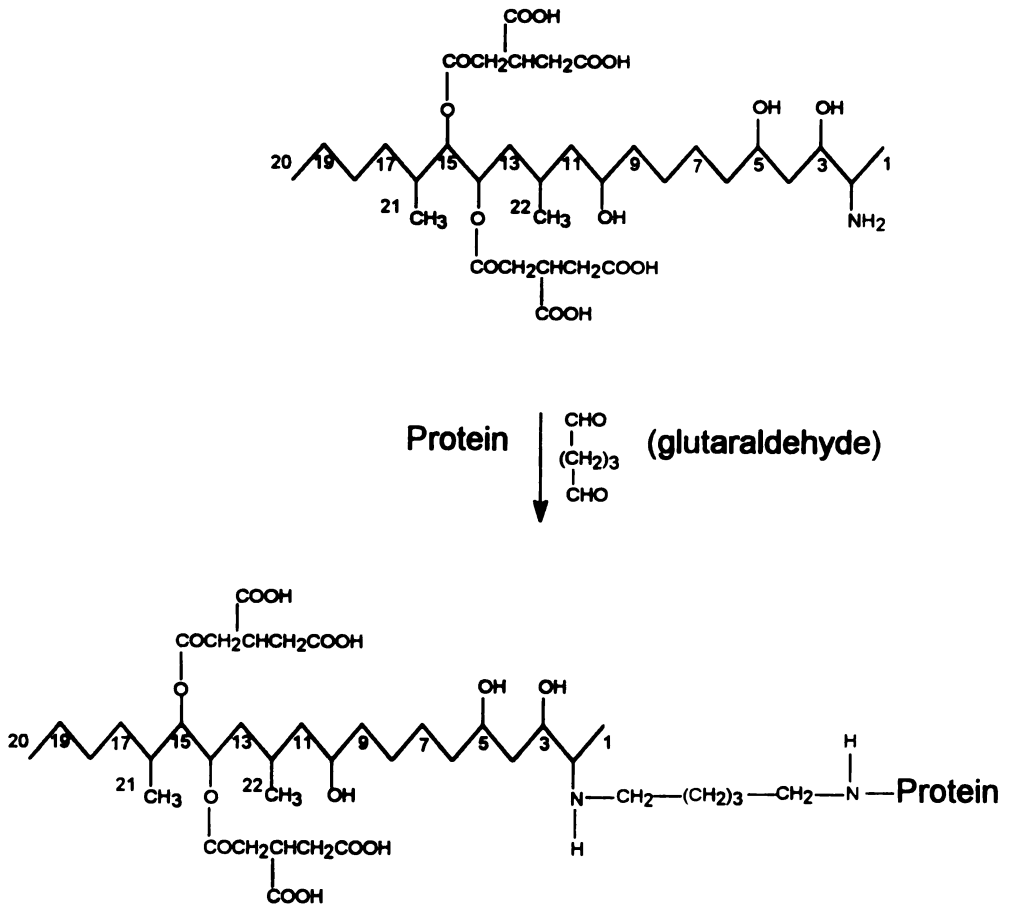

Figure 3.1. Preparation of fumonisin B_1 conjugates with glutaraldehyde linkage via a free amino group (Pestka et al., 1995).

ELISA

For antiserum titration, wells of polystyrene microtiter plates (Immunolon-4-Removawells) (Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight at 4°C with 10 μg/ml (100 μl/well) of FB_1-OVA, FB_1-BSA or FB_1-KLH in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6). Plates were washed four times with 300 μl of PBS. The wells were blocked for 1 hr at 37°C with 320 μl of 1% (wt/vol) OVA, BSA or KLH in PBS and then washed four times with 300 μl of PBS. Serially diluted serum (100 μl) was then added to each well and incubated for 1 hr at 37°C. Unbound antibodies were removed by washing six times with 0.1% (vol/vol) Tween 20 in PBS (PBS-T), and 100 μl of goat anti-mouse immunoglobulin G peroxidase conjugate (diluted 1/2000 in 1% OVA-PBS or 1% BSA-PBS) was added to each well. Plates were incubated for 1 hr at 37°C and washed six times with PBS-T. The bound peroxidase was determined by the addition of 100 μl/well of TMB substrate (Sigma Chemical Company) and incubation at 37°C for 15 min. The reaction was stopped by adding 100 μl/well of 10% H_2SO_4. The absorbance at 450nm was determined with a microtiter plant reader (Molecular Device Corporation, Menlo Park, CA).

A competitive indirect ELISA (CI-ELISA) was used to assess the presence of FB_1 specific antibodies in mouse sera. Microtiter plates were coated and blocked as described above. A 50-μl volume of FB_1 (at various concentrations) dissolved in PBS was added over FB_1-OVA (or FB_1-BSA) coated wells, followed by addition of a 50-μl volume of antiserum (appropriately diluted in 1% OVA-PBS or 1% BSA-PBS) and incubated at 37°C for 1 hr. Bound antibodies were determined as

described above. Relative antibody affinity was arbitrarily designated as the toxin concentration required to inhibit antibody binding by 50% (IC_{50}).

Spleen mRNA isolation and cDNA synthesis

Total cellular RNA was extracted by homogenizing the murine spleen tissue of immunized mice in RNA STAT-60 (TEL-TEST Inc., Friendswood, TX). The mRNA was purified by affinity chromatography on oligo (dT)-cellulose column according to the manufacturer's instructions. First-strand cDNA was synthesized from the mRNA template with Moloney murine leukemia virus reverse transcriptase and random hexadeoxyribonucleotide [pd$(N)_6$] primers (Pharmacia Biotech). Random hexamers were used because they eliminated the need for Ig-specific primers or oligo (dT) primers that require synthesis of a long cDNA for obtaining the sequences encoding the variable heavy or light chain.

PCR amplification and scFv cloning

The variable regions of heavy chain (V_H) and kappa light chain (V_K) were amplified from the first-strand cDNA as described previously (Yuan et al., 1997). Briefly, the variable regions of heavy chain (V_H) were amplified by using *Taq* DNA polymerase and VHBACK and du-VHFOR as primers (Table 2.1). The kappa light chains (V_K) were amplified with dU-VKBACK and VKFOR as primers (Table 2.1). A 93-bp DNA linker (Pharmacia Biotech, Piscataway, NJ), containing a sequence encoding a short flexible peptide, $(Gly_4Ser)_3$, was also amplified with LINKBACK and du-LINKFOR (Table 2.1). A two-step procedure described previously (Yuan et

al., 1997) was used for PCR assembly of scFv DNA fragment. The assembled scFv products were gel-purified and reamplified with restriction site tagged primers (RS Primers Mix) (Pharmacia Biotech) to add an *Sfi*I site on the 5' end and a *Not*I site on the 3' end of the scFv DNA. The scFv DNA products were then digested with *Sfi*I and *Not*I restriction enzymes, gel purified and ligated into the phagemid pCANTAB5E (digested with these same enzymes).

Affinity selection of phage-displayed anti-FB_1 scFv antibodies

E. coli TG1 competent cells, an amber suppressor strain (*sup*E), were transformed by the ligated DNA products above and plated on SOB medium (Sambrook et al., 1989) containing 100 μg/ml ampicillin and 2% glucose, and incubated at 30°C overnight. Colonies were pooled and infected with M13K07 helper phage as described previously (Yuan et al., 1997) to display scFv fusion protein on the surface of the recombinant phage. The recombinant phages from the supernatant were filtered through a 0.45 μm-pore-size filter (Gelman Sciences)

For panning-elution selection, wells of sterile polystyrene Immulon-4 microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight (4 °C) with 100 μl/well of FB_1-OVA or FB_1-BSA or FB_1-KLH conjugate (10 μg/ml) in 0.05 M carbonate-bicarbonate buffer (pH 9.6). The control wells were coated with the carrier proteins (10 μg/ml) alone. Plates were washed four times by filling each well with 300 μl of 0.01 M PBS and aspirating the contents. Non-specific binding was blocked by incubation at 37°C for 1 hr with 300 μl of 1% OVA, BSA or KLH (w/v) dissolved in PBS and then washing 6 times with 0.05% (v/v) Tween-20 in 0.01

M PBS (PBS-T). Recombinant phages (about 10^8 to 10^{10} pfu/ml) were then added (100 μl/well). The plates were incubated for various times (10 to 60 min) at 37 °C followed by ten washes with PBS-T and incubation with 300 μl/well of PBS at 37°C for 1 hr. The wells were then washed another ten times with PBS-T. One hundred microliter of free FB_1 (10, 50 and 100 μg/ml) diluted with PBS was added to the wells and incubated at 37 °C for 1 hr with rotary shaking (150 rpm) to elute the bound phages. PBS was used as a control elution reagent. The eluted phages were collected and passed through a 0.45 μm-pore-size low-protein-binding syringe filter (Gelman Sciences), and were used to reinfect *E. coli* TG1 cells for phage titration and reamplification.

Recombinant phages from selected clones were used to infect *E. coli* HB2151 for production of soluble scFv antibodies. Briefly, the infected *E. coli* HB2151 cells were cultured in SB medium (35 g/l tryptone, 20 g/l yeast extract, 5 g/l NaCl) supplemented with 100 μg/ml ampicillin at 30°C with 250 rpm shaking until they reached an A_{600} of 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to 1 mM final concentration and the cells were incubated on a shaker at 30°C overnight. Supernatants containing the extracellular soluble scFv proteins were separated from the cell pellets by centrifugation at 1500 x *g* for 15 min, and filtered through a 0.45 μm-pore-size filter. The sensitivities and specificities of the scFv antibodies (phage-associated or soluble forms) were determined with ELISA as described previously (Yuan et al., 1997). Briefly, microtiter plates were coated and blocked as described above. Next, 50 μl of FB_1 standard (at various concentrations) dissolved in PBS was added to the coated wells and simultaneously incubated with

50 μl of phage-associated (about 5 x 10^{10} pfu/ml in 2x YT medium) or soluble scFv antibody (various dilutions with 1% OVA-PBS or 1% BSA-PBS or PBS-2% non-fat dry milk). A monoclonal antibody (diluted 1:20,000) was included for comparison. The plates were incubated for 1 hr at 37 °C then washed 6 times with PBS-T. The amount of bound phage-associated scFv antibody was determined by the addition of 100 μl sheep anti-M13-HRP conjugate diluted 1:2500. The amount of soluble scFv antibody bound was determined by incubation with 100 μl mouse anti-E tag (1 μg/ml) at 37°C for 1 hr followed by washing (six times) and a subsequent incubation with goat anti-mouse IgG HRP conjugate (diluted 1:2000). The bound monoclonal antibody was also detected by the addition of 100 μl goat anti-mouse IgG HRP conjugate. The plates were incubated for 1 hr at 37°C and then washed 6 times with PBS-T. The amount of anti-M13-HRP and goat anti-mouse IgG HRP conjugates bound in the wells was assessed by the addition of 100 μl of 3,3',5,5'-tetramethyl-benzidine (TMB) at 37°C for 15 min. The absorbance was read at 450 nm with a microtiter plate reader (Molecular Devices Corporation, Menlo Park, CA) after the color development was stopped with 100 μl/well of 10% H_2SO_4.

RESULTS AND DISCUSSION

Mouse immunization and polyclonal antibodies

Eight mice were immunized and boosted with FB_1-KLH immunogen and another eight mice were injected intraperitoneally with FB_1-CT conjugate. Each mouse was bled one week after every boost and its serum was prepared and titrated. Titers of the mouse antisera after the second boost were detected in an indirect ELISA format (Figure 3.2A). The specificity and sensitivity of the antisera were determined with a competitive indirect ELISA (Figure 3.2B). Concentration of FB_1 required for 50% binding inhibition with antisera from FB_1-CT immunized mice (approximately 0.27 μg/ml) were much lower than that from FB_1-KLH immunized mice (>10 μg/ml) (Figure 3.2B). Like FB_1-BSA conjugate (Azcona-Olivera et al., 1992a), the conjugate of FB_1-KLH was generally ineffective in the production of FB_1 antibodies in mice. The apparent high antibody titers in mice immunized with FB_1-KLH might be due to the presence of antibodies that recognize the toxin-carrier bridge or a conjugate by-product, but which are not inhibited by free FB_1 in CI-ELISA. In contrast to immunization with FB_1-KLH, immunization with low FB_1-CT doses resulted in the rapid production of FB_1 specific antibodies with high relative affinity in all treated animals. Compared with the FB_1-KLH immunogen, the effectiveness of FB_1-CT conjugate in the production of FB_1 antibodies might be explained on the basis of the CT adjuvant effect (Azcona-Olivera et al., 1992a).

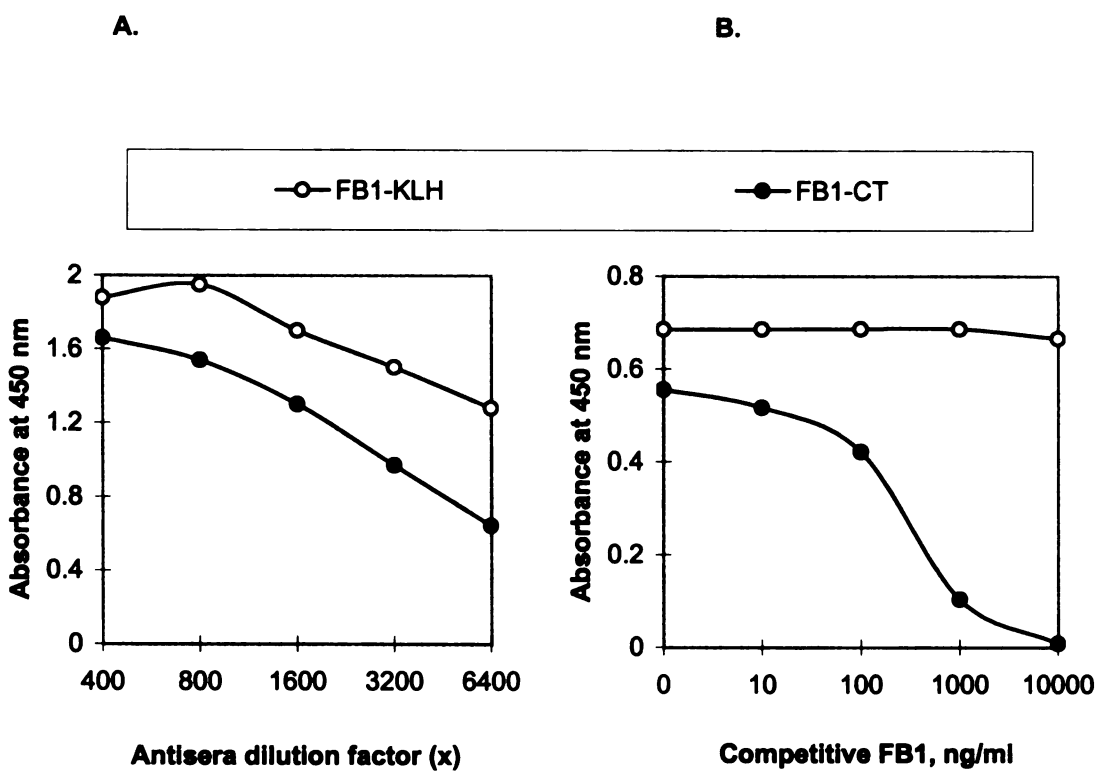

Figure 3.2. Polyclonal anti-FB_1 antibody production from mice immunized with FB_1-cholera toxin (FB_1-CT) and FB_1-keyhole limpet hemocyanin (FB_1-KLH) conjugates (Each group had one representative out of eight mice). Sera were obtained one week after the second boost with the immunogens. A: Titration of antisera measured in FB_1-ovalbumin (FB_1-OVA) conjugate coated Immulon-4 microtiter wells. Bound mouse antibodies were detected by horseradish peroxidase conjugated goat anti-mouse IgG. B: Competitive indirect ELISA for FB_1 with the antisera (diluted 1:60,000 and 1:3,200 respectively for FB_1-KLH and FB_1-CT immunized mice).

