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Molecular Cloning, Expression, and Characterization of a Functional Single-Chain Fv Antibody to the Mycotoxin Zearalenone

QIAOPING YUAN,[1] JAMES R. CLARKE,[2] HUI-REN ZHOU,[2] JOHN E. LINZ,[2] JAMES J. PESTKA,[2] AND L. PATRICK HART[1]*

Departments of Botany and Plant Pathology[1] and of Food Science and Human Nutrition,[2] Michigan State University, East Lansing, Michigan 48824

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The heavy-chain and kappa light-chain variable region genes of an antizearalenone hybridoma cell line (2G3-6E3-2E2) were isolated by PCR 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 into a phagemid (pCANTAB5E) and expressed as a fusion protein with E tag and phage M13 p3 in *Escherichia coli* TG1. In the presence of helper phage M13K07, the scFv fusion protein was displayed on the surfaces of recombinant phages. High-affinity scFv phages were enriched through affinity 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 a high zearalenone-binding affinity (concentration required for 50% inhibition of binding, 14 ng/ml), similar to that of parent monoclonal antibody in a competitive indirect enzyme-linked immunosorbent assay. However, scFv QY1.5 exhibited higher cross-reactivity with zearalenone analogs and had greater sensitivity to methanol destabilization than the parent monoclonal antibody did. Nucleotide sequence analyses revealed that the light-chain portion of scFv QY1.5 had a nucleotide sequence identity of 97% to a mouse germ line gene $V_K23.32$ in mouse kappa light-chain variable region 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 complementarity-determining region sequences. The potential of soluble scFv QY1.5 for routine screening of zearalenone and its analogs was demonstrated with zearalenone-spiked corn extracts.

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 (8, 19, 21). When fed to animals, the compound causes hyperestrogenism with symptoms such as enlargement of the uterus and nipples, vulvar swelling, vaginal prolapse, and infertility (10, 20). 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 (5, 30, 31), gas-liquid chromatography (30, 34), high-pressure liquid chromatography (11, 30, 36), and, more recently, immunoassays (2, 4, 15, 32, 35, 37). Compared with other methods, immunoassays have several advantages for rapid field tests, including high specificity, sensitivity, facile sample preparation, and ease of use (27). 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 molecule. One of the most successful approaches is to display single-chain Fv (scFv) antibodies on filamentous phage (1, 3, 6, 16–18). 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. When expressed with phage protein p3 (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 (9, 16). In this report, we describe the molecular cloning, expression, and characterization of a functional scFv with high specificities and sensitivities to zearalenone and its analogs 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, Ind.). Zearalenone-ovalbumin and -keyhole limpet hemocyanin (KLH) conjugates were prepared as described by Dixon et al. (4). Antizearalenone hybridoma cell line 2G3-6E3-2EZ (4) was kindly supplied by M. A. Abouzied (Neogen Company, Lansing, Mich.). Plasmid pCANTAB5E, *Escherichia coli* TG1 and HB2151, M13K07 helper phage, mouse anti-M13 antibody, and mouse anti-E tag antibody were obtained from Pharmacia Biotech (Piscataway, N.J.). Cell culture components and reagents, goat anti-mouse immunoglobulin G (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. Restriction enzyme *Sfi*I was purchased from New England BioLabs (Beverly, Mass.). All DNA manipulations, if not described, were carried out by standard procedures (29).

mRNA isolation. Hybridoma cell line 2G3-6E3-2EZ producing antizearalenone antibody (4) was cultured in macrophage-conditioned Dulbecco modified Eagle medium containing 20% (vol/vol) fetal bovine serum supplemented with 100 U of penicillin per ml and 100 μg of streptomycin per ml in a 5% CO_2 humidified chamber. After the cells were grown to a density of 10^6 cells/ml, they

* Corresponding author. Mailing address: Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824. Phone: (517) 353-9428. Fax: (517) 353-5598. E-mail: 22771LPH @msu.edu.

