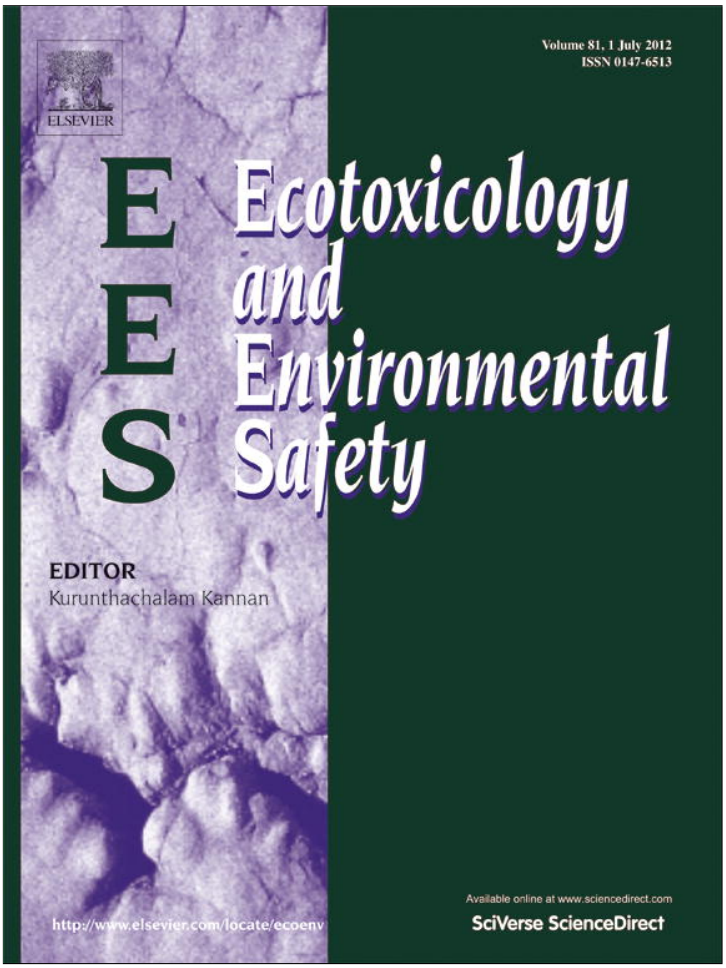

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Rapid isolation of single-chain antibodies from a human synthetic phage display library for detection of *Bacillus thuringiensis* (Bt) Cry1B toxin

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ABSTRACT

Single chain variable fragment antibody (scFv) is capable of binding its target antigens and is one of the most popular recombinant antibodies format for many applications. In this study, a large human synthetic phage displayed library (Tomlinson J) was employed to generate scFvs against Cry1B toxin by affinity panning. After four rounds of panning, six monoclonal phage particles capable of binding with the Cry1B were isolated, sequenced and characterized by Enzyme-Linked Immunosorbent Assay (ELISA). Two of the identified novel anti-Cry1B scFvs, namely H9 and B12, were expressed in *Escherichia coli* HB2151 and purified by Ni metal ion affinity chromatography. Sodium dodecyl sulfate polyacrylamine gel electrophoresis (SDS-PAGE) indicated that the relative molecular mass of scFv was estimated at 30 kDa. The purified scFv-H9 was used to develop an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) for Cry1B toxin. The linear range of detection for standards in this ic-ELISA was approximately 0.19–1.1 μg mL^{-1} and 50% inhibition of control (IC_{50}) was 0.84 μg mL^{-1} for Cry1B. The affinity of scfv-H9 was $(1.95 \pm 0.12) \times 10^7$ M^{-1} and showed cross-reactivity with Cry1Ab toxin and Cry1Ac toxin (8.53% and 7.58%, respectively), higher cross-reactivity (12.8%) with Cry1C toxin. The average recoveries of Cry1B toxin from spiked leaf and rice samples were in the range 89.5–96.4%, and 88.5–95.6%, respectively, with a coefficient of variation (C.V) less than 6.0%. These results showed promising applications of scfv-H9 for detecting Cry1B toxin in agricultural and environmental samples.

1. Introduction

Bacillus thuringiensis (Bt) bacteria produce insecticidal proteins named Cry toxins, which are effective in killing some insect pests. These toxins are used worldwide, being delivered as sprays or expressed in some genetically modified crops (Pardo-Lòpez et al., 2009). Though they have long been known as a safe insecticidal crystal protein due to their insect specificity, and have great potential in agriculture and for the control of disease-related insect vectors (Bravo et al., 2007). The results showed that the Cry toxins have been reported to kill non-target insect pest, such as wasps and bees (Craig et al., 2008) and the main concerns regarding genetically modified crop include toxin or allergen production, changes in nutrient levels, and development of antibiotic resistance (Kaeppler, 2000). The potential broad applications and risk of Cry toxins make it necessary to establish a simple and inexpensive method for detecting Cry toxins.