Construction of phage-display scFv library

Mice immunized with FB_1-CT conjugate were sacrificed one week after the second boost and their spleens were homogenized in RNA STAT-60 (TEL-TEST Inc.). After mRNA was purified by affinity chromatography on oligo (dT)-cellulose column, first-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase and random hexadeoxyribonucleotide [$pd(N)_6$] primers (Pharmacia Biotech). PCR amplification from the mRNA yielded a V_H DNA fragment with a size close to the expected 340 bp (lane 3 in Figure 3.3) and a V_K DNA fragment with a size close to the expected 324 bp (lane 2 in Figure 3.3). A linker DNA (lane 1 in Figure 3.3) was also produced from a template containing sequence encoding short peptide $(Gly_3Ser)_4$ by PCR amplification with LINKBACK and du-LINKFOR as primers. The DNA fragments of V_K and linker were successfully joined by PCR assembly (lane 4 in Figure 3.3) and then joined with V_H DNA forming the scFv DNA fragments (lane 5 in Figure 3.3). After addition with an *Sfi*I site on the 5' end and a *Not*I site on the 3' end, the assembled scFv DNA products were digested with *Sfi*I and *Not*I restriction enzymes, gel purified and ligated into the phagemid pCANTAB5E. The ligated products were introduced into *E.coli* TG1 competent cells by chemical transformation. Approximately 10,000 transformants were produced for the construction of scFv phage-display library. Restriction analysis of the recombinant phagemid DNA isolated from the transformants showed an expected size of scFv insert DNA in pCANTAB5E.

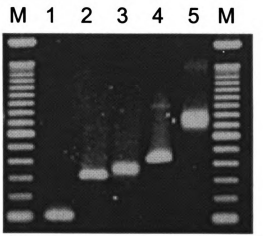

Figure 3.3. PCR amplification of V_H and V_K genes from mouse splenic mRNA and the assembly of scFv. Lanes are: (1): Linker PCR product; (2): V_K PCR product; (3): V_H PCR product; (4): Linker-V_K PCR product; (5):V_H-Linker-V_K (scFv) PCR product; Lane M:100-bp ladder DNA marker (Gibco BRL).

Affinity selection of phage-display scFv antibodies

In a preliminary experiment, the newly constructed recombinant scFv phage library was incubated in FB_1-OVA (1 μg/well) coated wells for FB_1 specific binding and in OVA coated wells as a negative control of nonspecific binding. After incubation for 60 min with the scFv phage library and extensive washing with PBS-T, FB_1 (100 μg/ml) or PBS were used to elute the bound phages from the wells. After five rounds of panning-elution selection, the number of phages eluted by FB_1 from FB_1-OVA coated wells did not differ significantly (less than one order difference) from that eluted by PBS. On the other hand, FB_1 eluted approximately the same number of phages from both FB_1-OVA coated wells and OVA coated wells. This indicated that FB_1-specific scFv phages were not enriched in this panning-elution selection.

To search for a suitable panning-elution selection procedure for specific binding and elution, the effects of different FB_1-carrier protein conjugates (i.e. FB_1-OVA, FB_1-BSA, and FB_1-KLH, Figure 3.4 A), scFv phage library incubation time (i.e. 60, 20, and 10 min, Figure 3.4 B) and FB_1 concentration (i.e.10, 50, and 100 μg/ml, Figure 3.4C) in the elution solution were investigated. No significant enrichment for FB_1-specific scFv phage (phage eluted by FB_1 from FB_1-carrier proteins coated wells) was found in any condition tested (Figure 3.4). Although the number of phages eluted by FB_1 (50, 100 μg/ml in the elution solution in most cases) from FB_1-OVA or FB_1-BSA coated wells was greater than that eluted by PBS, the difference appeared not due to the presence of FB_1 specific scFv phages because the number

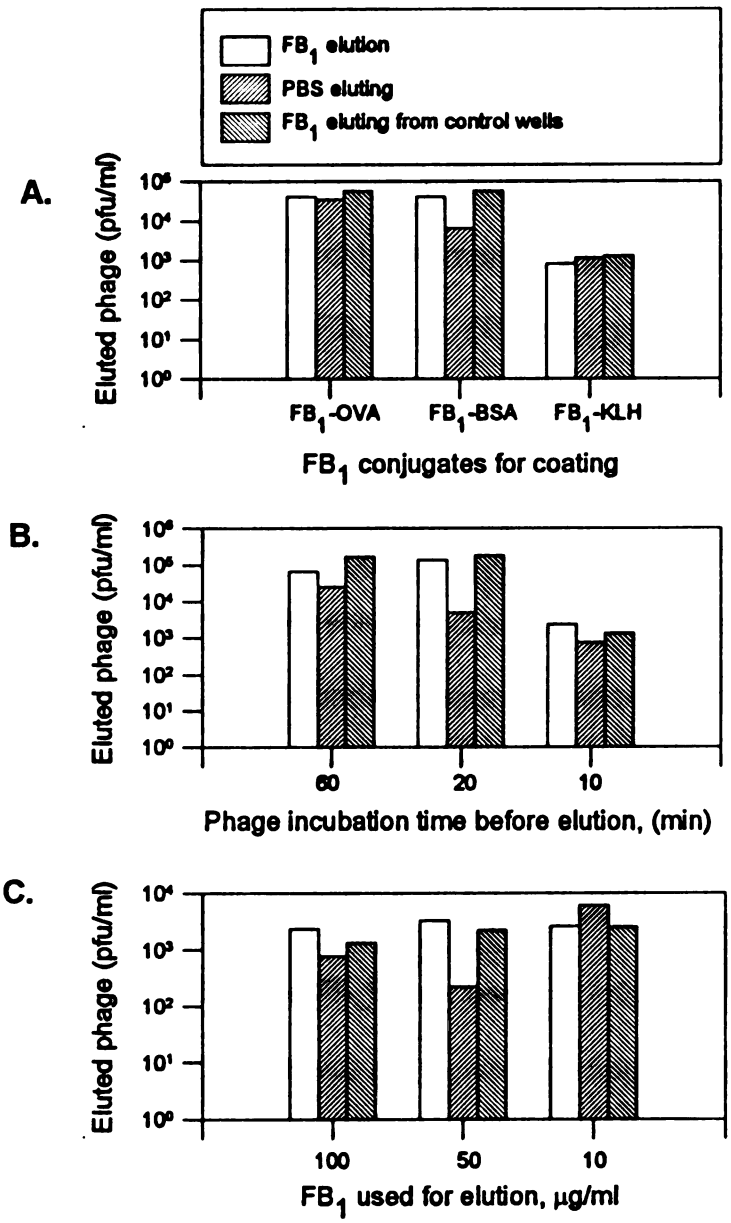

Figure 3.4. Phage titers after panning-elution selection of the phage library. Standard procedure was: coating: 1 µg/well of FB_1-OVA conjugates or OVA alone (in control wells); phage incubation: 60 min; bound phage elution: by PBS or 100 µg/ml of FB_1 in PBS. Modifications were different conjugates for coating (A), phage incubation time (B), and FB_1 concentration for elution (C).

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of phages eluted by FB_1 from the FB_1-carrier protein conjugates coated wells was similar to that eluted by FB_1 from wells coated only carrier proteins. Reducing the phage library incubation time in the coated wells, or decreasing the FB_1 concentration in the elution solution did not improve the differentiation between specific elution (phages eluted by FB_1 from FB_1-conjugate coated wells) and nonspecific elution (phages eluted by FB_1 from carrier proteins coated wells) (Figure 3.4).

After failing to enrich FB_1 specific scFv phages by panning-elution selection, identification of individual clones with soluble scFv proteins that specifically bound to FB_1 was also investigated. Two hundred seventy randomly selected individual phage plaques from the unselected original scFv phage library and 39 individual phage plaques selected after four rounds of panning-elution selection (In each round of selection, phages were incubated in FB_1-OVA coated wells for 60 min, washed, and eluted with 100 μg/ml FB_1) were used to infect *E. coli* HB2151 for soluble scFv protein production. None of the isolates bound FB_1 specifically in ELISA.

Although the mice whose splenic mRNAs were used to construct the scFv phage library in this study produced relatively high affinity antibodies to FB_1 , no FB_1 specific scFv antibody clone has been selected from this scFv phage library. Sequence changes during the scFv construction procedure and conformation differences between scFv protein and the native antibodies may cause changes in specificity and sensitivity for the scFv proteins. These changes may result in the absence of FB_1 specific clones in the original scFv phage library. Even if there were

a few clones specifically binding to FB_1 in the library, the high degree of nonspecific binding of the phage library to the carrier proteins alone plus nonspecific elution activity by FB_1 in the panning-elution selection make the enrichment for FB_1 specific phages impossible. Considering that phage panning selection also failed to generate any scFv antibody clone with high affinity to FB_1 from an scFv phage library constructed from a hybridoma cell line producing FB_1 specific monoclonal antibody (Zhou et al., 1996), an alternative way to clone a functional FB_1 scFv antibody is needed. For example, the V_H and V_L can be cloned using PCR-based RACE (Rapid Amplification of cDNA Ends) technology with primers which are independent of the variability within the V regions (Ruberti et al., 1994) and with high fidelity enzymes such as *Pfu* DNA polymerase. They are then assembled into an scFv fragment and tested for their activities. The specificity and sensitivity of the cloned scFv antibody could be improved further by mutagenesis or chain shuffling (Hoogenboom et al., 1992) or CDR-grafting (Smith et al., 1995; Jung and Pluckthun, 1997).

CONCLUSION

Mouse polyclonal antibodies to FB_1 were readily generated after immunization with FB_1-CT conjugate but not produced with FB_1-KLH conjugate immunization. The relative affinity (measured by FB_1 concentration required for 50% binding inhibition) of mouse polyclonal antibodies to FB_1 was 0.27 μg/ml. From the splenic cDNA of mice that were immunized with FB_1-CT, variable region genes of heavy- and kappa light-chain were successfully amplified by PCR with previously designed primers (Yuan et al., 1997). The V_H and V_K genes were assembled with a linker DNA to form scFv DNA fragments by PCR and cloned into a phage-display vector. However, panning-elution selection from this scFv phage-display library did not result in any enrichment of FB_1 specific phages. Different FB_1-protein conjugates, phage incubation times, and FB_1 concentration in the elution solution were tested for their effects on specific binding and elution. No conditions were found suitable for FB_1 specific scFv phage selection in the panning-elution procedure. The soluble form of scFv protein from approximately 300 randomly selected individual clones did not show FB_1 specific binding activity either. An alternative method is needed to clone functional FB_1 specific scFv antibodies.

PART IV

EXPRESSION OF A FUNCTIONAL ANTI-ZEARALENONE SINGLE-CHAIN FV ANTIBODY IN TRANSGENIC *ARABIDOPSIS* PLANTS

ABSTRACT

EXPRESSION OF A FUNCTIONAL ANTI-ZEARALENONE SINGLE-CHAIN FV ANTIBODY IN TRANSGENIC *ARABIDOPSIS* PLANTS

The efficacy of cloning a recombinant mycotoxin antibody in plants was tested using *Arabidopsis* as a model. An anti-zearalenone scFv DNA fragment was first cloned in the newly constructed phage-display vector (pEY.5) and then recloned in the plant transformation vector, pKYLX71::35S^2. After transformation, constructs of anti-zearalenone scFv were introduced into immature *Arabidopsis* seeds via *Agrobacterium tumefaciens* mediation by vacuum infiltration. Only plants transformed with construct containing a PR-1b signal peptide sequence produced transgenic offspring. The anti-zearalenone scFv plantibody from these transgenic plants bound zearalenone with a high affinity (IC_{50}, 11.2 ng/ml) comparable to bacteria-produced scFv antibody and the parent monoclonal antibody (mAb) from hybridoma cell culture. By electron microscopic immunogold labeling, the presence of anti-zearalenone scFv was mainly detected in the cytoplasm and only occasionally outside the cell. Like bacteria-produced scFv antibody, anti-zearalenone scFv plantibody exhibited greater sensitivity to methanol destabilization than the parent monoclonal antibody did. The sensitivity of anti-zearalenone scFv plantibody to acidic disassociation was similar to bacterial-produced scFv antibody and mAb. Expression of specific plantibodies in crops might be useful to neutralize mycotoxins in animal feeds and to reduce mycotoxin-associated plant diseases.

INTRODUCTION

Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone] is a mycotoxin produced by members of the genus *Fusarium* after infection of corn and small grains (Mirocha et al., 1971; 1977; Hart et al., 1982). When fed to animals, the compound causes hyperestrogenism with symptoms such as enlargement of the uterus and nipples, vulvar swelling, vaginal prolapse, and infertility (Hidy et al., 1977; Mirocha et al., 1974).

Recently, we developed a single-chain Fv (scFv) antibody with high affinity to zearalenone (Yuan et al., 1997). Our experience and others (Kramer and Hock, 1996; Dr. Chu in UW, personal communication) showed that scFv assembly from V_H , linker and V_K DNA fragments by PCR is one of the most problematic steps in scFv cloning. The assembly often results in undetectable amplificates or undefined DNA amplified products with various sizes. To facilitate the cloning and chain shuffling, and to increase the efficiency of scFv assembly from V_H and V_K cDNA fragments, a new phage-display vector was constructed by replacing a 52-bp *Sfi* I/*Not* I fragment in pCANTAB5E (Pharmacia Biotech, Piscataway, NJ) with a DNA fragment encoding the $(Gly_4Ser)_3$ linker peptide plus restriction sites which rarely exist in V gene regions. To confirm the feasibility of the new vector in scFv antibody cloning, an anti-zearalenone scFv DNA fragment was cloned and expressed with this new vector in *Escherichia coli*.

In the last ten years, the expression of specific antibodies or antibody fragments in plant has attracted great interest (reviewed by Hiatt and Ma, 1992;

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Conrad and Fiedler, 1994; Ma and Hein, 1995; Whitelam, 1995; 1996; Smith, 1996) and has shown some potential in improving plant resistance against pathogens (Tavladoraki et al., 1993) and altering plant metabolic pathways (Owen et al., 1992; Artsaenko et al., 1995). To explore the possibility of using plantibodies to neutralize mycotoxin through passive immunization of animals by feeding, as a first step, we used the newly cloned anti-zearalenone scFv DNA fragment to transform *Arabidopsis* plants. In this report, we demonstrated that the expression of anti-zearalenone scFv gene in transgenic *Arabidopsis* plants leads to the accumulation of a soluble scFv plantibody with high affinity to the mycotoxin zearalenone.

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METHODS AND MATERIALS

General

All chemicals and solvents were reagent grade or better. Chemicals were purchased from Sigma Chemical Company (St. Luis, MO) unless otherwise noted. All DNA manipulations, if not described, were carried out by standard procedures (Sambrook et al., 1989).

Construction of scFv cloning vector

A DNA fragment (Pharmacia Biotech, Piscataway, NJ), encoding a short peptide containing the structurally flexible peptide linker $(Gly_4Ser)_3$, was amplified by polymerase chain reaction (PCR) with *Taq* DNA polymerase using a sense primer (5'-TCTATGCGGCCCAGCCGGCCGGCACTAGTGTCACCGTC-3') containing *Sfi* I and *Spe* I restriction sites (underlined) and an antisense primer (5'-AGCACCTGCGGCCGCCTGAGTGAGCTCGATGTCCGATCC-3') containing *Not* I and *Sac* I sites (underlined). After electrophoresis in 1.5% low melting point agarose gel (Boehringer Mannheim Biochemicals, Indianapolis, IN), the amplified fragment was excised from the gel and purified with QIAEXII gel extraction kit (QIAGEN, Chatsworth, CA). The purified DNA fragment was then digested with *Sfi* I and *Not* I and ligated into the same enzyme digested pCANTAB5E (Pharmacia Biotech), creating pEY.5 (Figure 4.1).

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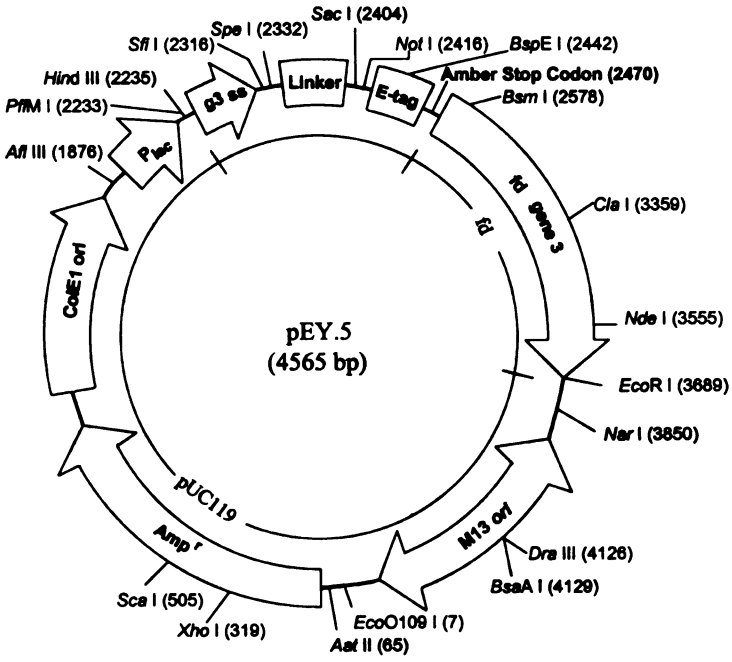

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GCG GCC CAG CCG GCC GGC ACT AGT GTC ACC GTC TCC TCA **GGT GGA**

GGC GGT TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA TCG GAC ATC

2424

GAG CTC ACT CAG GCG GCC GCA **E tag-Amber stop codon -fd gene 3**

Figure 4.1. Map of phage-display vector pEY.5. (A). Restriction map with unique restriction sites marked. (B). The nucleotide sequence of linker (bold sequence) and the V_H and V_K cloning sites (underlined) in pEY.5.

