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# Selection of a Single Chain Variable Fragment Antibody against Ivermectin from a Phage Displayed Library

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Single chain variable fragment antibodies (scFvs) have considerable potential in immunological detection of pesticides, veterinary and other residues. In this study, a large human synthetic phage displayed library was used to select scFvs against ivermectin (IVM) by subtractive panning. After four rounds of panning, seven monoclonal phage particles capable of binding with the IVM were obtained. One of the identified novel anti-IVM scFv, hsIVM8, was expressed in *Escherichia coli* HB2151 and purified with Ni metal ion affinity chromatography. Sodium dodecyl sulfate polyacrylamine gel electrophoresis (SDS−PAGE) indicated that the relative molecular mass of hsIVM8 was estimated at 28 kDa. The purified hsIVM8 was used to develop a competitive indirect enzyme-linked immunosorbent assay (ci-ELISA) for IVM. The linear range of detection for standards in this ci-ELISA was approximately 0.1−5 $\mu$g/mL and 50% inhibition of control ($IC_{50}$) was 4.11 $\mu$g/mL for IVM. These results showed promising applications of hsIVM8 for detecting IVM in agricultural and environmental matrices.

**KEYWORDS: IVM; scFv; detection; phage displayed library**

## INTRODUCTION

Ivermectin (IVM), a semisynthetic macrolide antibiotic derived from *Streptomyces avermitilis*, is now used extensively as an effective veterinary, agricultural and human antiparasitic agent to control and treat a broad spectrum of infections caused by parasitic nematodes and arthropods (*1*−*5*). It interferes with the nervous system and muscle function by activating glutamate-gated chloride channels in the parasite leading to membrane hyperpolarization and muscle paralysis (*6*). Though it has long been known as a safe antiparasitic drug, the significant considerations concerning the effects of IVM on the ecological system were investigated. The results showed that the IVM residues could decrease pasture productivity (*7*), could affect aquatic mesocosms (*8*), and has significant risk to the environment (*9*−*11*). The potential broad applications and risk of IVM make it necessary to establish a simple and inexpensive method for detecting IVM.

Current methods including hollow fiber-supported liquid membrane extraction and liquid chromatography−mass spectrometry/mass spectrometry (*12*, *13*) for detecting IVM are expensive and requiring lengthy cleanup procedures. In contrast, immunoassay methods, in which monoclonal and polyclonal antibodies are commonly used, generally are faster and much less expensive. The development of antibody engineering technology provided the tools necessary to generate novel antibodies for the detection of residues in food and environment. Recombinant approaches have led to the development of single chain variable fragment antibodies (scFvs), which are monovalent and formed by the variable region of heavy chain ($V_H$) and light chain ($V_L$) joined by a flexible linker. The obvious advantages of scFv are that it maintains the specific affinity to the antigen, and can be produced in large scale at low cost (*14*). Former studies indicated that scFvs may have comparable or better affinity than that of monoclonal or polyclonal antibodies (*15*), and could be applied in detecting agricultural samples (*16*).

Phage displayed technology is a system which makes it possible to generate scFvs with desired binding affinity and specificities. We report here for the first time the selection of anti-IVM scFvs from a human synthetic phage displayed library by subtractive panning. The expressed scFvs, for which the culture conditions need to be optimized due to complex secretion mechanisms and packing system of bacterial (*17*, *18*), were purified and applied in a ci-ELISA, and showed promising application in detection of IVM from agricultural and environmental samples.

## MATERIALS AND METHODS

Tomlinson J library provided by MRC (Cambridge, England) HGMP Resource Center is a human synthetic phage displayed scFv library (total diversity, $1.37 \times 10^8$) cloned into pIT2 phagemid. The library is based on a single human framework for $V_H$ and $V_\kappa$ with side chain diversity incorporated at positions in the antigen binding site that make contact

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to antigens in known structures and is highly diverse in the mature repertoire. The scFv fragments comprise a single polypeptide with the $V_H$ and $V_L$ domains attached to one another by a flexible Gly-Ser linker. Culturing flask was purchased from Corning (Beijing, China), and IVM from AEPI (Tianjin, China). IVM-Bovine Serum Albumin (IVM−BSA) was synthesized by our laboratory. Other reagents were all purchased from GE Healthcare (Beijing, China).