Despite the large number of Cry toxins, only about a dozen (Cry1Aa, Cry1Ab, Cry1Ac, Cry1C, Cry1D, Cry1E, Cry1F, Cry2Aa, Cry2Ab, Cry3A, Cry3B and Cry34/Cry35) are used commercially as sprays or in Bt crops (Bravo and Soberón, 2008). The Cry1B toxin, which was isolated from Bt strain HD2 by Brizzard and whiteley, has also widely used in insect–pest control (Brizzard and Whiteley, 1988). Breitler et al. (2000) firstly reported of Striped Sten Borer (SSB) control using the Cry1B gene, which directed by the maize ubiquitin promoter proved to afford full SSB (*Chilo suppressalis* Walker) control in transgenic *japonica* rice varieties Ariete and Senia, with an accumulation reaching up to 0.4% of the total soluble proteins. López-Pazos et al. (2009) reported the Bt δ-endotoxins such as Cry1B and Cry3A are active against *Hypothenemus hampe*i Ferrari (Coleoptera: Scolytidae). Martins et al. (2010) had cloned, sequenced and expressed a cry1Ba toxin gene from the *B. thuringiensis* S601 strain which was previously shown to be toxic to *Anthonomus grandis*, a cotton pest. Polymerase chain reaction (PCR) technique has been used to

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determine the presence or absence of particular Cry genes, it is highly sensitive and fast, and therefore can be easily used on a routine basis, either to identify Cry-type genes (Martinez et al., 2005; Ben-Dov et al., 1997), or to detect new Cry genes (Juárez-Pérez et al., 1997; Kuo and Chak, 1996). However, PCR techniques are expensive, time-consuming, and require sophisticated instrumentation and trained personnel. Thus, they are not adequate for the extensive high-throughput screening of crops and foodstuffs samples (Roda et al., 2006). On the other hand, Enzyme-linked immunosorbent assay (ELISA) methods have been used for detection and quantization of proteins expressed by most biotechnology-derived crops in commercial production, and many immunoassay kits are commercially available with detection in samples (Roda et al., 2006; Asensio et al., 2008). It is the most convenient method of detecting the specific antigen. The development of antibody engineering technology provided the tools necessary to generate novel antibodies (such as scFvs) for the detection of Bt toxin in food and environment. ScFvs are monovalent and formed by the variable region of heavy chain (V_H) and light chain (V_L) joined by a flexible linker (Raag and Whitlow, 1995; Wen et al., 2010). The obvious advantages of scFv are that it maintains the specific affinity to the antigen, and can be produced in large scale without immunization procedure (Dai et al., 2003; Weisser and Hall, 2009).

Phage displayed technology is a system which makes it possible to generate scFvs with desired binding affinity and specificities (Garet et al., 2010; Pande et al., 2010; Li and Caberoy, 2010). Some researchers have employed the phage displayed libraries to successfully obtain scFvs against Bt Cry1Ab toxin (Gómez et al., 2001, 2006). We firstly reported the selection of anti-Cry1B scFvs from a human synthetic phage displayed library (Tomlinson J). The expressed scFvs were purified and applied in an indirect competitive ELISA, and showed promising application in detection of Cry1B toxin from agricultural and environmental samples.

2. Materials and methods

Cry1B toxin was purchased from Youlong Chemicals (Shanghai, China) and dissolved in sodium carbonate buffer (CBS, pH 9.6), aliquoted and stored at −20 °C. 25 cm^2 plastic cell culturing flasks were acquired from Corning (Beijing, China); other reagents were all purchased from GE Healthcare (Beijing, China).

2.1. Libraries, helper phage and bacterial strains

The human single-fold scFv Tomlinson J libraries, KM13 helper phage and *Escherichia coli* TG1 and HB2151 were all provided by MRC (Cambridge, England) HGMP Resource Center. The library is based on a single human framework for VH (V3-23/DP-47 and JH4b) and Vk (O12/O2/DPK9 and Jk1), which encodes the most common human canonical structure. The size of the Tomlinson J library is 1.37×10^8. The libraries have a high proportion of functional antibodies with 88% of clones containing inserts. The antibodies are displayed as scFv fragments on the minor coat protein pIII of filamentous phage, cloned in an ampicillin resistant phagemid vector (pIT2). 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.

2.2. Preparation of phage antibodies and panning

A total of 500 μL of the glycerol stock of Tomlinson J library was grown in 200 mL of 2 × TY medium (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl in 1 L aqueous solution) containing 1% glucose and 100 μg mL^{-1} ampicillin, and incubated with shaking at 37 °C until an optical density at 600 nm (OD_{600}) of 0.4–0.6 (2–4 h) was obtained. 10^{10} helper phage KM13 was added to 50 mL of the culture and incubated without shaking in 37 °C water bath for 30 min. Subsequently, infected cells were collected by centrifugation, resuspended in 100 mL 2 × TY (containing 0.1% glucose, 100 μg mL^{-1} ampicillin and 50 μg mL^{-1} kanamycin), and incubated with shaking overnight at 30 °C. Centrifuged at 3300*g* for 30 min and added 20 mL polyethylene glycol (PEG) 6000 in 2.5 M NaCl (20% w/v), and stored at −80 °C with 15% glycerol, as depicted by the MRC protocol.