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Anti-zearalenone scFv antibody cloning in pEY.5

V_H and V_K DNA fragments were re-amplified from an anti-zearalenone scFv clone pQY1.5 phagemid DNA (Yuan et al., 1997) using the following restriction site attached primers. The primers used for V_H DNA re-amplification were: VHFORY (5'-TGAGGAGACGGTGACACTAGTGCCTTGGC-3') and VHBACKY (5'-ATGACTCGCGGCCCAGCCGGCCATGGCCSAGGTSMARCTGCAGSAGTCWGG-3'); S= C or G; M=A or C; R=A or G; W=A or T. The primers used for V_K DNA re-amplification were: VKBACKY (5'-TCGGACATCGAGCTCACTCAGTCTCC-3') and VKFORY (5'-GAGTCATTCTGCGGCCGCCCGTTTBAKYTCCARCTTKGTSCC-3'); B=T or C or G; K=T or G; Y=C or T. The re-amplified V_H DNA fragments were digested with *Sfi* I and *Spe* I and ligated into pEY.5 digested with the same enzymes. The ligated products were then digested with *Sac* I and *Not* I and ligated with the same enzyme digested V_K re-amplified DNA fragments, yielding pEY.5HL. The pEY.5HL phagemid DNA was introduced into *E. coli* TG1 cell for the production of recombinant phages in the presence of helper phage M13KO7 and into *E. coli* HB2151 for soluble scFv antibody production as described previously (Yuan et al., 1997). The soluble scFv antibody from *E. coli* cultures was characterized with ELISA as described (Yuan et al., 1997).

Construction of plant scFv expression plasmids

The anti-zearalenone scFv DNA fragment was amplified from a zearalenone binding positive clone, pEY.5HL3 (reconstructed in pEY.5 from pQY1.5), by polymerase chain reaction (PCR) using *Pfu* DNA polymerase (Stratagene, La Jolla,

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CA) with the following primers: Sense primer (5'-TATCCGCGGTATGGCCCAG GTGAAACTGC-3') containing an *Sst* II site and antisense primer (5'-CCCTCTAGACCAGACGTTAGTAAATGAA-3') containing an *Xba* I site. The amplified DNA fragment was isolated from a low melting point agarose gel, purified with QIAEXII, digested with *Xba* I, and ligated into *Hinc* II/*Xba* I -digested pUC19, yielding pQY2. For expression without a signal peptide, a *Hind* III-*Xba* I fragment from pQY2 was ligated into the *Hind* III-*Xba* I sites of pKYLX71::35S^2 directly, creating pQY4.1-1. For expression with a plant protein signal sequence, a 110-bp *Hind* III/*Sst* II fragment containing tobacco pathogenesis-related protein PR-1b signal peptide (sp) sequence from pSIG3, a clone with the PR-1b signal peptide sequence (Gopalan et al., 1996) ligated in the *Eco*R V site of Stratagene's pBluescript SK, was ligated into the *Hind* III-*Sst* II sites of pQY2, generating pQY3. Finally, a *Hind* III-*Xba* I fragment containing the sp-scFv fusion fragment from pQY3 was ligated into the *Hind* III-*Xba* I sites of pKYLX71::35S^2 (Maiti et al., 1993), creating pQY4.4-1. Both pQY4.1-1 and pQY4.4-1 were introduced from *E. coli* DH5α into *Agrobacterium tumefaciens* strain GV3850 (Zambryski et al., 1983) by triparental mating (Figurski and Heliniski, 1979).

Plant transformation

Arabidopsis thaliana ecotype Columbia (Col, *GLABROUS1*) plants were transformed using a vacuum infiltration protocol similar to the one described by Bechtold et al. (1993). Mature seeds from these infiltrated plants were germinated and selected on plant nutrient agar plates (Bechtold et al., 1993) supplemented with

50 μg/ml kanamycin. Kanamycin resistant seedlings were transferred into soil and leaves from two-month-old kanamycin resistant plants were tested for scFv antibody binding activity with ELISA.

Expression of scFv plantibody and ELISA analysis

Immulon-4 microtiter wells (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 1 μg/well of zearalenone-OVA (ZEN-OVA) conjugate in 100 μl 0.05M carbonate-bicarbonate coating buffer (pH9.4) at 4°C overnight. The wells were washed and blocked as described previously (Yuan et al., 1997). Two to three leaves from two-month-old transgenic plants were cut and ground in a 1.5 ml microcentrifuge tube on ice with a pestle. After centrifugation at 13,000 rpm in a microcentrifuge for 10 min at 4°C, the sap (supernatant) was collected. Fifty microliters of the leaf sap was diluted 1:1 (vol/vol) with 2% non-fat dry milk in PBS and added to each well, followed by incubation at 37°C for 1 hr. After washing 6 times with 320 μl/well of PBS-0.1% Tween 20, 100 μl/well of mouse anti-E tag antibody (1 μg/ml) was added, followed by addition of the goat anti-mouse IgG HRP conjugate (diluted 1:2000 in 2% non-fat dry milk in PBS). Finally, 100 μl/well of TMB substrate (Sigma Chemical Company) was added and incubated at 37°C for 15 min. The reaction was stopped by adding 100 μl/well of 10% H_2SO_4. The absorbances at 450nm were determined with a microtiter plant reader (Molecular Device Corporation, Menlo Park, CA).

For competitive ELISA, 50 μl/well of free zearalenone at various concentrations (0, 1, 10, 100, and 1000 ng/ml in PBS containing 1% methanol) was

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added into zearalenone-OVA coated wells, then 50 μl/well of diluted (1:49) plant sap (from GV3850/pQY4.4-1 transformed plants) was added. After 1 hr incubation, the bound plantibodies were detected in the same way as described above. For comparison, a CI-ELISA with diluted (1:9) bacterial anti-zearalenone scFv (produced from *E. coli* HB2151/pEY.5HL3) was performed simultaneously.

Methanol effects on the binding activity were measured for mAb, bacterial scFv antibody and scFv plantibody (diluted 10 folds in 10 % non-fat milk-PBS) as described previously (Yuan et al., 1997). The effect of the pH environment on these antibodies was also tested. The antibodies were diluted (1:5 vol/vol) in pH 2.3 and pH 6.3 (adjusted with 0.05 M citric acid and 0.05 M Na_2HPO_4) buffer solution, and incubated at 4°C, overnight. A portion of the pH 2.3 treated sample was adjusted back to pH 6.3 and incubated at room temperature for one hour. To zearalenone-KLH conjugate (1 μg/well) coated wells, 100 μl/well of pH treated antibody samples was added. Bound antibodies were determined as described above.

Protein analysis

Proteins in plant leaf sap from independent transgenic plants were separated in a 12% SDS-polyacrylamide gel. Two identical gels were run at the same time. After electrophoresis, one gel was stained in Coomassie brilliant blue G-250. The proteins in another gel were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Mouse anti-E tag antibody, goat anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Company, St. Louis, MO) and NBT/BCIP

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substrate (Pierce, Rockford, IL) were used to detect plantibodies in a procedure similar to that previously described (Yuan et al., 1997).

ScFv antibody cytolocation with immunogold labeling

Segments (approximate 0.5 x 1.0 mm) of leaves from 2-month-old transgenic *Arabidopsis* plants were fixed by vacuum infiltration in 2% glutaraldelhyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 hr at room temperature. After 3 washes in the above buffer, the samples were post-fixed with 1% osium tetroxide in 0.1 M sodium cacodylate buffer at room temperature for 2 hr, followed by dehydration with a graded acetone series. The samples were then embedded by infiltration with Resin "Spurtol" (Spurr, 1969; Kushida, 1974). Ultrathin sections were cut and supported on formvar-coated grids.

The grids containing ultrathin sections were pretreated by incubation in a saturated aqueous solution of sodium metaperiodate as described by Bendayan and Zollinger (1983) After washed throughly in distilled water, the pretreated grids were blocked for 1 hr at room temperature in 20 mM Tris-buffered saline (TBS, pH 7.5), containing 0.05 % Tween 20 , 1 % wt/vol bovine serum albumin and 0.01 % NaN_3. The samples on the grids were then incubated with the primary antibody (mouse anti-E tag antibody) diluted in the blocking buffer for 2 hr at room temperature. In the case of testing for the cross-reactivity of anti-zearalenone scFv antibody to *Arabidopsis*, samples from non-transformed control plants were incubated with anti-zearalenone scFv antibody first, then with mouse anti-E tag antibody. After four 5 min washings with TBS, the grids were incubated with goat

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anti-mouse IgG gold conjugate (10 nm gold particle) (1: 20 dilution) for 2 hr at room temperature. After four 5 min washings with TBS and two 5 min washings in distilled water, the grids were post-stained with 1 % uranyl acetate for 30 min, rinsed in distilled water, and then stained with Hanaichi's lead solution (Hanaichi et al., 1986) for 5 min. The grids were then rinsed in 20 mM NaOH and distilled water and examined with a CM10 Transmission Electron Microscope (Philips, The Netherlands). Immunogold labeling of sections of non-transformed *Arabidopsis thaliana* ecotype Columbia was performed as a control.

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RESULTS

Cloning of anti-zearalenone scFv in pEY.5

To determine the feasibility of cloning vector pEY.5 for scFv antibody expression, the DNA fragments of V_H and V_K from pQY1.5 (Yuan et al., 1997) were amplified with the above primers and re-cloned into pEY.5. In *E. coli* HB2151 , one new clone (pEY.5HL3) produced soluble scFv antibody with high affinity to zearalenone (Figure 4. 2). The IC_{50}, concentration of free zearalenone required for 50% inhibition of binding, was 15.5 ng/ml, similar to that previously described for pQY1.5. Either V_H or V_K alone cloned in pEY.5 is in frame with E-tag and g3p, therefore this vector is also suitable for selecting single domain Fv antibodies and chain shuffling. However, either V_H or V_K alone from pQY1.5 (Yuan et al., 1997) did not show any zearalenone binding activity when they were cloned into pEY.5 separately (data not shown), indicating that both V_H and V_K were important for the scFv binding to zearalenone. It was notable that *E.coli* HB2151/pEY.5HL3 grew much slower than HB2151/pEY.5, which suggests the functional anti-zearalenone scFv expression may affect the growth of the *E. coli* host cells.

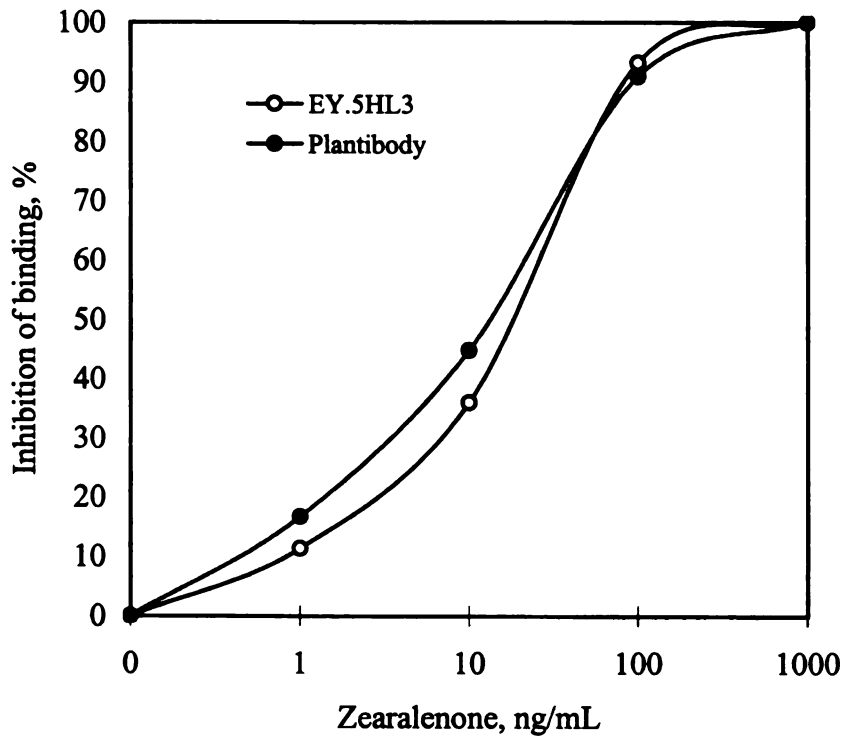

Figure 4.2. Sensitivity and specificity of anti-zearalenone scFv antibodies measured by competitive indirect ELISA using zearalenone-ovalbumin coated wells. Bound soluble scFv antibody was detected by mouse anti-E tag antibody, followed by horseradish peroxidase conjugated goat anti-mouse IgG. EY.5HL3 was produced in *E. coli* and plantibody was produced in *Arabidopsis*.

Expression of anti-zearalenone scFv plantibody

The anti-zearalenone scFv DNA fragment from pEY.5HL3 was re-cloned into a plant transformation vector to generate pQY4.1-1 and pQY4.4-1(Figure 4.3). About 1% of 20,000 seeds from plants transformed with GV3850/pQY4.4-1 were kanamycin resistant, whereas plants transformed with GV3850/pQY4.1-1 produced no kanamycin resistant seedlings from approximately 20,000 seeds (Figure 4.4). In the putative transgenic plants (kanamycin resistant) transformed with GV3850/pQY4.4-1, approximately 20% of the tested plants produced functional antibody for zearalenone as determined by ELISA. Western analysis showed that GV3850/pQY4.4-1 transformed *Arabidopsis* plants produced a 32-KDA scFv plantibody protein (Figure 4.5), similar in size to the scFv antibody produced in E. coli (Yuan et al., 1997). The specificity and sensitivity to zearalenone of the plantibody was determined by CI-ELISA (Figure 4.2). The plantibody had an IC_{50} of 11.2 ng/ml for zearalenone, showing similar affinity to zearalenone as the scFv antibody produced from bacteria and monoclonal antibody from hybridoma cell culture (Yuan et al., 1997).

From each of the T_0 transgenic *Arabidopsis* plants, 15 to 20 T_1 offspring seedlings were tested with ELISA for zearalenone binding. Some plants had an approximately 1:1 segregation ratio of functional anti-zearalenone scFv plantibody producing offspring to non-producing offspring; others had a 1:0 segregation ratio. This suggests that both single and double integrations of plant chromosomes by the anti-zearalenone scFv DNA fragment occurred. In the T_2 generation, all plants

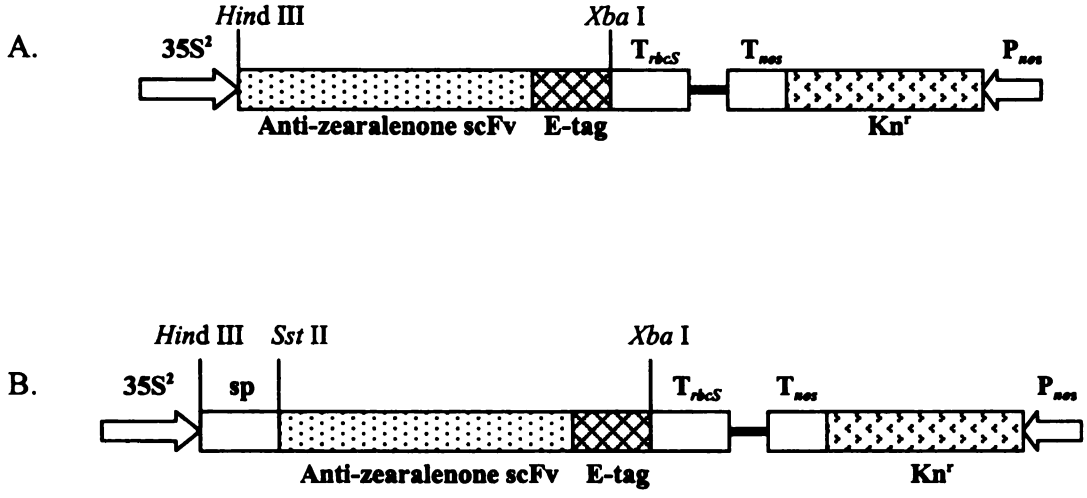

Figure 4.3. The transgene in plant transformation constructs pQY4.1-1 (A) and pQY4.4-1 (B). $35S^2$: CaMv 35S promoter with a duplicated enhancer; sp: plant pathogenicity-related protein PR-1b signal peptide sequence; T_{rbcs} and T_{nos}: transcription termination sequences for *rbc*S and *nos* respectively; P_{nos}: nos promoter; Kn^r: kanamycin resistance gene.