**Subtractive Biopanning on Culturing Flask.** Phages ($10^{12}$ cfu/mL) of the Tomlinson J were preadsorbed on 25 $cm^2$ plastic cell culturing flasks coated with bovine serum albumin solution (BSA in 50 mM sodium carbonate buffer, pH 9.6) for 1 h at room temperature. The phage supernatant was transferred to new cell culturing flasks coated with 4 mL 10−100 $\mu$g/mL IVM−BSA. The remaining steps of panning were performed as described in the Human Single Fold scFv Library I + J handbook (http://www.geneservice.co.uk/products/proteomic/datasheets/tomLinsonIJ.pdf).

**Selecting Phage Particles by Monoclonal Phage ELISA.** The individual clones from panned library were selected for binding to IVM by monoclonal phage ELISA. Briefly, 96-well plates were coated overnight at 4 °C with 100 $\mu$L of IVM−BSA (10 $\mu$g/mL IVM−BSA in sodium carbonate buffer). The wells were blocked with 200 $\mu$L of 2% skim milk in phosphate buffered saline (PBS) for 2 h at 37 °C. After washing three times with 0.05% Tween-20 in PBS (PBST), wells were incubated with 100 $\mu$L of monoclonal phage supernatant for 1 h at 37 °C. Bound phages were detected by horseradish peroxidase (HRP) conjugated anti-M13 antibody with incubation for 1 h at 37 °C, and revealed by adding ready-to-use 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution. Negative control reactions were performed by coating with native BSA and probing as described above. Color development was performed for 5−15 min at room temperature and stopped with 50 $\mu$L/well 2 M sulfuric acid. All analyses were performed in triplicate. $OD_{450}$ was measured with an automatic microplate reader (Thermo, USA).

**Colony PCR.** The selected phage particles were inoculated into *Escherichia coli* strain HB2151 (a nonsuppressor strain) for 30 min at 37 °C. Colony PCR (94 °C 4 min, then 94 °C 1 min, 58 °C 30 s, 72 °C 2 min for 25 cycles, and final extension at 72 °C for 10 min) was carried out to check individual clones for the presence of full length $V_H$ and $V_\kappa$ insert (935 bp). The PCR products were examined by electrophoresis on 1% agarose gel.

**DNA Sequencing and Analysis.** The single-stranded DNAs of the chosen monoclonal phages were sequenced by Yinjun (Shanghai, China). Two sequencing primers were used: LMB3 (5′-CAG GAA ACA GCT ATG AC-3′) and pHEN seq (5′-CTA TGC GGC CCC ATT CA-3′). Sequences of scFvs were aligned with the human germline sequences (on NCBI Web site), and complementarity determining region (CDR) regions of $V_H$ were analyzed (on VBASE Web site).

**Expression of Soluble scFv Antibody Fragments.** The full length and well sequenced clones were picked into 2TY medium (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl in 1 L aqueous solution) with 100 $\mu$g/mL ampicillin and 0.1% glucose to an optical density (OD) of 1.0 at 600 nm. Expression of the scFvs was induced by adding of isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.2−0.6 mM, and cultures were further grown at 20−37 °C for 6−20 h.

**Purification of Soluble scFv.** Cultures were centrifuged at 4 °C and 3300*g* for 15 min to separate the supernatant and cells. The supernatant was treated with solid ammonium sulfate to 65% saturation. The precipitated proteins were resuspended in buffer A (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 20 mM imidazole) and dialyzed against buffer A to remove ammonium sulfate. The cells were treated as follows to get soluble scFvs in periplasmic: cell pellets from 400 mL culture ( < 2 g wet weight cells) were resuspended in 15 mL of buffer A. After sonication at 400 W for 12 min (sonication 4 s, intermission 8 s, for 60 cycles), supernatants of cell lysate were collected by centrifugation at 12000*g* for 15 min.