TABLE 1. Oligonucleotide PCR primer sequences

Primer	Sequence[a]
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′

[a] U is deoxyuracil, R is A or G, Y is C or T, M is A or C, K is T or G, S is C or G, W is A or T, and B is T or C or G.

were pelleted by centrifugation at 1,000 × *g* for 5 min. For total RNA isolation, the pelleted cells were homogenized in RNA STAT-60 (TEL-TEST Inc., Friendswood, Tex.) at 5×10^6 cells/ml and then subjected to chloroform extraction and isopropanol precipitation. mRNA was purified by affinity chromatography with an oligo(dT)-cellulose column according to the manufacturer's instructions.

cDNA synthesis, PCR amplification of immunoglobulin variable regions, and scFv cloning. First-strand cDNA was synthesized from mRNA template with Moloney murine leukemia virus reverse transcriptase and random hexadeoxyribonucleotides [pd$(N)_6$] primers (Pharmacia Biotech). 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 (1 cycle is 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C). The primers used in the PCR amplification were based on those of Orlandi et al. (25) and Clackson et al. (3) and modified for facilitating ligase-free uracil DNA glycosylase (UDG) ligation (24) (Table 1). Primers VHBACK and du-VHFOR were used for amplification of V_H; primers dU-VKBACK and VKFOR were used for amplification of V_K. A 93-bp DNA linker (Pharmacia Biotech), containing a sequence encoding a short flexible peptide, $(Gly_4Ser)_3$, was also amplified with primers LINKBACK and du-LINKFOR. After gel purification with a QIAEX gel extraction kit (QIAGEN Inc., Chatsworth, Calif.), 25 ng of linker DNA and 75 ng of V_K DNA were mixed and treated with 1 U of UDG (U.S. Biochemicals, Cleveland, Ohio) at 37°C in a 20-μl reaction mixture with 1× PCR buffer for 1.5 h. The UDG-treated fragments were then scaled up to a 40-μl PCR mixture without primers and cycled 15 times (1 cycle is 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C) for joining the linker DNA with V_K DNA. The joined linker-V_K DNA was amplified in a 100-μl PCR mixture for 30 cycles (1 cycle is 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C) with 75 pmol (each) of 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 mixture after UDG treatment. The assembled scFv DNA was amplified with 75 pmol (each) of VHBACK and VKFOR in a 100-μl PCR amplification reaction mixture for 30 cycles (1 cycle is 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C). Finally, the assembled scFv products were gel purified and reamplified with restriction site-tagged primers (RS Primers Mix) (Pharmacia Biotech) 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).

ScFv antibody expression and affinity selection. To display scFv as a fusion protein with E tag and M13 p3 (there is an amber stop codon between E tag and fd g3 in pCANTAB5E), the ligated products were used to transform competent cells of *E. coli* TG1, which is an amber suppressor strain (*supE*). Transformed cells were plated on SOB medium (29) containing 100 μg of ampicillin per ml and 2% glucose and incubated at 30°C overnight. Colonies were pooled and infected with M13K07 helper phage in 2× YT medium (29) containing 100 μg of ampicillin per ml and 50 μg of kanamycin per ml to rescue the phagemid with its scFv gene inserts and to display scFv fusion protein on the surfaces of the recombinant phages. The recombinant phages from the supernatant were filtered through a 0.45-μm-pore-size filter.

For affinity selection, 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 phosphate-buffered saline (PBS) with 0.05% (vol/vol) Tween 20 (PBS-T) and aspirating the contents. Nonspecific binding was blocked by incubation 300 μl of 1% ovalbumin (wt/vol) dissolved in PBS (PBS-OVA) in each well for 1 h 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 h at 37°C and then washed 10 times with PBS and 10 times with PBS-T. One-hundred-microliter samples of free 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 h 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, Mich.). 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 were tested with the modified competitive indirect enzyme-linked immunosorbent assay (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 of tryptone per liter, 20 g of yeast extract per liter, 5 g of NaCl per liter) supplemented with 100 μg of ampicillin per ml 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 1,500 × *g* for 15 min and filtered through a 0.45-μm-pore-size filter. The affinities of soluble scFv antibodies were detected with the modified CI-ELISA described below.