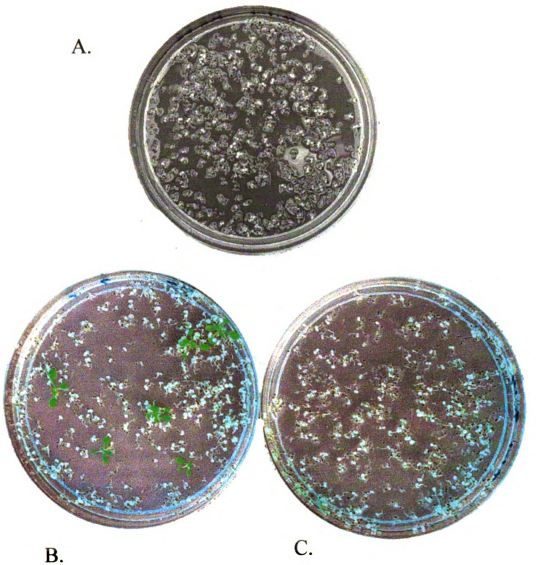

Figure 4.4. The germination of *Arabidopsis* tansformant seeds on kanamycin selection medium plates. A. Seeds from un-transformed control plants; B. Seeds from plants transformed with pQY4.4-1, targeting scFv antibody to the apoplastic space with signal peptide sequence. C. Seeds from plants transformed with pQY4.1-1, targeting scFv antibody to the cytoplasm.

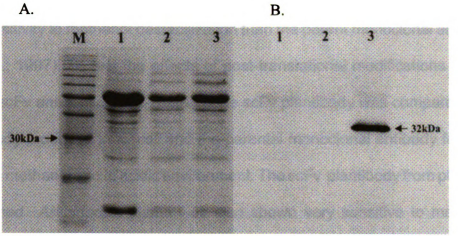

Figure 4.5. SDS-polyacrylamide gel electrophoresis and Western blot analysis of anti-zearalenone scFv plantibody expression. Soluble proteins of *Arabidopsis* from un-transformed plants (lane 1), vector pKYLX71::35S^2 transformed plants (lane 2), and pQY4.4-1 transformed plants (lane 3) were subjected to SDS-PAGE and stained with Coomassie brilliant blue G-250 (A). Western blot of duplicate SDS-polyacrylamide gel detected by anti-E tag antibody (B). Lane M: protein molecular size marker with 10KDa-ladder.

produced anti-zearalenone scFv plantibody without segregation. This indicates that the anti-zearalenone scFv antibody gene was stably integrated into the genome of *Arabidopsis*.

The effects of methanol and low pH on plantibody

As shown previously, anti-zearalenone scFv antibody from bacteria exhibited greater sensitivity to methanol destabilization than the parent monoclonal antibody (Yuan et al., 1997). To test the effects of post-translational modifications on the stability of scFv antibodies, anti-zearalenone scFv plantibody was compared with scFv antibody produced in *E. coli* and the parental monoclonal antibody for their stabilities in methanol and in acidic environment. The scFv plantibody from pQY4.4-1 transformed *Arabidopsis* plants was also shown very sensitive to methanol destabilization (Figure 4.6), whereas the parent monoclonal antibody exhibited tolerance to much higher concentrations of methanol. In the low pH environment (pH 2.3), however, all three antibodies (monoclonal antibody, bacterial scFv antibody and scFv plantibody) lost binding activity to zearalenone (Table 4.1). When the pH was adjusted back to neutral (pH 6.3), the binding activity recovered.

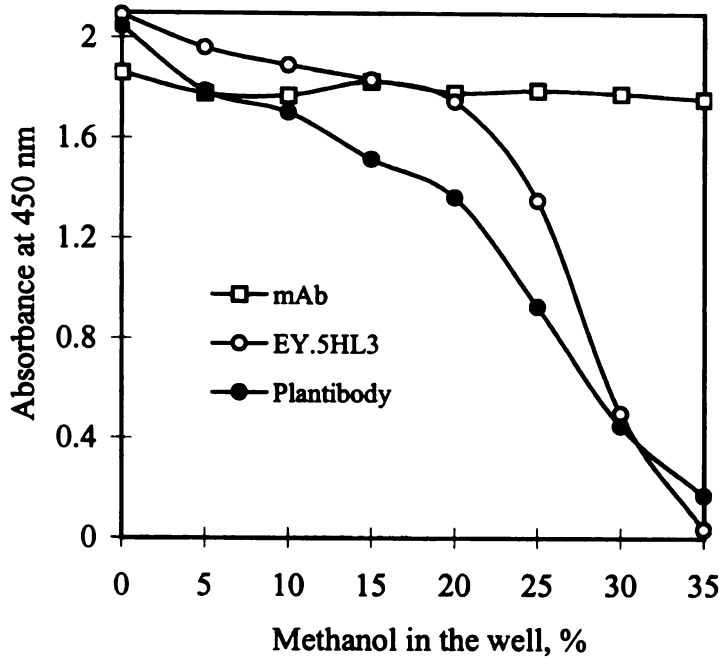

Figure 4.6. Effect of methanol on the binding of anti-zearalenone monoclonal antibody (mAb) and bacterial scFv antibody (EY.5HL3) and plant scFv antibody (plantibody) to immobilized zearalenone-KLH conjugate.

Table 4.1. Effect of pH environment on antibody binding activity.

Anti-zearalenone antibody	Relative binding activity in different pH environment, %*		
	pH 6.3	pH 2.3	pH 2.3 then pH 6.3
mAb	100	0	99
Bacterial scFv	100	0	98
scFv plantibody	100	0	89

*: The binding activity at pH 6.3 was set as 100%.

Localization of anti-zearalenone scFv plantibody

Transgenic *Arabidopsis* plants expressing anti-zearalenone scFv antibody were processed and labeled for electron microscopic immunolocalization as described in materials and methods. Gold particle deposition indicate the presence of scFv antibody mainly in the cytoplasm with a few labeling signal in the intercellular spaces (Figure 6A,B). Deposition was not found in the non-transformed control plant samples (Figure 4.7D). However, incubation of non-transformed plant sections with the anti-zearalenone scFv antibody then with mouse anti-E tag antibody followed by goat anti-mouse IgG gold conjugate caused many labeling signals evenly distributed in the cytoplasm (Figure 4.7C), indicating that anti-zearalenone scFv antibody can bind to *Arabidopsis* cell components.

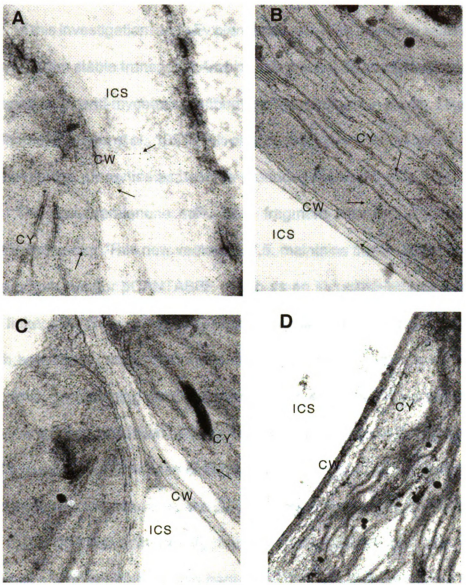

Figure 4.7. Cytolocation of anti-zearalenone scFv plantibody by immunogold labeling and transmission electron microscopy. Leaf sections from plants expressing anti-zearalenone scFv plantibodies (A, B) and non-transformed control plants (D) were detected by mouse anti-E tag antibody followed by goat anti-mouse IgG gold conjugate. (C): Leaf sections from non-transformed control plants were pre-incubated with anti-zearalenone scFv antibody, then with mouse anti-E tag antibody followed by goat anti-mouse IgG gold conjugate. CW, cell wall; CY, cytoplasm; ICS, intercellular space; Arrows: gold labeling; Magnification x 39,000.

DISCUSSION

In this investigation, an scFv plantibody with high affinity to zearalenone was produced from stable transgenic *Arabidopsis* plants . To our knowledge, this is the first report of an anti-mycotoxin antibody gene expressed in plants. Like other scFv plantibodies (Bruyns et al., 1996), this anti-zearalenone scFv plantibody had similar antigen-binding properties as bacteria-produced scFv antibody.

The anti-zearalenone scFv DNA fragment was first cloned in a newly constructed vector. This new vector, pEY.5, maintains the advantageous features of the original vector pCANTAB5E , such as an inducible *lac* promoter, a widely used linker $(Gly_4Ser)_3$, a highly specific E-tag, an amber stop codon allowing the switch between expression of membrane anchored scFv-g3p fusion and soluble scFv simply by changing the expression host. It also allows the direct cloning of V_H and V_K, with the introduction of two rare cutting restriction enzyme sites (*Spe* I and *Sac* I). In the Kabat Database of Sequences of Proteins of Immunological Interest (http://immuno.bme.nwu.edu), the *Spe* I recognition sequence was found in less than 1% of 4701 known murine V_H chains and 1% of 2492 known murine V_K chains. *Sac* I has a slightly higher cutting frequency, but accounts for only 4.5% and 2.3% of known V_H and V_k chains, respectively. This indicates that the majority of variable region cDNAs will be free from internal cutting. We tested the feasibility of this new vector by cloning and expressing an anti-zearalenone scFv in *E. coli*. By utilizing the *Sfi* I/*Spe* I or *Sac* I/*Not* I sites, this new vector will also facilitate chain shuffling,

which will be useful for cloning compatible variable chains and improving antigen binding ability (Kang et al., 1991; Marks et al., 1992a).

For plant transformation, two constructs of the anti-zearalenone scFv were made by fusion with or without the N-terminal signal peptide from tobacco pathogenesis related protein PR-1b, which has been used to secrete foreign proteins to the plant apoplast (Dixon et al., 1991; Lund and Dunsmuir, 1992). The PR-1b signal sequence was included in the expectation that it would target the fusion protein to the endoplasmic reticulum where the signal peptide would be cleaved and the scFv protein transported to the apoplast via the default pathway (Denecke et al., 1990). Plants transformed with this signal sequence containing construct produced many transformants expressing scFv plantibody with high affinity to zearalenone. However, electron microscopic immunolocalization indicated the presence of the scFv plantibody mainly in the cytoplasm. Only low level of labeling signals existed in the intercellular space and was limited to those parts of intercellular space that were filled with electron-dense material. The labeling signals in the cytoplasm might reflect a binding activity of anti-zearalenone scFv to some plant cell components. As a matter of fact, immunogold labeling showed that anti-zearalenone scFv antibody binds to non-transformed *Arabidopsis* cell components in the cytoplasm. Although some antibodies or antibody fragments fused with plant signal sequences have been confirmed by immunogold electron microscopy to be secreted through the cell wall into intercellular spaces, despite limitations in cell wall porosity (De Wilde et al., 1996), others were retained in endoplasmic reticulum compartment or in the cytoplasm (Düring et al., 1990; Artsaenko et al., 1995;

Schouten et al., 1997). The construct without signal sequence failed to generate any transgenic plants in this study. One explanation might be that high accumulation of anti-zearalenone scFv in the plant cytoplasm and the sequential binding to plant cell components was toxic to the host plant cell.

As previously reported, anti-zearalenone scFv antibody produced from bacteria had greater sensitivity to methanol destabilization than its parent mAb. One of the possibilities may be the differences between prokaryote and eukaryote in protein post-translational modifications, especially glycosylation. N–linked glycosylation of proteins in plants is very similar to the glycosylation process in mammals (Jones and Robison, 1989; Sturm et al., 1987). Possible plant post-translational changes in addition to glycosylation include fatty acylation, phosphorylation, sulfation, or modifications that change the p*I* of the protein (Jones and Robinson, 1989). The comparison between antibody produced in bacteria and plants may help elucidate the role of post-translational modifications in stabilizing antibody. Unfortunately, it was not possible to test the hypothesis that differences between anti-zearalenone scFv antibody and its parent mAB in methanol sensitivity reflect structural differences derived from post-translational modifications because the anti-zearalenone scFv lacks the canonical Asn-X-Ser/Thr signal sequence for glycosylation.

Some mycotoxins not only cause toxic effects to animal and humans, but also serve as virulence factors for the producing fungi in causing plant diseases. The mycotoxin zearalenone has been reported to cause certain phytotoxicities (Wakulinski, 1989; Vianello and Macri, 1981; Nedelnik, 1993). The expression of

anti-zearalenone antibody in plants might be useful to reduce the severity of plant diseases associated with this mycotoxin.

Antibodies have been used successfully as antidotes for some low molecular weight toxins through passive immunization (reviewed by Baud et al., 1997). However, large quantities of antibody are generally required for passive immunization *in vivo*. The prospect of harvesting antibody on an agricultural scale offers the potential for economic production of almost limitless amounts of antibody. The production of antibody in edible plants makes it possible to deliver large amount of toxin specific antibody through food and feed.

Feed-delivered antibody could theoretically bind zearalenone in the intestine providing it can survive passage through the acidic stomach environment. When the anti-zearalenone antibody was subjected to low pH, the mAb and scFv antibody from both bacteria and plants lost their binding ability. However, all three antibodies recovered their binding activity when the pH was adjusted back to neutral (pH 6.3). This suggests that the pH effects on the binding activity is not irreversible denaturation. Thus, providing these antibodies survive the proteolysis in the gut, they could recover binding to zearalenone in the small intestine, where the pH is close to neutral. Whether the anti-zearalenone plantibody reported in this study will neutralize zearalenone in animal feed and reduce toxicity will be determined in the future.

The expression of mycotoxin specific plantibody may also be useful in reducing mycotoxin-associated plant diseases. Especially, the catalytic plantibodies expressed in crops may degrade mycotoxins when they are produced.

PART V

IDENTIFICATION OF MIMOTOPE PEPTIDES WHICH BIND TO THE MYCOTOXIN DEOXYNIVALENOL-SPECIFIC MONOCLONAL ANTIBODY

ABSTRACT

IDENTIFICATION OF MIMOTOPE PEPTIDES WHICH BIND TO THE MYCOTOXIN DEOXYNIVALENOL-SPECIFIC MONOCLONAL ANTIBODY

A monoclonal antibody (mAb) 6F5 that recognizes the mycotoxin deoxynivalenol (DON, vomitoxin) was used to select for peptides that mimic the mycotoxin by employing a library of filamentous phage which display random 7-mer peptides on their surface. Two phage clones were selected from the random peptide phage-display library and found to code for the amino acid sequences SWGPFPF and SWGPLPF. These clones were named DONPEP.2 and DONPEP.12, respectively. A competitive enzyme-linked immunosorbent assay (ELISA) strongly suggested that these two phage-displayed peptides bound to mAb 6F5 specifically in the deoxynivalenol binding site. The amino acid sequence of DONPEP.2 plus a structurally flexible linker at the C-terminus, i.e. SWGPFPFGGGSC, was synthesized and tested for its ability to bind to mAb 6F5. This synthetic peptide (C430) and deoxynivalenol competed with each other for mAb 6F5 binding. When translationally fused with bacterial alkaline phosphatase, DONPEP.2 bound specifically to mAb 6F5 while retaining enzyme activity. The potential use of these peptides as immunochemical reagents in deoxynivalenol immunoassay was evaluated with a deoxynivalenol-spiked wheat extract. When peptide C430 was conjugated to bovine serum albumin, it elicited antibodies in mice and rabbits that were specific only to the peptide C430 but not to deoxynivalenol. In an *in vitro* translation system containing rabbit reticulocyte lysate, the synthetic peptide C430 did not inhibit protein synthesis but did show antagonism toward deoxynivalenol-

induced protein synthesis inhibition. These data suggest that the peptides selected in this study bind to both mAb 6F5 and to the ribosome at the same sites as deoxynivalenol does, but possibly in a slighter different orientation.