Soluble scFv antibodies obtained from culture supernatants and periplasmic were applied to Ni metal ion affinity chromatography (1 mL HisTrap column) equilibrated in distilled water and buffer A. The column was washed sequentially with buffers B (20 mM Tris-HCL, pH 7.4, 500 mM NaCl, 20−60 mM imidazole). The bound proteins were eluted with 500 mM imidazole followed by extensive dialysis against PBS. Chromatography was performed at 1.0 mL/min and 4 °C. Purity of the eluted proteins was analyzed by 12% SDS−PAGE and stained with Coomassie brilliant blue.

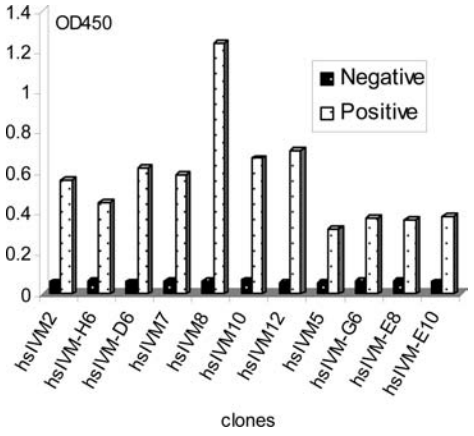


**Figure 1.** Monoclonal phage ELISA. The black column represents OD values of monoclonal phage particles to carrier protein BSA, and the white column represents values of that to IVM−BSA.

**ci-ELISA for Purified scFv.** The 96-well plates were coated overnight at 4 °C with IVM−BSA, and blocked in 200 $\mu$L of 3% BSA in PBS for 2 h at 37 °C. After washing three times with PBST, wells were incubated with 100 $\mu$L of a mixture of IVM standard (0, 0.01, 0.1, 2, 5, 10 $\mu$g/mL) and scFv for 2 h at 25 °C. Bound scFvs were probed by anti-His-HRP antibodies for 1 h at 25 °C and revealed by adding TMB substrate solution. Color development was performed for 5−15 min at room temperature and stopped with 50 $\mu$L/well 2 M sulfuric acid. All analyses were performed in triplicate. $OD_{450}$ was measured with an automatic microplate reader. The inhibition of control of the purified anti-IVM scFv was calculated by using the formula $[(P - S - N)/(P - N)] \times 100$, where $P$ is the OD value of the positive control (50 $\mu$L of scFv + 50 $\mu$L of PBS), $N$ is OD value of the negative control (100 $\mu$L of PBS), and $S$ is the OD value of the standard (50 $\mu$L of scFv + 50 $\mu$L of serial IVM standard). The data was analyzed by ELISACalc (designed by Wange Chen) for establishing standard curves and calculating the $IC_{50}$.

## RESULTS

Low molecular weight antigens (haptens) applied in phage panning are usually conjugated to a carrier molecule for immobilization. A problem associated with selecting scFvs against haptens is that the scFvs commonly bind to the combination of the hapten and carrier but not to the free hapten, which would make the selected scFvs ineffective in detection applications (*19*). In this paper, subtractive panning was used to remove the phage binders which showed specificity to the carrier proteins. To monitor the enrichment of the panning process, input and output phages of each round were titrated (data not show). The increased phage recovery percentage (quantity of eluted phage divided by quantity of input phage) indicated that phage clones bound to IVM were enriched after 4 rounds of subtractive panning. Eleven individual clones, in which hsIVM8 exhibited the best interaction with IVM−BSA, were selected using monoclonal phage ELISA (**Figure 1**). The selected clones were identified for full length single chain fragments with 7 among them identified positive (**Figure 2**).

Single-stranded DNAs of the 7 monoclonal phage clones were sequenced, and the deduced amino acid sequences were all different aligned with human germline VH3-23 (**Table 1**). The DNA sequence of hsIVM8 was submitted to the GenBank database under Accession No. FJ535062. The germline genes and hsIVM8 differed especially in the CDR2 and CDR3 regions of both heavy and light chain. These regions indicated probably the most important contact-associated epitopes. The secondary structure and hydrophobicity of hsIVM8 were predicted using related computer software and database. Results showed that hsIVM8 was a secretory, unstable and hydrophilic protein,