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 analogs) dissolved in PBS containing 1% methanol was added to each coated well and simultaneously incubated with 50 μl of phage-associated (about 5×10^{10} PFU/ml in 2× YT medium) or soluble scFv antibody (various dilutions with PBS-OVA) or parent monoclonal antibody (diluted 1:4 with PBS-OVA) from the supernatant of hybridoma cell culture for comparison. The plates were incubated for 1 h at 37°C and 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-horseradish peroxidase (HRP) conjugate diluted 1:2,500 with PBS-OVA. The amount of soluble scFv antibody bound was determined by incubation with 100 μl of mouse anti-E tag (diluted 1:2,500 with PBS-OVA) at 37°C for 1 h, 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 of goat anti-mouse IgG-HRP conjugate (diluted 1:500 with PBS-OVA). The plates were incubated for 1 h 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) substrate (26). 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, Calif.).

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 at a final concentration of 10% on ice for 20 min. After centrifugation in a microcentrifuge at full speed 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 the addition of 5 μl of 4× loading buffer, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) with a 0.5-mm-thick gel in a Bio-Rad Mini-Protein II Electrophoresis System (Bio-Rad Laboratories, Hercules, Calif.). 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, and those in the other gel were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) 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) and then incubated first with rabbit anti-mouse IgG-alkaline phosphatase conjugate (Sigma Chemical Company) as described by the manufacturer's instructions and then with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine (Pierce, Rockford, Ill.).

Sequence analysis. Plasmid DNAs from anti-zearalenone-producing clones were 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) 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.

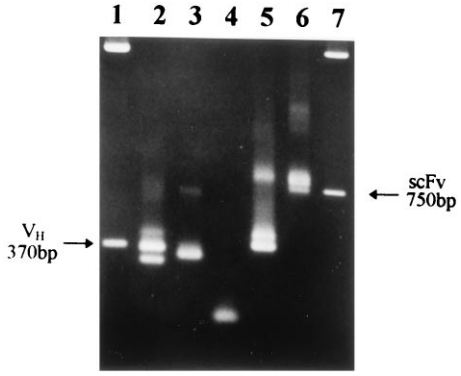

FIG. 1. V_H and V_K amplification and assembly of scFv. Lanes: 1, V_H marker (pUC18/V_H, *Eco*RI/*Xba*I digested; Pharmacia Biotech); 2, V_H PCR product; 3, V_K PCR product; 4, linker PCR product; 5, linker-V_K PCR product; 6, V_H-Linker-V_K (scFv) PCR product; 7, scFv marker (pUC18/A10B, *Sfi*I/*Not*I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

CI-ELISA for spiked corn extracts. The procedures for extraction from corn and spiking were similar to that described by Liu et al. (15). Briefly, zearalenone-free homogeneous ground corn was extracted with 5 volumes (wt/vol) of methanol-water (70:30) for 5 min, with constant stirring. The mixture was immediately filtered through Whatman no. 4 filter paper, and the filtrate was spiked directly with zearalenone, or the filtrate was diluted 1:1 with 1× 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 procedure similar to that 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 from this clone for V_H and V_K 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 (Fig. 1). 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 Fig. 1) 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 Fig. 1) to form the scFv fragments in lane 6 of Fig. 1. 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.

Phage display of scFv antibody and affinity selection. Two major scFv DNA fragments in lane 6 of Fig. 1 were collectively isolated, purified, digested, then ligated into pCANTAB5E, and translationally fused with the E tag DNA fragment (Fig. 2). 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-p3 fusion protein. In the presence of helper phage (M13K07), scFv fusion products were displayed on the recombinant phage tips, allowing for affinity selection. 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, which was used for elution in the following affinity selection. Prior to affinity selection, the pooled recombinant phage library exhibited very low zearalenone-binding activity (Fig. 3). After one round of affinity selection, a clear enrichment for scFv clones expressing zearalenone binding was observed (Fig. 3). The concentrations required for 50% inhibition of binding (IC_{50}) for preselection phage, first-round selected phage, and second-round selected phage were >1,000, 31, and 28 ng/ml, respectively. The second-round selection mainly increased yield of functional scFv fusion protein. Following each round of affinity selection, individual clones (in TG1) were randomly isolated and the presence of 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 (*supE*), 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 into the culture supernatants. The zearalenone-binding pattern of soluble scFv antibodies from these nine HB2151 clones was the same as that for the parent phage scFv antibodies. CI-ELISA results for four typical scFv clones are shown in Fig. 4A. 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 (Fig. 4B). IC_{50} for scFv QY1.5, QY1.12, and their parent monoclonal antibody were 14, 35, and 17 ng/ml, respectively. Compared with the parent monoclonal antibody, soluble scFv QY1.5 exhibited similar sensitivities but higher relative cross-reactivities to hydroxylated zearalenone analogs (Table 2).