INTRODUCTION

Deoxynivalenol (DON, vomitoxin, Figure1.3) is one of the sesquiterpene mycotoxins classified as 12,13-epoxy-trichothecenes (Mirocha et al., 1977). It occurs naturally in infected corn (Hart et al., 1982; Mirocha et al., 1976), small grains (Hart and Braselton, 1983; Neish and Cohen, 1981), and mixed feeds (Mirocha et al., 1976). DON is mainly produced by the fungus *Gibberella zeae* (Schwein.) Petch (anamorph =*Fusarium graminearum* Schwabe). At the cellular level, the main toxic effect of DON is inhibition of protein synthesis via binding to the ribosome and by interfering with peptidyltransferase (Ueno, 1983; Thompson and Wannemacher, 1986; Betina, 1989b). In animals, DON can cause anorexia and emesis (vomiting) (Mirocha et al., 1976; Rotter et al., 1996); Other toxic effects of DON include skin irritation, hemorrhaging, hematological changes, human lymphocyte blastogenesis impairment, radiomimetic effects, apoptosis (cytotoxicity) and immunotoxicity (Ueno, 1983; Forsell and Pestka, 1985; Forsell et al., 1986; Pestka et al., 1987; Pestka et al., 1994b; Pestka and Bondy, 1994; Ueno et al., 1995; Rotter et al., 1996).

A major means for eliminating DON from human and animal food, like for most other mycotoxins, is to detect and divert contaminated raw materials from feed and finished food use. Conventional methods for DON detection (Pathre and Mirocha, 1977; Scott, 1982), including gas chromatography, gas chromatography-mass spectrometry, high-pressure liquid chromatography, and thin-layer chromatography, involve considerable sample preparation, are time-consuming, and

require technical expertise. Compared with other methods, immunoassays have several advantages for rapid field tests, including high specificity, sensitivity, facile sample preparation, and ease of use (Pestka et al., 1995). Following the development of a reliable monoclonal antibody to DON (Casale et al., 1988), immunological methods, mainly enzyme-linked immunosorbent assay (ELISA), have been used widely for detection of DON (Pestka et al., 1995). Such developments have led to a great demand for specific antibodies and related immunochemical reagents for the assay. However, chemical conjugation of DON to a carrier protein or an enzyme has low efficiency because it involves extensive modification and blocking stages and causes substantial bridge-group interferences and un-wanted cross-reactions (Casale et al., 1988; Wilkinson et al., 1992; Xiao et al., 1995; Pestka et al., 1995). Secondly, when DON is conjugated to a carrier protein, it is weakly immunogenic. Finally, since DON is toxic and is included as standard and conjugate in immunoassay, it potentially poses a toxicity risk to the kit user.

A possible alternative to the use of mycotoxins as immunochemical reagents would be to develop protein or peptide mimics that serve the same function. One approach for this is through generation of anti-idiotype antibodies (Chanh et al., 1989; 1990; 1991;1992; Hsu and Chu, 1994; Chu et al., 1995), which mimic the surface structure of low-molecular-weight biological toxins. Sometimes the sensitivity of original antibody can be improved via generation anti-anti-idiotype antibody (Chanh et al., 1990). However, these techniques are time consuming and costly. A new technique, namely phage display, has been developed more recently. In this procedure, foreign peptides and proteins can be genetically fused to the N-

terminus of the minor coat protein g3p of the filamentous phage fd, resulting in display of the peptides and proteins on the surface of the virion (Smith, 1985). By insertion of random oligonucleotide sequences into the g3 gene of filamentous phage fd, phage display provides a means of constructing extensive peptide libraries that may be screened to select peptides with specific affinities or activities (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990). Phage-displayed short peptide libraries have been widely used in a number of applications (Cortese et al., 1995; Lowman, 1997; Katz, 1997), including epitope mapping (Scott and Smith, 1990; Cwirla et al., 1990), mapping of protein-protein contacts (Hong and Boulanger, 1995), identification of protease substrate (Smith et al., 1995), identification of integrin (O'Neil et al., 1992) and other receptors (Doorbar and Winter, 1994), antagonists (Pierce et al., 1996), and ligands (Katz, 1997). Few studies, however, have used this technology to select peptide mimics of non-proteinaceous chemicals with the exception of lectin (Oldenburg et al., 1992; Scott et al., 1992), biotin (Weber et al., 1992), and carbohydrate (Hoess et al., 1993).

Given the feasibility of selecting peptides that mimic non-proteinaceous chemicals, we were interested in determining whether peptide mimics of low-molecular-weight mycotoxins could be identified. Once selected, these peptide mimics might be useful in mycotoxin immunoassay and for development of new antibodies against mycotoxins. Here we describe two closely related heptapeptide mimotopes for DON selected from a phage-display random peptide library and their application to the analysis of DON by ELISA. The mimotope peptide was also

compared with DON for cytotoxicity in cell culture and *in vitro* protein synthesis inhibition.

MATERIALS AND METHODS

Reagents

All inorganic chemicals and organic solvents were reagent grade or better. Ovalbumin (OVA) (crude), bovine serum albumin (BSA)(fraction V), 3,3',5,5'-tetramethyl-benzidine (TMB), phenylmethylsulfonyl fluoride (PMSF), polyoxyethylene sorbitan monolaurate (Tween 20), deoxynivalenol (DON), mouse immunoglobulin G, and goat anti-mouse IgG peroxidase conjugate were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) and methanol were purchased from J. T. Baker Inc. (Phillipsburg, NJ). Instant nonfat dry milk (Commander Foods, Inc., Syracuse, NY) was purchased from a local grocery store. DON-BSA, DON-OVA, and DON-horseradish peroxidase (DON-HRP) conjugates at DON's 3-hydroxyl group were made though an activated *N*-hydroxysuccinimide ester as described by Casale et al. (1988) (Figure 5.1) and kindly provided by Frank E. Klein (Neogen Corporation, Lansing, MI). Sheep anti-M13 horseradish peroxidase (HRP) conjugate was purchased from Pharmacia Biotech Inc. (Piscataway, NJ). *m*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester (Sulfo-MBS) and *p*-nitrophenyl phosphate disodium salt tablets (pNPP) was purchased from Pierce Chemical Company(Rockford, IL). Anti-DON monoclonal antibody from hybridoma cell line 6F5 was prepared as described previously (Casale et al., 1988).

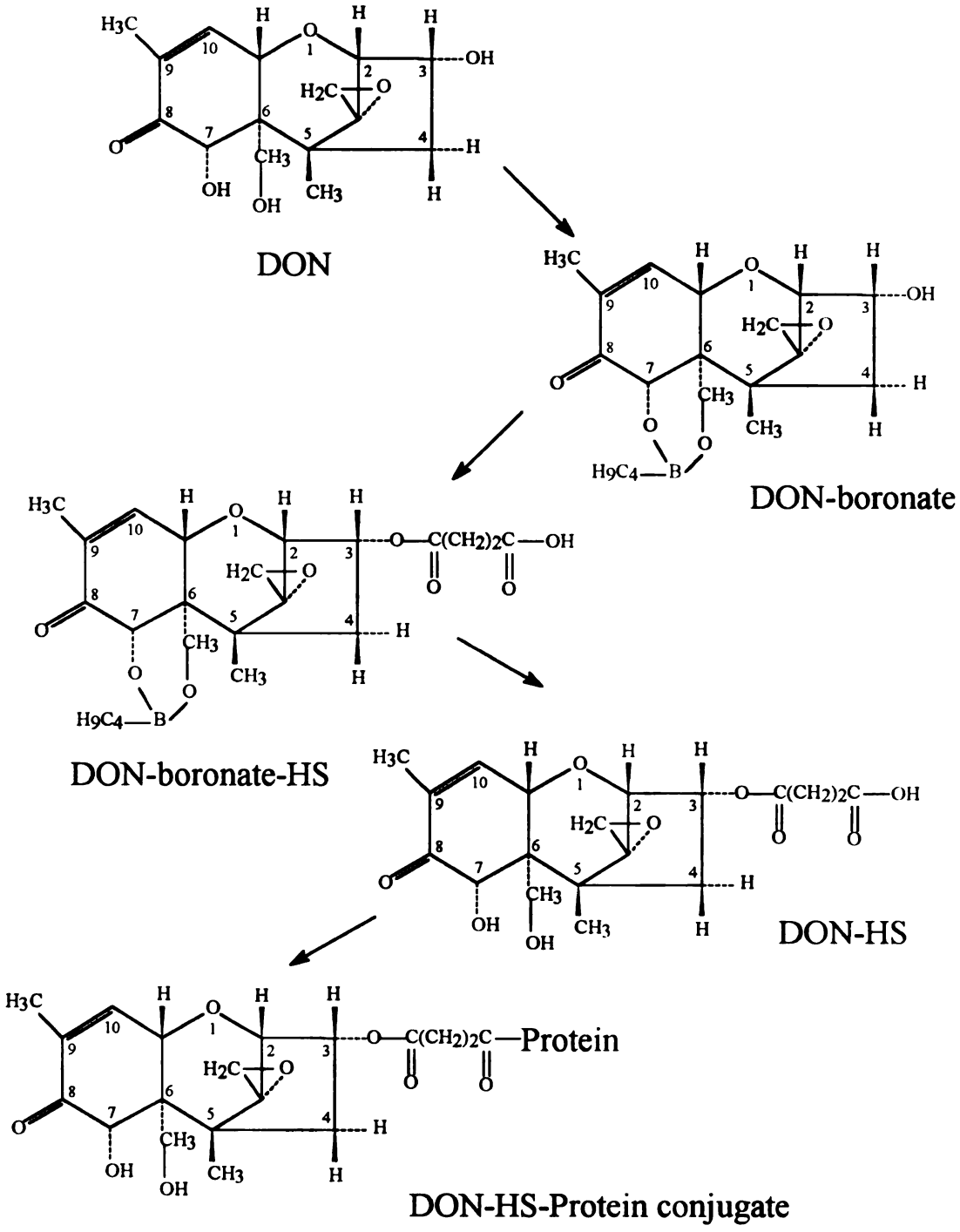

Figure 5.1. Preparation of deoxynivalenol (DON) conjugates via 3-hydroxyl group (Pestka et al., 1995).

Phage-display peptide library and amplification

A phage-display heptapeptide library with 2 x 10^9 sequence complexity (random peptide 7-mers fused to the minor coat protein g3p of the filamentous coliphage M13) was purchased from New England Biolabs, Inc. (Beverly, MA). The library was amplified by adding 5 μl (= 10^{11} pfu) recombinant M13 phage from the stock library into 100 ml of *Escherichia coli* ER2537 (New England Biolabs, Inc.) (grown in LB medium with 250 rpm shaking at 37°C to OD_{600} approximately 0.4) and incubated 6.5 hr at 37°C with shaking at 250 rpm. After the culture was centrifuged at 1,200 x *g* for 20 min, the supernatant containing amplified recombinant M13 phage was collected and filtered through an Acrodisc Syringe Filter (pore size 0.45μm, low protein binding) (Gelman Science, Ann Arbor, MI). The titer of the recombinant phage was determined by plaque counting after infection of *E. coli* ER2537 with serial 10-fold dilutions of the phage in LB top agar and plating on LB agar plates.

Phage selection by panning-elution selection

One hundred microliters of mAb 6F5 (15 μg/ml) in 0.01M phosphate buffered saline (PBS) (pH 7.4) was dispensed into each well of disposable Immulon-4 microtiter strips (Dynatech Laboratories, Inc., Chantilly, VA). The antibody was dried onto the wells in a forced-air oven at 40°C, overnight. Blank wells were coated with an equal concentration of mouse immunoglobulin G. The wells were washed 4 times by filling each well with 300 μl PBS and aspirating the contents. Nonspecific binding was blocked by incubating 320 μl of 10% non-fat dry milk dissolved in PBS

(10% milk-PBS) in each well for 1 hr at 37°C and then washing 4 times with PBS. For the panning-elution selection (Figure 5.2), the recombinant phage-display peptide library (diluted with 10% milk-PBS to approximately 10^{10} pfu/ml) was then added into the wells (100 μl/well) and incubated at 37°C for 1 hr. The wells were washed 20 times by filling each well with approximately 300 μl PBS containing 0.1% (vol/vol) Tween 20 (PBS-T) followed by incubation with 300 μl/well of PBS at 37°C with shaking at 150 rpm for 1 hr. The wells were washed again 10 times with PBS-T (300 μl/well). To elute the bound phages in the microtiter wells, 100 μl of DON (100 μg/ml in PBS containing 1% methanol) or PBS containing 1% methanol was added to each well and incubated at 37°C with shaking at 150 rpm for 1 hr. The eluted phages were then collected from the microtiter wells and used to reinfect *E. coli* ER2537 cells for phage titering and amplification. After four rounds of panning-elution selection, individual plaques were picked from LB plates and used to infect 2.5 ml of *E. coli* ER2537 culture described above. After 6 hr incubation at 37°C with shaking at 250 rpm, the culture was centrifuged at 10,000 x *g* for 10 min and the supernatant containing recombinant phages was collected. The binding activity to mAb 6F5 was tested for each individual phage clone by ELISA. The bound phages in mAb 6F5 coated microtiter wells was determined by incubation with 100 μl/well of sheep anti-M13 HRP conjugate (5000 times diluted in 10% non-fat dry milk-PBS) at 37°C for 1 hr followed by incubation with 100 μl/well of TMB substrate at 37°C for 15 min. The absorbance was read at 450nm after stopping the reaction with 100 μl/well of 10% H_2SO_4 .

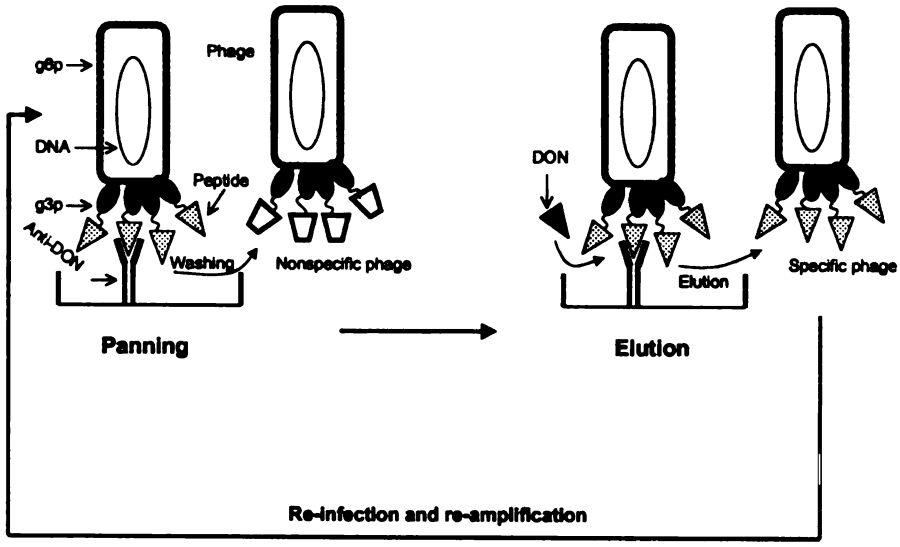

Figure 5.2. Panning-elution selection procedure for deoxynivalenol (DON) mimotopes in anti-DON monoclonal antibody coated Immulon-4 microtiter wells.

Titration and amplification of panning-elution selected phages

A 10-fold dilution series of the selected phages was made in LB medium and 100 µl of the diluted phages was mixed with 100 µl of *E. coli* ER2537 (grown in LB with shaking (250 rpm) at 37°C to OD_{600} approximately 0.2) and incubated at room temperature for 30 min for infection. The infected *E. coli* ER2537 cells were mixed with 4 ml LB top agar and spread onto LB agar plates. After solidification, the plates were incubated at 37°C overnight and plaques were counted. To amplify the phages, approximately 2 ml of phage solution eluted from monoclonal antibody 6F5 coated wells was added into 50 ml *E. coli* ER 2537 (grown in LB with shaking at 250 rpm at 37°C to OD_{600} 0.2) in a 250 ml flask and incubated for 6 hr at 37°C with shaking (250 rpm). The supernatant from this culture was collected after centrifugation at 10,000 x *g* for 10 min. In order to avoid the interference of a high concentration of free DON in the phage solution, 100 µl of the supernatant containing amplified phages was added into 50 ml *E. coli* ER 2537 for re-amplification as described above. The re-amplified phages, containing approximately 8 ng/ml of DON, was collected after centrifugation and used for a subsequent round of panning-elution selection.