**Table 1.** Deduced Amino Acid Sequence of Chosen Clones Compared with Human Germline VH3-23[a]

| name | FR1 | CDR1 | FR2 | CDR2 | FR3 | CDR3 | FR4 |
|---|---|---|---|---|---|---|---|
| hsIVM2 | EVQLLESGGGLVQPGGSLRLSCAAS | GFTFSSYA | MSWVRQAPGKGLEWVSS | ILSAGYST | YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC | AKLKALFDY | WGQGTLVTVSS |
| hsIVM-d6 | EVQLLESGGGLVQPGGSLRLSCAAS | GFTFSSYA | MSWVRQAPGKGLEWVSS | ILSAWTST | YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC | AKRHAPFDY | WGQGTLVTVSS |
| hsIVM-h6 | EVQLLESGGGLVQPGGSLRLSCAAS | GFTFSSYA | MSWVRQAPGKGLEWVSS | LLSAWYST | YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC | AKLKALFDY | WGQGTLVWSS |
| hsIVM7 | EVQLLESGGGLVQPGGSLRLSCAAS | GGGGLNLL | MSWVRQAPGKGLEWVSS | LLSAWYCA | YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC | AKYSAHFDY | WGQGTLVTVSS |
| hsIVM8 | EVQLLESGGGLVQPGGSLRLSCAAS | GFTFSSYA | MSWVRQAPGKGLEWVSS | ICHEGKPT | YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC | AKSDHTFDY | WGQGTLVTVSS |
| hsIVM1O | EVQLLESGGGLVQPGGSLRLSCAAS | GFTFSSYA | MSWVRQAPGKGLEWVSL | IDE IGSST | YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC | AKKMNVFDY | WGQGTLVTVSS |
| hsIVM12 | EVQLLESGGGLVQPGGSLRLSCAAS | GATFSLYA | MSWVRQAPGKGLEWVSS | ICLEASPT | YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC | AKIKPHFDY | WGQGTLVTVSS |
| VH3-23 | EVQLLESGGGLVQPGGSLRLSCAAS | GFTFSSYA | MSWVRQAPGKGLEWVSA | ISGSGGST | YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYC | AK | |

[a] The amino acid sequencing results of seven active clones were all different, but shared some similarities in CDR loops. The high variation in CDR2 and CDR3 of heavy chain indicated the possible binding epitopes.

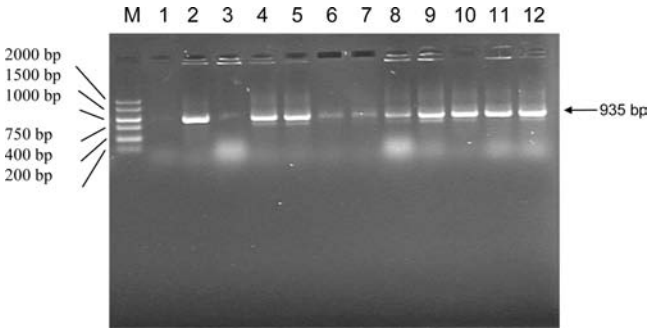


**Figure 2.** PCR amplification of the chosen clones. Clones with full length scFv gene had bands at 935bp. Lane M: DNA marker. Lanes 1−12: empty HB2151; hsIVM2, hsIVM-G6, hsIVM-H6, hsIVM-D6, hsIVM-E8, hsIVM-E10, hsIVM5, hsIVM7, hsIVM10, hsIVM12, hsIVM8.

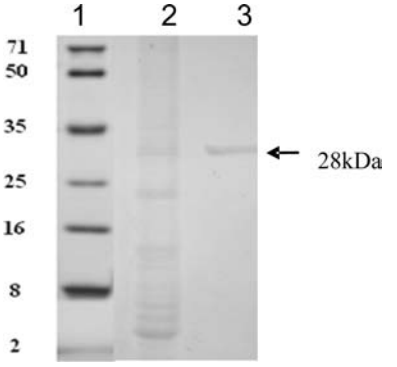


**Figure 3.** SDS−PAGE analysis of hsIVM8 expressed in *E. coli* HB2151. Proteins were visualized by Coomassie brilliant blue staining. The numbers on the left refer to the molecular masses of the markers. Lane 1: marker. Lane 2: unpurified culture supernatant of the infected *E. coli* HB2151. Lane 3: purified hsIVM8 protein.