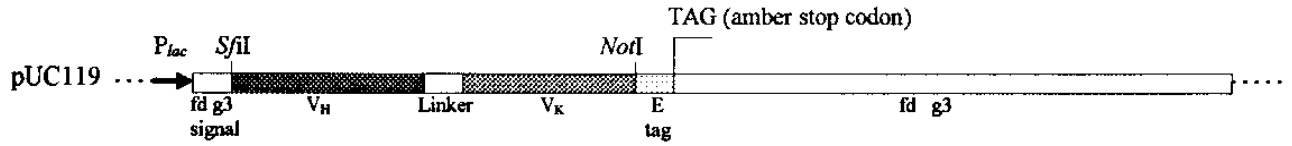

FIG. 2. pQY1 construct. PCR-amplified scFv was cloned into *Sfi*I and *Not*I sites of pCANTAB5E, a pUC119-based vector from Pharmacia Biotech. The linker DNA sequence was 5′ GGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGATCG 3′, which encoded a short peptide [$(Gly_4Ser)_3$].

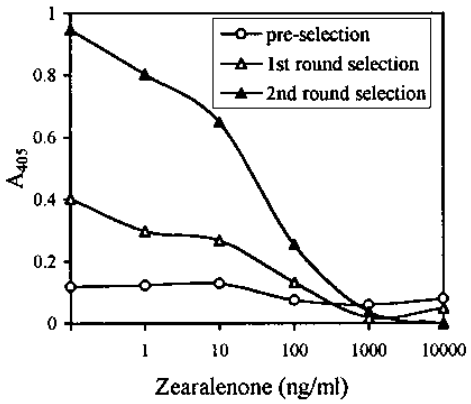

FIG. 3. Zearalenone-binding activities of recombinant phages following each round of affinity selection measured by CI-ELISA.

SDS-PAGE and Western blot analyses. An approximately 32-kDa p3 signal-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 (Fig. 5). 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 (Fig. 3B).

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 Fig. 6. According to the amino acid 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). 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 germ line gene with no identified binding specificity in the V_K23 family (14). 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 framework region 1 (FR1) and were attributed to the use of a degenerate VKBACK primer, 3 were in FR3, and a single silent nucleotide change (causing no amino acid sequence change) occurred in both complementarity-determining regions 2 and 3 (CDR2 and CDR3, respectively). This implied that V_K23.32 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 antizearalenone 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 (13), 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 FRs at both nucleotide and amino acid levels.

TABLE 2. Comparison of cross-reactivities of scFv antibody and monoclonal antibody to zearalenone analogs

Analog	Monoclonal antibody		scFv QY1.5	
	IC_{50} (ng/ml)	Cross-reactivity[a] (%)	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

[a] Cross-reactivity defined as (IC_{50} of zearalenone/IC_{50} of analog) × 100%.