Competitive ELISA with phage-displayed peptide

For competitive direct ELISA (CD-ELISA) with phage-displayed peptide, microtiter wells were coated with mAB 6F5 and blocked as described for panning-elution selection. Various concentrations (0-10,000 ng/ml in 1% methanol-PBS) of DON were mixed with an equal volume of phage-displaying peptide (diluted 10

times in 10% non-fat dry milk-PBS). The mixtures were added into mAB 6F5 coated microtiter wells at 100 μl/well and incubated at 37°C for 1 hr. After washing 6 times with PBS-T (300 μl/well), the bound recombinant phages were determined by incubation with 100 μl/well of sheep anti-M13 HRP conjugate (diluted 1:5000 in 10% non-fat dry milk-PBS) at 37°C for 1 hr. Bound enzyme was determined as described above. For comparison, 50 μl/well of DON-HRP was also mixed with 50 μl/well of DON at various concentrations (0-10,000 ng/ml in 1% methanol-PBS) and incubated in mAB 6F5 coated microtiter wells at 37°C for 1 hr. Bound enzyme was determined as described above.

For competitive indirect ELISA (CI-ELISA) with phage-displayed peptide, 100 μl/well of phage-displayed peptide was dispensed into each well of disposable Immulon-4 microtiter strips and dried onto the wells in a forced-air oven at 40°C overnight. Plates were washed and blocked as in the CD-ELISA. Various concentrations (0-10,000 ng/ml in 1% methanol-PBS) of DON (50 μl/well) were added into the wells followed by 50 μl/well of anti-DON mAB 6F5 (10 μg/ml in 10% non-fat dry milk-PBS). Wells were incubated at 37°C for 1 hr. After washing 6 times with PBS-T, the bound anti-DON mAB 6F5 was determined by incubation with goat anti-mouse IgG HRP conjugate (diluted 2000 times with 10% non-fat dry milk-PBS) at 37°C for 1 hr. Bound enzyme was determined as described above.

DNA sequencing

After confirmation of the specific binding to mAB 6F5 by ELISA, 10 ml of recombinant phage (10^{11} pfu/ml in LB) from each positive phage clone was used for

single stranded DNA (ssDNA) isolation with QIAprep Spin M13 Kit (QIAGEN Inc., Chatsworth, CA). The ssDNA was sequenced with -28 gIII sequencing primer and -96 gIII sequencing primer (New England Biolabs, Inc.) by *Taq* cycle sequencing and dye terminator chemistry at the Michigan State University Sequencing Facility.

Translational fusion with bacterial alkaline phosphatase (AP)

A 179 bp DNA fragment encoding the g3p signal peptide, DON mimotope peptide and first 17 amino acids of M13 phage g3p protein was amplified by polymerase chain reaction (PCR) with *Pfu* DNA polymerase using a sense primer (5'-GCCAAGCTTAGATCTTGGAGCCTTTTTTTTGGAG-3') and an anti-sense primer (5'-CCGGTCGACCTGTATGGGATTTTGCTAAACAACT-3'). After gel purification, the amplified DNA was digested with *Bgl* II and *Sal* I and cloned into *Bam*H I-*Sal* I digested pLIP5 (Carrier et al., 1995) to generate plasmid pQY7. The ligated product was used to transform *E. coli* DH11S competent cells (Gibco BRL, Gaithersburg, MD), generating DH11S/pQY7. For DONPEP-AP fusion protein production, DH11S/pQY7 was grown in SB supplemented with ampicillin (100 μg/ml) and 2 % glucose at 30°C with shaking (250 rpm) to absorbance (600nm) (A_{600}) approximately 0.5. After the culture was centrifuged at 1000 x *g* for 10 min and the medium was discarded, the cells then were resuspended in SB containing ampicillin (100 μg/ml) and 1 mM isopropyl-β-D-thiogalctopyranoside (IPTG) and incubated with shaking (250 rpm) at 30°C overnight. The supernatant containing the extracellular soluble DONPEP-AP fusion protein was separated from the cell pellets by centrifugation at 7,000 x *g* for 15 min and filtered through a 0.45 μm-pore-size

filter. The periplasmic DONPEP-AP fusion protein was extracted by suspending the bacterial cells (1:5 vol/vol) in lysis buffer (50 mM Tris-HCl, pH 8.0, 20% sucrose, 10 mM EDTA , 0.1 mg/ml of lysozyme, 0.5 mM PMSF), and left on ice for 1 hr with agitation before centrifugation for 30 min at 7,000 x *g*. The supernatant was filtered through a 0.4 μm porous filter. The activity of DONPEP-AP fusion protein was determined by CD-ELISA as described below.

CD-ELISA with DONPEP-AP fusion protein

Microtiter wells were coated with mAB 6F5 and washed as described above. Various concentrations (0-10,000 ng/ml in 0.05M Tris buffered saline, pH7.4) of DON were mixed with an equal volume of DONPEP-AP fusion protein (periplasmic extract diluted 1:7 with 2% non-fat dry milk-TBS). The mixtures (100 μl/well) were added into each mAB 6F5 coated microtiter well and incubated at 37°C for 1 hr. After washing 6 times with 300 μl TBS containing 0.1% Tween 20 (TBS-T), the bound DONPEP-AP was determined by incubation with pNPP substrate solution at 37°C for 45 min. The absorbance was read at 405 nm.

CD-ELISA using peptide C430 and its HRP conjugate

One DON mimotope peptide plus a structurally flexible linker and a cysteine residue, NH_2- SWGPFPFGGGSC-COOH, was synthesized via Fmoc chemistry at Bio-Synthesis, Inc. (Lewisville, TX) and was named C430. The synthetic peptide C430, at various concentrations, was used to compete with DON-HRP for binding to mAB 6F5 in CD-ELISA. C430 was also chemically conjugated to HRP with a

sulfo-MBS cross linker. For CD-ELISA with C430-HRP conjugate, the procedure was the same as CD-ELISA with DON-HRP described above, except that C430-HRP (diluted 1:5000 in blocking buffer) replaced DON-HRP.

Immunization of animals with C430-BSA conjugate

The synthetic peptide C430 was conjugated to bovine serum albumin (BSA) with a sulfo-MBS cross linker. The conjugate (approximately 29 : 1 molar ratio of peptide C430 to carrier protein BSA) was used to inject 7-weeks-old BALB/c female mice intraperitoneally or 6-month-old New Zealand white rabbits subcutaneously. Initial injections for mice were 100-200 μg of C430-BSA conjugate in 200 μl of saline-Freund's complete adjuvant (1:1), followed at 3-week intervals by boosts of 100-200 μg conjugate in 200 μl saline-Freund's incomplete adjuvant (1:1). The initial injection for rabbits was 1 mg of C430-BSA conjugate in 1 ml of saline-Freund's complete adjuvant (1:1), followed at 4-week intervals by boosts of 250 μg conjugate in 1 ml saline-Freund's incomplete adjuvant (1:1). Animals were bled 1 week after each boost. Antisera were screened for specific binding in phage-displayed DONPEP.2 coated wells by CI-ELISA using C430 or DON as competitive antigens.

Cytotoxicity and effects on protein synthesis *in vitro*

B6C3F1 mice (10-weeks-old) were euthanized by cervical dislocation and femurs were removed. Bone marrow cells were flushed from the femurs using a 1 cc syringe and 25 gauge needle. Erythrocytes were lysed with 0.83% ammonium chloride. Cell number and viability were determined by trypan blue dye exclusion

using a hemacytometer. The cells were cultured at 1 X 10^6 cells/ml in 96 well flat bottom plates in RPMI-1640 in a 5% CO_2 humidified incubator. RPMI-1640 was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 2 mM glutamine and 10% fetal bovine serum. DON and synthetic peptide C430 were diluted in RPMI-1640. Duplicate cultures were treated. After 18 hr of exposure to DON and synthetic peptide C430, cell viability was determined using an MTT conversion assay as described by Marin et al. (1996).

To determine the effects on protein synthesis, DON or synthetic peptide C430 (0 or 3.4 μM in the final 50 μl reaction) was added to 30 μl biotin translation mix (Boehringer Mannheim Corporation, Indianapolis, IN) containing reticulocyte lysate, 10 pmol biotin-lysine-$tRNA^{lys}$, 42 μM amino acids without lysine, spermidine, energy mix, dithiothreitol, 83 mM K-acetate, and 0.83 mM Mg-acetate. The mixtures (46 μl) were incubated on ice for 10 min, followed by the addition of 4 μl of γ-globulin mRNA (0.5 μg/μl). The final reaction (50 μl) was incubated at 30°C for 1 hr. The translated protein samples were stored at -80°C.

For Western blot analysis, 3 μl of protein translated *in vitro* were boiled for 10 min after mixing with SDS-PAGE loading buffer. The samples then were loaded into a mini SDS-10% polyacrylamide gel and subjected to electrophoresis at 80 V for 2 hr. The newly synthesized proteins were detected by electrotransfer to a PVDF transfer membrane (DuPont NEN Research Products, Boston, MA), followed by incubation with streptavidin-peroxidase conjugate (Boehringer Mannheim Corporation). Bound enzyme was visualized by incubation with SuperSignal®

ULTRA chemiluminescent substrate (Pierce Chemical Company) and exposing a Kodak XAR5 autoradiography film (Kodak, Rochester, NY).

RESULTS

Panning-elution selection of specific phages

After each round of panning-elution selection, the numbers of eluted phages were monitored (Figure 5.3). Enrichment for the specific phages with affinity to mAb 6F5 was observed after two rounds of panning-elution selection. The phages with affinity to mAb 6F5 were enriched approximately six orders of magnitude after the fourth round of panning-elution. Fifteen individual phage plaques from the fourth round of panning-elution selected phages were randomly isolated to infect *E. coli* ER2537 for phage reamplification. Each of the 15 phage isolates was tested for their binding activity to mAb 6F5 by ELISA. Five of these phage isolates, named as DONPEP.1, DONPEP.2, DONPEP.3, DONPEP.4, and DONPEP.12, showed binding activity to mAb 6F5.

Single stranded phage DNA was isolated and the nucleotide sequences were determined for each of the five positive phage isolates. Four of five sequenced isolates had identical DNA sequence (5'-AGTTGGGGTCCTTTTCCGTTT-3') encoding the consent peptide sequence SWGPFPF, while isolate DONPEP.12 differed by 4 nucleotides (5'-**TC**TTGGGGTCC**GC**TTCCTTTT-3') resulting in a change of the fifth amino acid from F^5 to L^5.

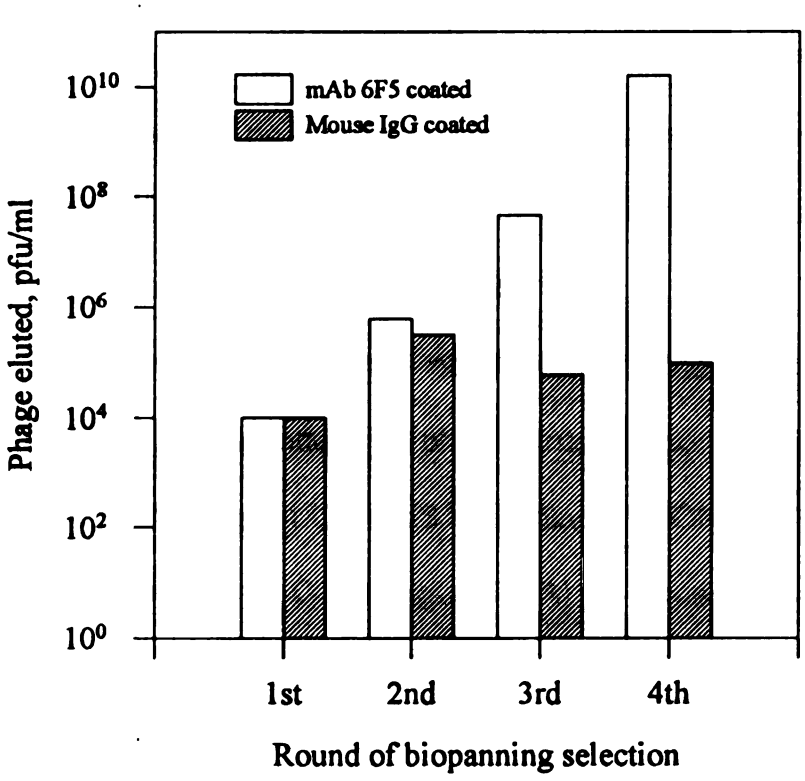

Figure 5.3. Enrichment of mAb 6F5 specific phage. Phage were titered after elution with DON (100 μg/ml) from mAb 6F5 coated Immulon-4 microtiter wells or from mouse IgG coated Immulon-4 microtiter wells in each round of panning-elution selection.

Application of DON mimotope peptide in competitive ELISA

To demonstrate whether the positive recombinant phage peptides actually mimic the epitope recognized by mAB 6F5 or just bind non-specifically to the surface of the antibody molecule outside the antigen binding site, two different positive phage clones were tested for binding to mAB 6F5 in a CD-ELISA (Figure 5.4). Binding of phage clone DONPEP.2 and DONPEP.12 to immobilized mAB 6F5 was competitively inhibited by free DON. This strongly suggested that these two phage clones were binding to the antigen binding site of the monoclonal antibody, mimicking, in part, the structural epitope of DON.

To confirm that the sequence obtained from the phage library binds specifically to mAB 6F5 independently of phage structural context, synthetic peptide C430 was tested for binding in CD-ELISA. Binding of DON-HRP to immobilized mAB 6F5 was inhibited by free C430 (Figure 5.5A) while the binding of C430-HRP conjugate to immobilized mAB 6F5 was conversely inhibited by free DON (Figure 5.5B). This suggested that the peptide was by itself sufficient for binding to the antibody, independent of the phage structural context. When C430 was used to compete DON-HRP or C430-HRP for binding to mAB 6F5, the IC_{50}'s were 0.64 to 0.8 μM, whereas 3.4 μM of free DON was required to reach 50% inhibition of DON-HRP or C430-HRP binding to the same antibody (Table 5.1). This indicates a higher affinity of mAB 6F5 for C430 than for DON. In a similar CD-ELISA, none of the individual amino acids (at concentrations up to 34 μM) in C430 showed any significant binding inhibition to DON-HRP (Figure 5.6). This suggests that the

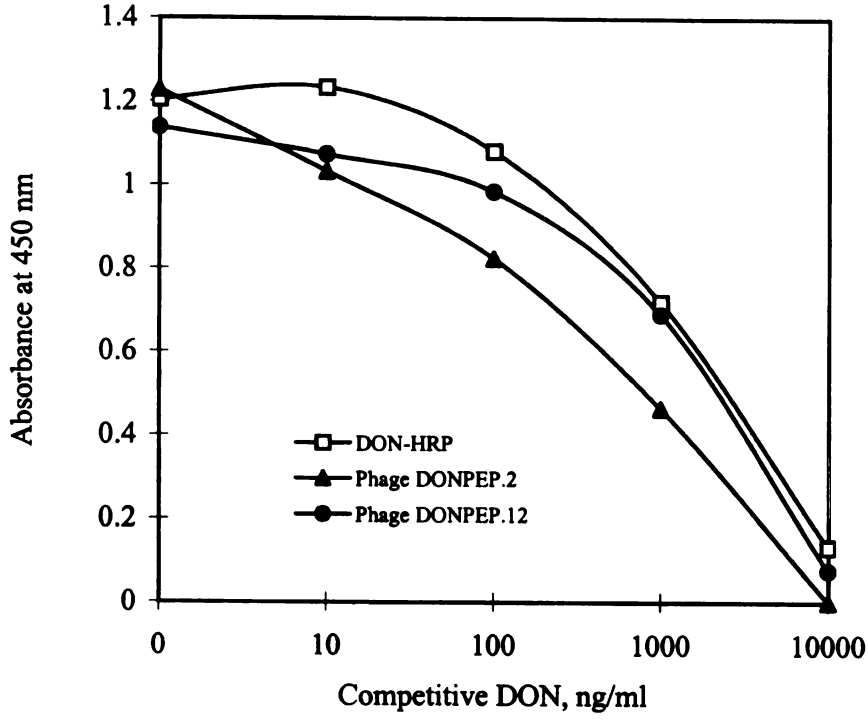

Figure 5.4. Competition between phage-displayed mimotope peptides and DON for binding to immobilized mAb 6F5 in CD-ELISA. Various concentrations of free DON competed with an equal volume of phage-displayed peptides (at constant concentration) for binding to immobilized mAb 6F5. Bound phage peptide was detected by horseradish peroxidase conjugated sheep anti-M13 IgG and then measured by absorbance. DON-HRP was included as a positive control.