with sorts of phosphorylation and proteinase sites (on Expasy Web site).

hsIVM8 clone was subcloned into the host strain *E. coli* HB2151 for expression as a soluble scFv. The optimized soluble expression conditions were cultured 19 h with 0.2 mM IPTG at 25 °C. Since all scFvs contain a C-terminal six residue his-tag, the soluble scFvs could be purified using Ni metal ion affinity chromatography. After purification, a band of 28 kDa representing anti-IVM scFv was observed on 12% SDS−PAGE, contained 18.3% of total cell protein and reached a purity of more than 94% (**Figure 3**). We obtained 4.4 mg scFv from 400 mL of culture.

In order to evaluate the sensitivity of hsIVM8 for detecting IVM, an immunoassay of ci-ELISA was carried out. A standard curve was established in a range of 0.01−10 $\mu$g/mL. Competition was observed with increasing concentrations of IVM, which indicated that hsIVM8 recognized free IVM. The linear range of standard ci-ELISA was 0.1−5 $\mu$g/mL, and the largest inhibition of hsIVM8 was 58%, as shown in **Figure 4**.

## DISCUSSION

The major result of this study is the successful selection of a scFv against IVM from phage displayed library which has been widely used in selecting of variety regents (*20*−*22*). To our knowledge, this is the first report about selecting anti-IVM scFv. In contrast to the conventional monoclonal and polyclonal antibodies, the newly identified scFv described here has lower molecular weight and combines genotype and phenotype.

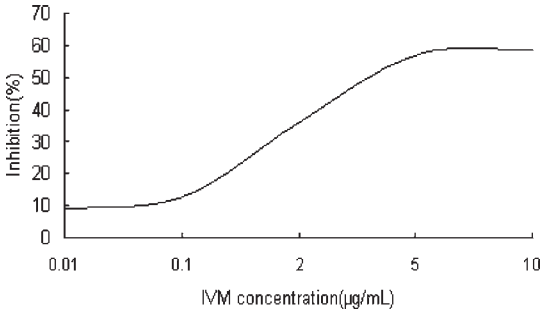


**Figure 4.** hsIVM8-based standard inhibition curve by ci-ELISA. Ivermectin standards were diluted in PBS−10% ethanol into different concentrations between 0.01 and 10 $\mu$g/mL. The linear range of detection was between 0.1 and 5 $\mu$g/mL and the calculated 50% inhibition of control ($IC_{50}$) valued 4.11 $\mu$g/mL.

However, their problems must be overcome in our study. The target IVM, a small molecule, could hardly be immobilized to the culturing flask. To overcome this problem, IVM−BSA conjugate was used, which meanwhile raised background binding of nonspecific phages especially phages binding to the carrier proteins BSA. To decrease the background binding, subtractive panning methods were used. Panning on culturing flasks was successfully developed, but the selected scFv phages had a high risk of weak interaction, which was similar with panning on immunotubes (*23*, *24*).

Most scFvs derived from phage displayed libraries have been selected using purified antigens or synthetic antigens immobilized on physical surfaces. This method may select scFvs that do not recognize or show weak interaction to free hapten in natural context. In this paper, the sensitivity of hsIVM8 is lower than that of monoclonal antibodies (*25*). The reason may be that the library we used is nonimmunized, and the modified hapten may be different from the natural conformation. The preliminary test of hsIVM8-based ci-ELISA was carried out to detect IVM residue in padder water (data not show). The results showed potential application of hsIVM8 in environmental sample detection.

To improve the sensitivity of hsIVM8, we have extensive interest in the in vitro affinity maturation in CDR3 of heavy chain with a number of studies having shown the feasibility (*26*−*28*). In the postgenomic era, scFvs selected from phage display libraries will be widely used in the detection of residues in agricultural and environmental matrices.

## ABBREVIATIONS USED

IVM, ivermectin; BSA, bovine serum albumin; PBS, phosphate-buffered saline, pH 7.4; IPTG, isopropyl $\beta$-D-1-thiogalactopyranoside; TY, tryptone yeast medium; CDR, complementarity determining region; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; scFv, single chain variable fragment; $V_H$, variable region of heavy chain; $V_L$, variable region of light chain.

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