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 (Fig. 7). Corn extracts containing as little as 10 ng of 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),

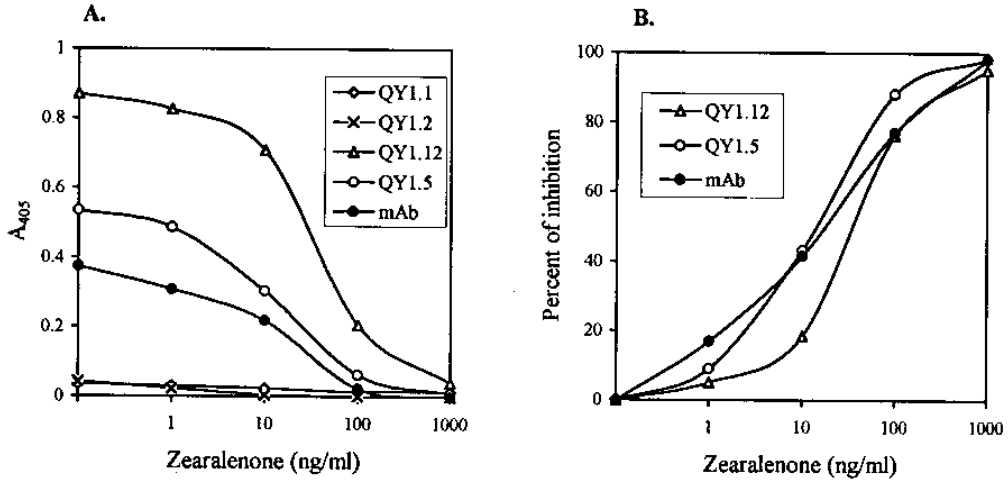

FIG. 4. Sensitivities of soluble scFv antibodies tested in CI-ELISA. (A) Absorbance reading at 405 nm; (B) percent binding inhibition determined by dividing sample absorbance by control absorbance × 100. QY1.1 and QY1.2 (no dilution) were from preselection; QY1.5 (1:4 dilution) was from the first round of affinity selection; QY1.12 (1:9 dilution) was from the second round of affinity selection; monoclonal antibody (mAb) (supernatant of hybridoma cell culture) was diluted 1:4.

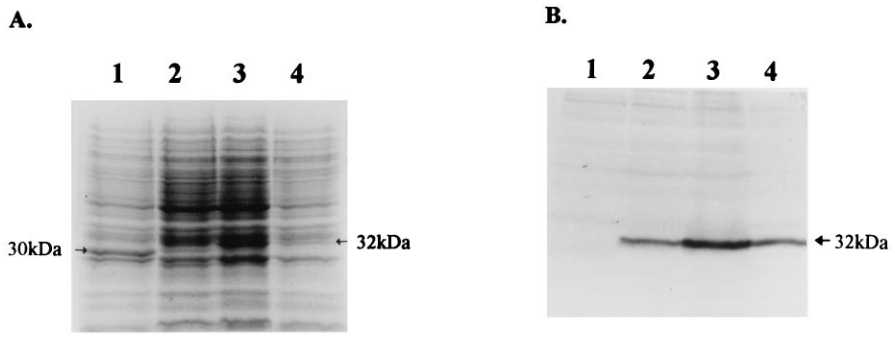

FIG. 5. SDS-polyacrylamide gel and Western blot analysis of scFv expression. (A) Supernatants of QY1.1 (lane 1; 0.3 ml), QY1.2 (lane 2; 0.3 ml), QY1.12 (lane 3; 0.3 ml), and QY1.5 (lane 4; 0.6 ml) concentrated in 10% trichloroacetic acid were subjected to SDS-PAGE and stained with Coomassie brilliant blue G-250. (B) Western blot of duplicate SDS-polyacrylamide gel.

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 (Fig. 8). In contrast, the parent monoclonal antibody exhibited much higher tolerance to methanol destabilization (Fig. 8). 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.

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 an mRNA source for construction of scFv antibody, it is most desirable to use a cell line that produces a high-affinity antibody for the target hapten or antigen. The hybridoma cell line we used in this study produced a 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-sensitivity scFv antibody specific for the mycotoxin fumonisin B_1 was developed in our previous study because, in part, the hybridoma cell line used produced a lower-affinity antibody specific to fumonisin B_1 (38).