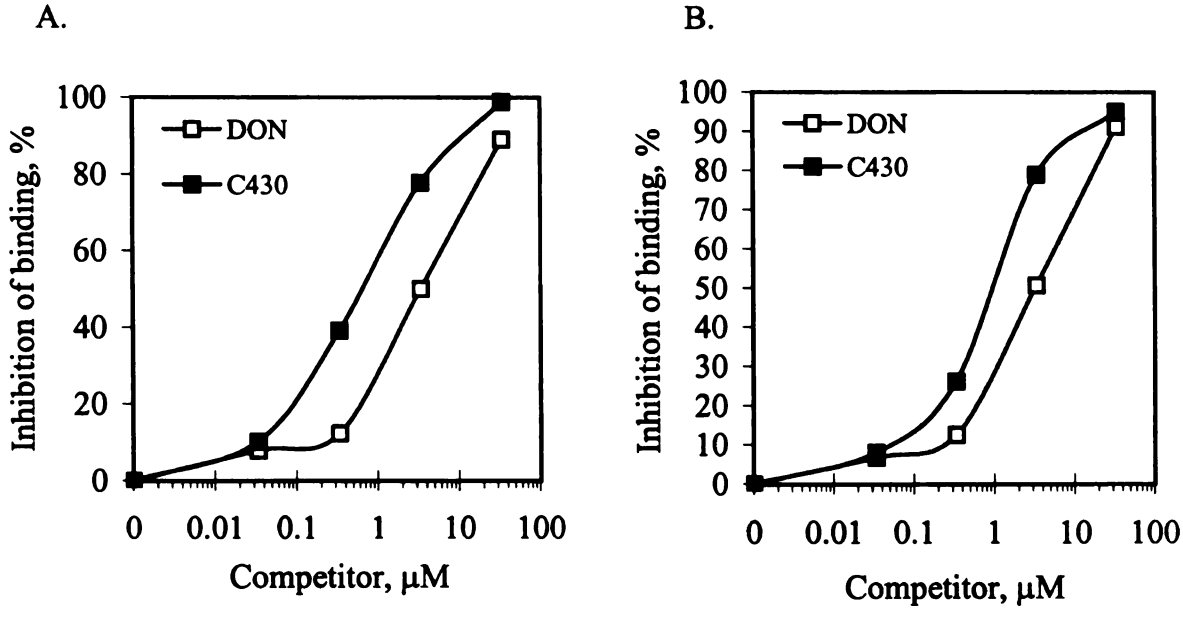

Figure 5.5. Competition between synthetic peptide C430 and DON for binding to mAb 6F5. (A). DON and synthetic peptide C430 compete with DON-HRP for binding to immobilized mAb 6F5. (B). DON and synthetic peptide C430 compete with C430-HRP for binding to immobilized anti-DON mAb 6F5.

Table 5.1. 50% binding inhibition of DON-HRP and C430-HRP by DON and C430 in competitive ELISA with monoclonal antibody 6F5 coated in microtiter wells.

Enzyme conjugate	Binding competitor (μM)	
	DON	C430
DON-HRP	3.43	0.64
C430-HRP	3.47	0.8

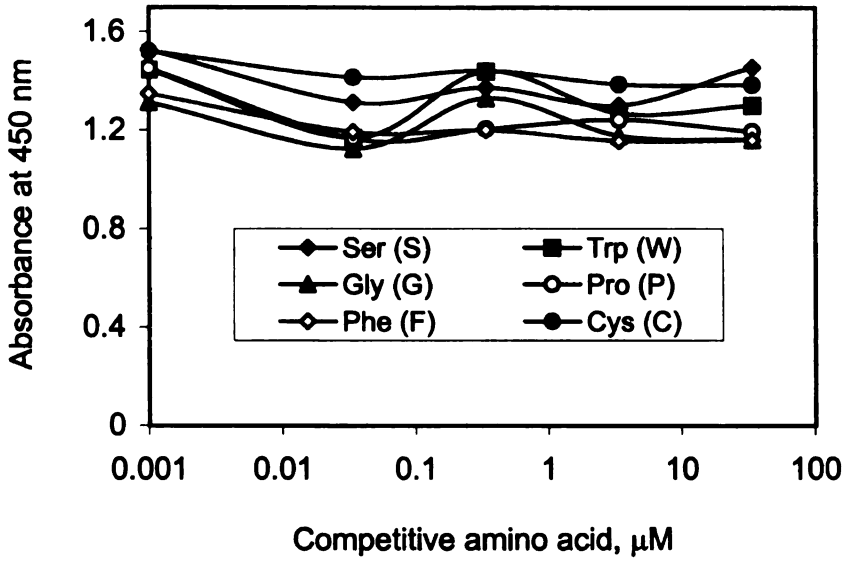

Figure 5.6. Competition between individual amino acids in synthetic peptide C430 and DON measured with CD-ELISA. Amino acids (at various concentrations) competed with DON-HRP for binding to immobilized mAb 6F5.

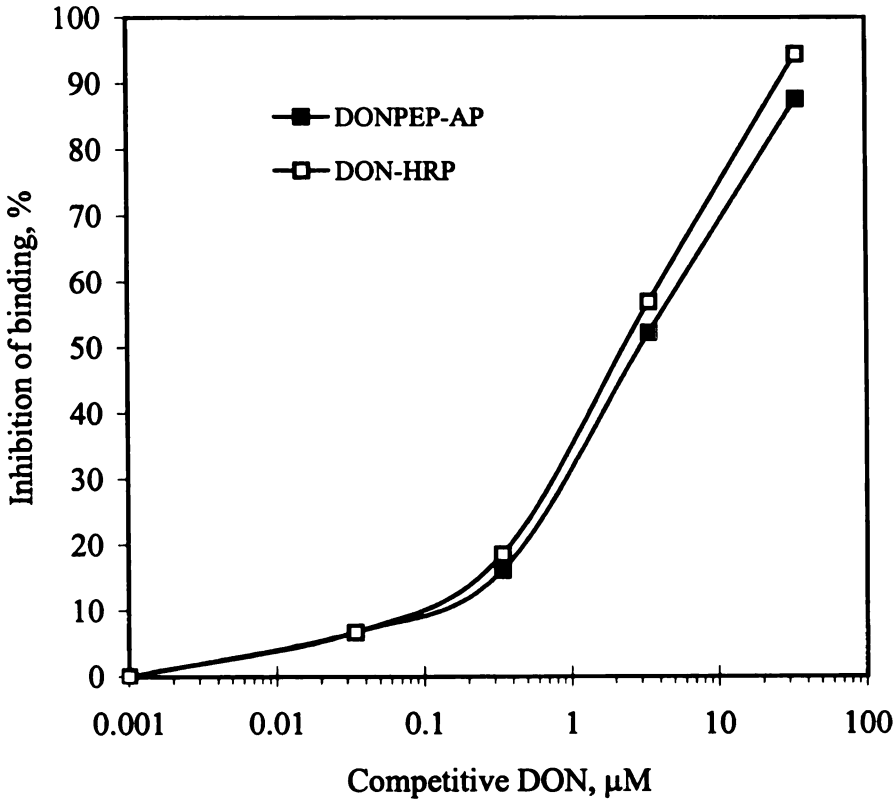

Figure 5.7. CD-ELISA with DONPEP-AP fusion protein. Binding of DONPEP-AP fusion protein to immobilized mAb 6F5 was inhibited by free DON. DON-HRP was included as a positive control.

sequence in C430 is important for the specific binding to mAB 6F5, while the individual amino acids can not bind to mAB 6F5 specifically.

The DONPEP-AP fusion protein collected from the culture supernatant or periplasmic extract showed alkaline phosphatase activity and had a similar specific binding to mAB 6F5 as DON-HRP (Figure 5.7). This suggests that the DON mimotope peptide sequence is structurally stable in a different protein structural context.

CD-ELISA for DON spiked wheat extract

To test the feasibility of DON mimotope peptide sequences as immunochemical reagents for a DON immunoassay in food and feed, a CD-ELISA was performed for a DON spiked wheat extracts. Both C430-HRP conjugate and DONPEP-AP fusion protein showed binding activity to immobilized mAB 6F5 in the wheat extract (Figure 5.8). All three conjugates had a similar linear inhibition curve at DON concentrations ranging from 0.1 μg/ml to 10 μg/ml in the wheat extract. However, a slightly lower absorbance was observed in the CD-ELISA with C430-HRP and DONPEP-AP in wheat extract than in PBS buffer, indicating that the wheat extract interfered with the binding of the mimotope peptide to anti-DON mAB 6F5 to some degree.

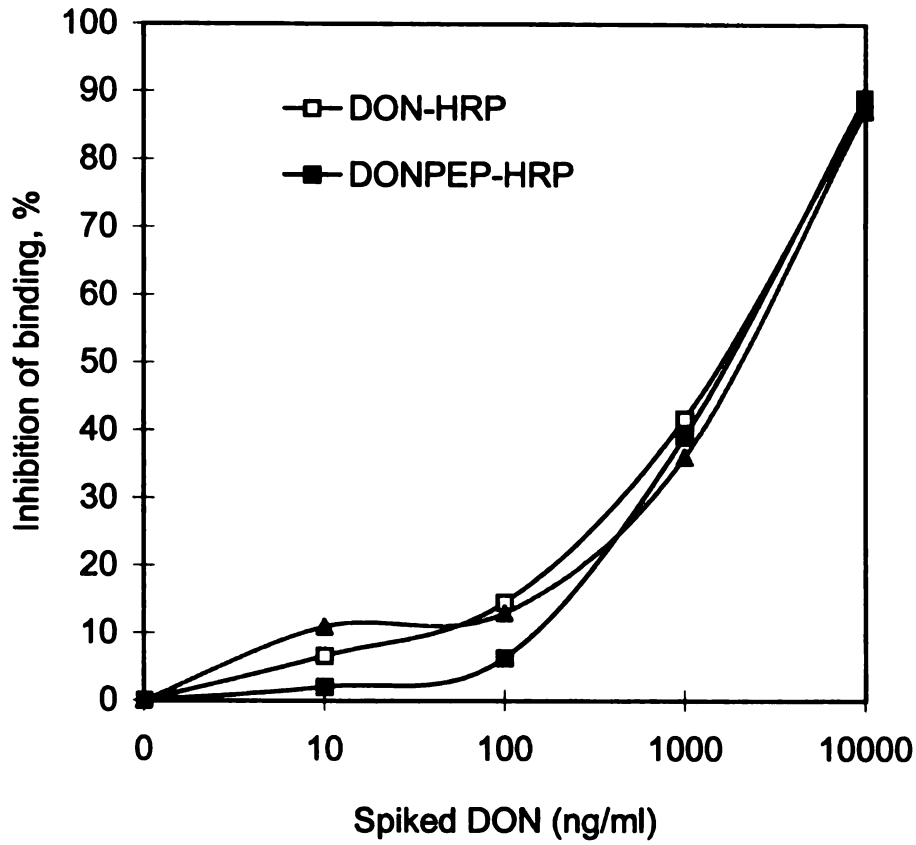

Figure 5.8. Application of C430-HRP and DONPEP-AP in DON immunoassay (CD-ELISA) in a DON-spiked wheat extract. Immulon-4 microtiter wells were coated with mAb 6F5 antibody. DON-HRP was included as a positive control.

Immunization with C430-BSA conjugate

If the selected peptides represent an true image of the DON surface structure, they might be able to elicit an immune response similar to that of the original DON epitope, and therefore they might serve as alternative antigens for DON. When rabbits and mice were immunized with C430-BSA conjugate, both produced antibody specific to the DON mimotope peptide sequence after the second injection of C430-BSA. After four injections, the antiserum showed a strong antibody response against the DON mimotope peptide sequence (Figure 5.9). A concentration of 0.39 μM of synthetic peptide C430 caused 50% inhibition of antiserum binding to immobilized phage-displayed peptide. However, binding of antisera to immobilized phage-displayed peptide was not inhibited by free DON in solution, indicating that antibodies in these immunized animals were not specific for DON.

Cytotoxicity and effects on protein synthesis *in vitro*

One of DON's effects is to cause cytotoxicity through cell apoptosis (Pestka et al., 1994b). To test if the DON mimotope peptide is cytotoxic, synthetic peptide C430 was compared with DON for their cytotoxic effects to bone marrow cells. As expected, DON at 3.4 μM caused 40-60% cell death after 18 hr incubation (Figure 5.10). Synthetic peptide C430, however, did not show any adverse effects on the viability of bone marrow cells in culture at concentrations up to 34 μM (Figure 5.10). When combined with DON, C430 at any of the tested concentrations did not

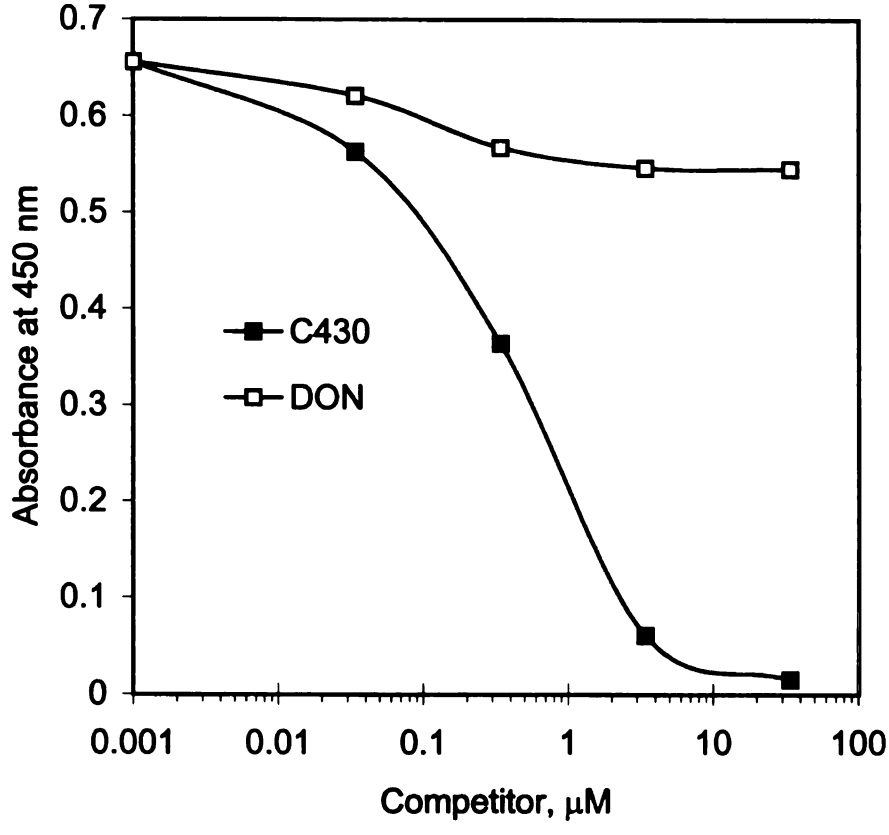

Figure 5.9. The specificity of antibody from C430-BSA immunized mice. DON and C430 at various concentrations were used to inhibit the binding of antisera to immobilized DONPEP.2 phage. Bound mouse antibodies were detected by horseradish peroxidase conjugated goat anti-mouse IgG and measure by absorbance.

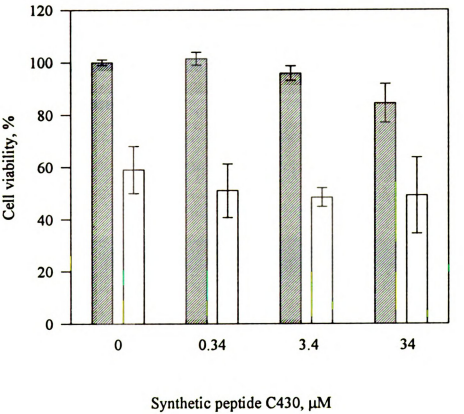

Figure 5.10. Cytotoxicity to bone marrow cells after 18 hr incubation with synthetic peptide C430 co-incubated with (white bar) or without (hatched bar) DON (3.4 μM). Cell viability was determined using a MTT conversion assay.

significantly increase or decrease cell viability caused by DON, suggesting that no significant synergism or antagonism between DON mimotope peptide and DON in causing bone marrow cell death.

In a cell free translation system, at a concentration 3.4 μM, DON significantly inhibited new protein synthesis, while C430 showed no inhibition (Figure 5.11). However, protein synthesis was not inhibited by DON when mixed with C430 both at 3.4 μM. This suggests that C430 was antagonistic to the inhibitory effects on protein synthesis caused by DON.