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 the FR3-CDR3 boundary (33). Other irrelevant V regions might possibly result from nonproductive 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

A. V_H sequence:

FR1 CDR1 FR2 CDR2
MA QVKLQESGGGLVQPGGSLKLSCAASGFTFS NYGMS WVRQTPDKRLEFVA NINGNGGKTYYPGSVKG

FR3 CDR3 FR4
RFTISRDNAKNTLYLQMSSLKSEDTAMYYCVR VAFDGYYDDF WGQGTTVTVSS

B. V_K sequence:

FR1 CDR1 FR2 CDR2
DIELTQSPATLSVTPGDRVSLSC RASQSISDYLH WYQQKSHESPRLLIK YASQSIS

FR3 CDR3 FR4
GIPSRFSGSGSGSDFTLSINSVEPEDVGVYYC QNGHSFPPT FGGGTKLEIK

FIG. 6. Deduced amino acid sequences of V_H and V_K in scFv QY1.5. CDRs and FRs are indicated.

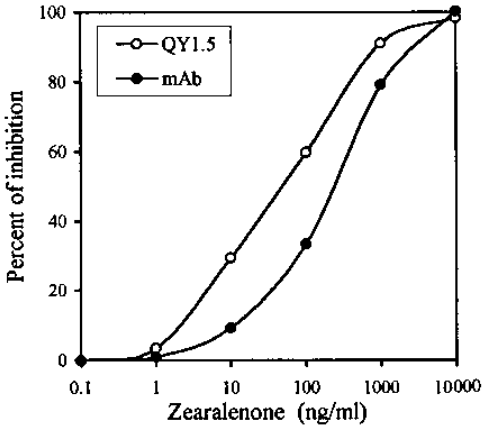

FIG. 7. Zearalenone sensitivities in spiked corn extracts with monoclonal antibody and soluble scFv antibody QY1.5 measured by CI-ELISA. Both scFv QY1.5 and monoclonal antibody (mAb) were diluted 1:4 in PBS containing 10% nonfat dry milk. Corn extracts were diluted 1:1 with PBS.

was therefore necessary for isolation of the variable regions with the desired specificity. Like the scFv antibodies before affinity selection in this study, the fumonisin B_1-specific recombinant scFv antibody (38), selected only by panning without affinity selection through hapten elution, was 10 to 100 times less sensitive than the antifumonisin B_1 polyclonal or monoclonal antibodies. Clackson et al. (3) also showed that affinity selection 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 affinity selection should select the desired V regions. In this study, a functional antizearalenone scFv protein with high affinity was isolated after only one round of affinity selection. Sequential selections mainly appeared to enrich clones with a higher yield of scFv protein, not necessarily improving the affinity.

Theoretically, an scFv will have a specificity and sensitivity similar to those of the 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 shown to have higher cross-reactivities to hydroxylated zearalenone analogs. Since these analogs are naturally occurring toxic metabolites of zearalenone (7, 22), the ability

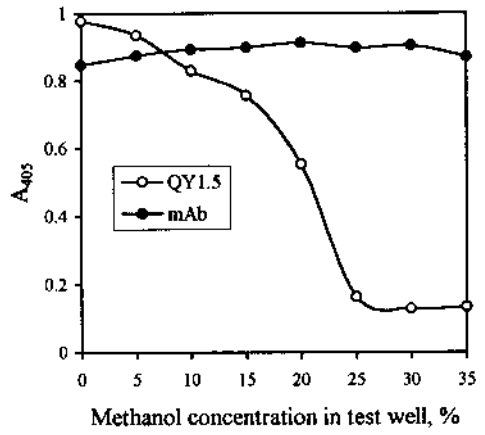

FIG. 8. Effects of methanol on the binding of monoclonal antibody (mAb) and scFv QY1.5 to plates coated with zearalenone-KLH conjugate.

of the scFv antibody to detect them is particularly advantageous when detection of these analogs is desirable. In general, changes 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 occurring 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. (23) 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, and metal binding sites and increasing hydrophobic area or electrostatic interactions (28).

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 toxins 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 desired specificity and sensitivity. The recombinant antibody scFv QY1.5 has high sensitivity and specificity to zearalenone and its analogs and should be readily applicable to the routine screening for these mycotoxins.

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