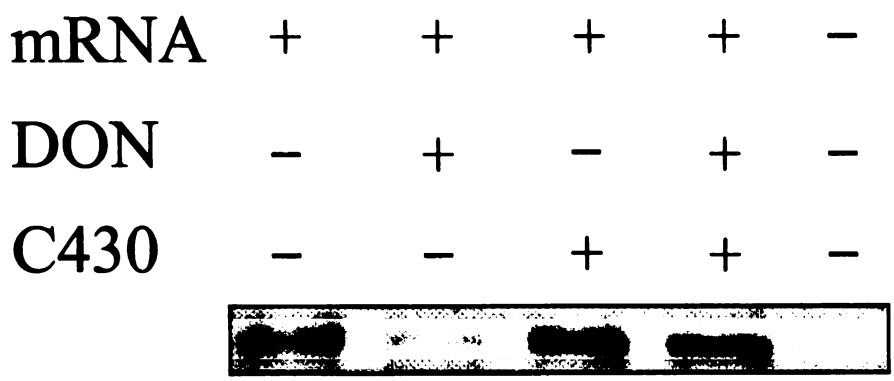

Figure 5.11. Effects of DON (3.4 μM) and synthetic peptide C430 (3.4 μM) on protein synthesis *in vitro* with rabbit reticulocyte lysate. The translation template was γ-globulin mRNA (2 μg). "+" or "-": with or without added, respectively.

DISCUSSION

In a search for DON mimotope peptides, we screened a phage-display random- heptapeptide library and identified two closely related peptide sequences that specifically bind with high affinity to anti-DON mAb 6F5. To our knowledge, this is the first example of a mimotope peptide for a nonproteinaceous, low-molecular-weight mycotoxin.

In a competitive ELISA, the binding of the selected peptides to mAb 6F5 could be competed by DON, while DON binding to the same antibody was also competed by the synthetic peptide, strongly suggesting that these clones bind to the same antigen-binding site of the monoclonal antibody. The demonstration that these peptides can be applied to ELISA has several implications. From a safety standpoint, it is advantageous to use a nontoxic peptide as an immunochemical reagent to replace toxic DON in the immunoassay. Second, the conjugation of peptide to peroxidase or other proteins will be much easier than the conjugation of DON to the same enzyme. Finally, by genetically fusing the peptide with reporter enzyme such as alkaline phosphatase, chemical conjugation can be avoided.

Although many mimotope peptides for protein antigens have been isolated from phage-displayed peptide libraries, only a few examples exist where peptides were selected as mimics to non-proteinaceous chemicals, such as lectins and carbohydrates (Oldenburg et al., 1992; Scott et al., 1992; Hoess et al., 1993). It is interesting to compare the peptide sequences selected with mAb 6F5 to other peptide sequences known to interact with carbohydrate binding sites. One can ask

whether there are general features common to these sequences. Our sequences are similar to those carbohydrate mimics in that there is a clear preference for aromatic groups and proline residues (Oldenburg et al., 1992; Scott et al., 1992; Hoess et al., 1993). This is not unreasonable since these amino acids resemble sugar moieties in their size and cyclic shape. Furthermore, the proline may serve an important structural role by orienting the aromatic side chains spatially in a manner similar to the branched carbohydrates they mimic.

If a small peptide completely mimics the epitope of a certain antigen, it can elicit antibodies directed against native antigen whose epitope the peptide represents after the peptide is conjugated on a suitable carrier protein or displayed on phage (Arnon, 1991; Minenkova et al., 1993; Di-Marzo et al., 1994; Motti et al., 1994). But peptides mimicking discontinuous epitopes were unable to elicit antibodies specific to the original antigen (Felici et al., 1993). Although our selected peptides and DON competed with each other for binding to mAB 6F5 and C430 can antagonize DON's inhibitory effects in an *in vitro* protein synthesis system, no specific antibodies for DON were produced from C430-BSA conjugate immunized animals when high affinity antibodies to the peptide were detected in these animals. It is not clear whether the selected peptides mimic the surface structure of DON in some respects or rather bind to the cleft by an entirely different mechanism (Figure 5.12). The latter possibility has been noted in the case of streptavidin-binding peptides discovered with a phage-display library (Weber et al., 1992), where the peptides associate with the binding site in a quite different fashion than the natural ligand, biotin. Turcatti et al. (1997) also observed that non-peptide antagonist and

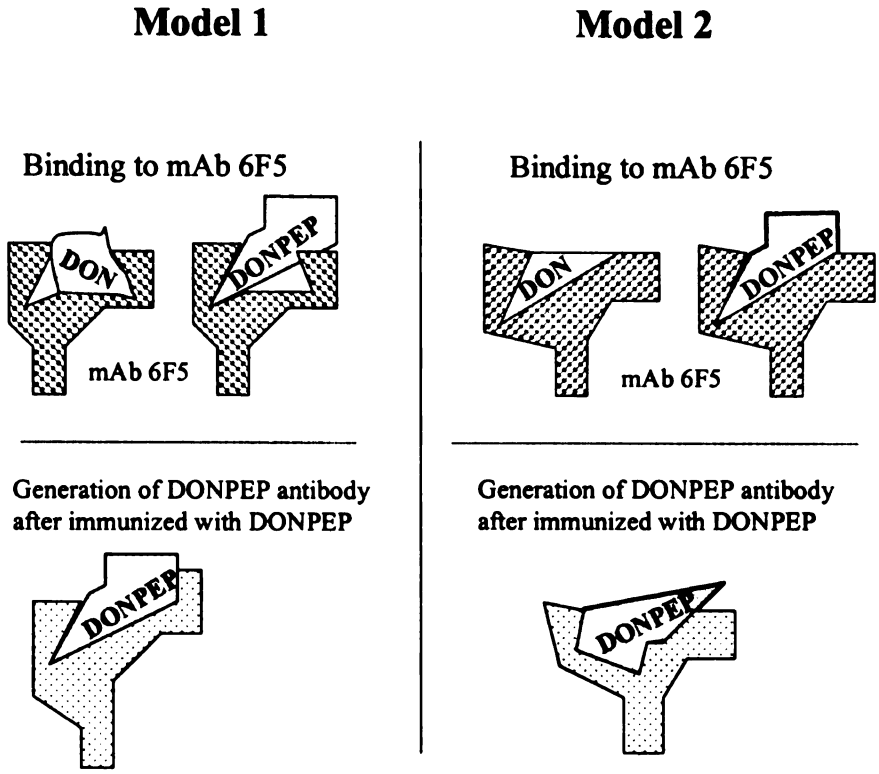

Figure 5.12. Possible mechanisms for the binding of DON mimotope peptides to mAb 6F5 and for the immunogenicity of C430-BSA. In Model 1, DON and DONPEP might bind to different portions at the antigen binding pocket of mAb 6F5. After immunization with DONPEP, only anti-DONPEP antibody is produced. In Model 2, DON and DONPEP bind to mAb 6F5 in the same fashion. But after immunization with DONPEP, only the epitope which is different from DON elicits antibody response.

peptide agonists were vastly different when bound to the NK1 receptor. Another possibility is that more than one epitope may exist in the peptide and the moiety which mimics DON has very poor immunogenicity (Figure 5.12). This proposed mechanism is supported by the observation that specific antibody for DON was rarely detected in large numbers of animals immunized with DON carrier protein conjugates (Casale et al., 1988). The toxic effects of trichothecenes are presumably related to their protein synthesis inhibition. The inhibitory activity of trichothecenes is due to their ability to bind the 60S subunit of eukaryotic ribosomes and to interfere with peptidyltransferase activity (Bamburg, 1983; Ueno, 1983; Thompson and Wannemacher, 1986; Betina, 1989b; Rotter et al., 1996). Stoichiometricaly, one trichothecene molecule binds one ribosome (Middlebrook and Leatherman, 1989). Presumably peptides will only be agonists if they undergo the same specific contacts and interactions with the ribosome as DON. The mimotope peptides selected from phage-display library in this study, however, showed an antagonism to DON-induced protein synthesis inhibition. This suggests that the synthetic peptide may bind to the same site in ribosome as DON does, but in a different fashion. It is commonly accepted that native ligands, agonists, or antagonists can partly bind to their receptors (Strader et al., 1995) although an inhibitory active site might be structurally distinct from the binding site. It is notable, however, that in a cell culture model, C430 did not potentiate or attenuate DON in causing cell death. This may be due to the difficulty of the peptide crossing the cell membrane (Burton et al., 1996; Firth et al., 1997). DON, with a 4-fold lower

molecular weight, absence of charged groups, and specific structure, may be more readily absorbed into the cell (Witt and Pestka, 1990).

In addition to the toxic effects to animals and humans, there is a growing body of evidence indicating that trichothothecenes, including DON, can enhance the virulence of plant-pathogenic species of *Fusarium* on plant hosts (Adams and Hart, 1989; Desjardins et al., 1989;1992; Bruins et al., 1993; Wang and Miller, 1988; Scholbrock et al., 1992; Atanassov et al., 1994; Proctor et al., 1995). These trichothecene-producing *Fusarium* fungi cause a broad range of plant diseases including head and seedling blight of small grains such as wheat and rye, ear and stalk rot of maize, stem rot of carnation, and seedling blight and root rot of a number of other plant species, including beans, clover, and tomato (Cook, 1981; Kommedahl and Windels, 1981). If the fungal virulence associated with DON is mediated through protein synthesis inhibition and the mimotope peptides presented here are antagonistic to DON activity in plant, a transgenic plant expressing the peptides (may need to be fused into another protein) might show reduced levels of wheat head scab and other plant diseases caused by DON-producing pathogenic *Fusarium*. Previously, synthetic peptide combinatorial libraries have been used to identify bioactive peptides against plant pathogens (Marcos et al., 1995; Blondelle and Houghten, 1996; Reed et al., 1997). Some antimicrobial peptides have been expressed in transgenic plants (De Bolle et al., 1996). Thus, phage-display peptide libraries may provide a new and innovative approach in developing antimicrobial peptides to control plant diseases.

In summary, we have identified two closely related mimotope peptides for DON and demonstrated their potential application in DON immunoassay. We also demonstrated an antagonistic activity of the mimotopes to DON-induced protein synthesis inhibition. Further understanding of the difference between the mimotope peptides and DON in the structure-function relationships might help in elucidation of the mechanisms of DON toxicity, and lead to prevention of plant and animal diseases associated with this mycotoxin.

SUMMARY

In this dissertation, phage-display technology was explored to develop recombinant antibodies and peptide mimotopes for *Fusarium* mycotoxins. Firstly, a functional scFv antibody with high affinity to the mycotoxin zearalenone was cloned and expressed in *E.coli*. A new phage-display vector which facilitates the cloning of genes for variable heavy chain, variable kappa light chain, and the assembly of scFv DNA fragment, was also constructed. However, attempts to generate scFv antibodies to the mycotoxin fumonisin B_1 from fumonisin B_1-cholera toxin conjugate immunized mice failed due to inefficient selection. Secondly, the anti-zearalenone scFv DNA fragment was transferred into *Arabidopsis* and functional anti-zearalenone scFv plantibody with high affinity to mycotoxin zearalenone was expressed. Finally, two highly homologous heptapeptide sequences from a phage-displayed random 7-mer peptide library were identified for specifically binding a deoxynivalenol-specific monoclonal antibody. The mimotope peptide appeared to be nontoxic and showed antagonistic to mycotoxin deoxynivalenol.

APPENDICES:

BUFFERS AND MEDIA

APPENDIX A: COMMONLY USED BUFFERS

Bacteria alkaline lysis buffer for mini-preparations of plasmid DNA (Sambrook et al., 1989):

Solution I
- 50 mM glucose
- 25 mM Tris.Cl (pH 8.0)
- 10 mM EDTA (pH 8.0)
- Autoclaved and stored at 4°C

Solution II (fresh prepared)
- 0.2 N NaOH (freshly diluted from a 10 N stock)
- 1% SDS

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

0.05 M carbonate-bicarbonate coating buffer (Sigma Chemical Company, St. Louis, MO):

1.59 g Na_2CO_3
2.93 g $NaHCO_3$
dissolved in 1 liter Milli-Q water
adjust pH to 9.6, if necessary
stored at 4°C

Citrate buffer (pH 4.0) for ABTS substrate preparation:

Add 9.6 g of citric acid monohydrate to 500 ml of Milli-Q water. Adjust the pH to 4.0 with NaOH. Scale up to 1 liter and check pH. Autoclave and store at 4°C.

DNA electrophoresis gel-loading buffer (Sambrook et al., 1989):

Buffer type I (6 x)
- 0.25% bromophenol blue
- 0.25%xylene cyanol FF
- 40% (w/v) sucrose in water
- 4°C storage

Buffer type II (6 x)
0.25% bromophenol blue
0.25%xylene cyanol FF
15% Ficoll (Type 400; Pharmacia) in water
room temperature storage

Buffer type III (6 x)
0.25% bromophenol blue
0.25%xylene cyanol FF
30% glycerol in water
4°C storage

Buffer type IV (6 x)
0.25% bromophenol blue
40% (w/v) sucrose in water
4°C storage

Phosphate-buffered saline (PBS) (Sambrook et al., 1989):

To 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, add Milli-Q water to a total volume of 1 liter. Adjust the pH to 7.4 with NaOH or HCl if necessary and autoclave.

0.05 M phosphate-citrate buffer for TMB substrate preparation (Sigma Chemical Company, St. Louis, MO):

Dissolve 3.65 g of Na_2HPO_4 and 2.55 g of citric acid in Milli-Q water, scale up the volume to 500 ml. Adjust pH to 5.0, if necessary.

Protein SDS-PAGE gel-loading buffer (2 x) (Sambrook et al., 1989)

100 mM Tris.Cl (pH 6.8)
200 mM dithiothreitol (add just before the buffer is used)
4% SDS (electrophoresis grade)
0.2% bromophenol blue
20% glycerol
room temperature storage (without dithiothreitol)

SDS-PAGE running buffer (5 x stock) (Sambrook et al., 1989):

15.1 g Tris base
94 g glycine
5 g SDS (electrophoresis-grade)
add Milli-Q water to 1 liter

SDS-PAGE protein transfer buffer

12.1 g Tris base
14.4 g glycine
add Milli-Q water to 1 liter

Tris-buffered saline (TBS) (Boehringer Mannheim Corporation, Indianapolis, IN):

Add 6.05 g Tris base (50mM), 8.76 g NaCl (150 mM) to 800 ml Milli-Q water, adjust pH to 7.5 with HCl or NaOH, scale up to a total volume of 1 liter with Milli-Q water.

Tris-EDTA (TE) buffer (Sambrook et al., 1989):

pH 7.4:
10mM Tris.Cl (pH7.4)
1mM EDTA (pH8.0)

pH 7.6:
10mM Tris.Cl (pH7.6)
1mM EDTA (pH8.0)

pH 8.0:
10mM Tris.Cl (pH8.0)
1mM EDTA (pH8.0)

Tris-acetate electrophoresis buffer (TAE, 50 x stock) (Sambrook et al., 1989):

242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8.0)
scale up to 1 liter with Milli-Q water

APPENDIX B: COMMONLY USED MEDIA

LB medium (Luria-Bertani medium) (Sambrook et al., 1989)

10 g bacto-tryptone
5 g bacto-yeast extract
10 g NaCl
dissolved in Milli-Q water, adjust pH to 7.4
scale up to 1 liter, autoclave.

Minimal medium (Pharmacia biotech):

6 g Na_2HPO_4 (dibasic)
3 g KH_2PO_4
1 g NH_4Cl
Dissolved in Milli-Q water (500 ml), adjust pH to 7.4, autoclave;

In a separate bottle, autoclave 15 g of Bacto-agar in 492 ml Milli-Q water, autoclave. Add 1 ml of 1 M $MgCl_2$.$6H_2O$, 1 ml of 1 M $CaCl_2$.$2H_2O$, 1 ml of 1 M thiamine hydrochloride and 5 ml of 20% glucose (all autoclave or filtration sterilized). Then combine with above salts.

SB medium (Pharmacia biotech):

35 g bacto-tryptone
20 g bacto-yeast extract
5 g NaCl
Dissolved in Milli-Q water, scale up to 1 liter, adjust pH to 7.4, autoclave.

SOB medium (Sambrook et al., 1989):

20 g bacto-tryptone
5 g bacto-yeast extract
0.5 g NaCl
10 ml 250 mM KCl
Dissolved in Milli-Q water, adjust pH to 7.4, scaled up to 990 ml with Milli-Q water. Autoclave, after cool down to 60°C, add 10 ml of 1 M $MgCl_2$.

2 X YT medium (Sambrook et al., 1989):

16 g bacto-tryptone
10 g bacto-yeast extract
5 g NaCl
Dissolved in Milli-Q water, adjust pH to 7.4, scale up to 1 liter, autoclave.

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