Tomlinson J library was taken through four rounds of panning on Cry1B toxin. Briefly, phages (10^8 cfu/mL) with 4 mL MPBS (2% (w/v) skimmed milk-PBS) were added into plastic cell culturing flasks coated with Cry1B toxin (the first round was 100 μg mL^{-1}, the remaining three rounds were 75, 50 and 25 μg mL^{-1}) for shaking 1 h and standing 1 h at room temperature. The remaining steps of panning were performed as described in the Human Single Fold scFv Library I t J handbook (http://www.geneservice.co.uk/products/proteomic/datasheets/tomlinsonI+J.pdf).

2.3. Screening of antibodies against Cry1B toxin

The ability of selected scFv phages to bind to Cry1B toxin was assessed by polyclonal phage ELISA as described in Garet et al. (2010). Briefly, 96-well plates (Costar, USA) were coated with 100 μL of Cry1B toxin (20 μg mL^{-1}) in CBS overnight at 4 °C and blocked for 2 h at room temperature with 200 μL per well of 3% skim-milk in phosphate buffered saline (MPBS). Phages precipitated at each round of panning (100 μL) were added to the wells in blocking solution. The plates were then incubated for 1 h at room temperature, and washed with PBST prior to adding 100 μL per well of a 1:5000 dilution of anti-M13 monoclonal antibody, and incubated at room temperature for 1 h. The plates were then washed and added tetramethylbenzidine substrate solution, color development was performed for 10 min at room temperature and stopped with 50 μL/well 2 M sulfuric acid. All analyses were performed in triplicate. OD_{450} was measured with an automatic microplate reader (Thermo, USA).

2.4. Selection of monoclonal phage antibodies

In monoclonal phage ELISA, individual colonies from round 4 of panning were grown in 100 μL of 2 × TY medium (100 mg mL^{-1} ampicillin and 1% glucose) in a 96-well plates shaking (250 rpm) overnight at 37 °C. 2 μL of each culture from plates was grown in a second plate with 200 μL of 2 × TY with ampicillin and 1% glucose shaking for 2 h at 37 °C. Each culture was infected with 10^{10} of helper phage and incubated at 37 °C for 1 h. Each plate was centrifuged and the pellet was resuspended in fresh 2 × TY containing 100 μg mL^{-1} ampicillin and 50 μg mL^{-1} kanamycin, shaking (250 rpm) overnight at 37 °C. After centrifugation of the cultures at 1800 rpm for 15 min, 50 μL of supernatant was used for ELISA as described above.

2.5. Colony PCR and DNA sequencing, analysis

The selected phage particles were inoculated into *E. coli* strain HB2151 (a non-suppressor strain) and grown with shaking for 2 h at 37 °C. Colony PCR (94 °C for 10 min, then 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min for 30 cycles, and final extension at 72 °C for 10 min) was carried out to check individual clone for the presence of full length V_H and V_L insert (935 bp). The PCR products were examined by electrophoresis on 1% agarose gel. The single-stranded DNAs of the chosen monoclonal phages were sequenced by Meiji Company (Shanghai, China). Two sequencing primers were used: LMB3 (5n-CAG GAA ACA GCT ATG AC-3GA and pHEN seq (5n-CTA TGC GGC CCC ATT CA-3A T Readily available web based tools were used for translation and alignment of DNA sequences.

2.6. Expression and purification of soluble anti-Cry1B scFvs

E. coli HB2151 strain, which can recognize the amber codon (TAG) as a stop signal, was infected by phage clones to express soluble scFvs. The full length and well sequenced clones (H9 and B12) were picked into 2 × TY with 100 μg mL^{-1} ampicillin and 0.1% glucose to an optical density (OD) of 1.0 at 600 nm. Expression of the scFvs were induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and cultures were further grown with shaking (200 rpm) overnight at 30 °C. Each overnight culture was divided into two equal volumes and centrifuged at 3300*g* for 30 min. The supernatant was clarified by filtration through disposable 0.22 μm filter and stored in 4 °C. For extracting protein from the whole cells, one fraction of the cell pellet was resuspended in 1 mL of 1 × PBS. The cells were ruptured by sonication and centrifuged at 12,000*g* for 15 min. The supernatant was collected to be tested for the presence of soluble scFv. To obtain soluble protein from the periplasm, the other fraction of cell pellet was resuspended in 0.5 mL ice-cold 1 × TES buffer (0.2 M Tris–HCl pH 8.0, 0.5 mM EDTA, 0.5 M sucrose). After adding 0.75 mL of TES (diluted 1:4 in sterile water), cells were vortexed for 30 s and incubated on ice for 1 h. The protein was collected as described previously. The control group was empty HB2151. The products were analyzed by 12% SDS-PAGE with Silver Straining.

Periplasmic soluble scFv (H9) was isolated as described earlier and dialyzed against 10 mM PBS (pH 7.4) at 4 °C. The dialyzed sample was clarified by centrifugation at 12,000*g* for 30 min at 4 °C and the clear supernatant was collected and applied to Ni metal ion affinity chromatography (1 mL HisTrap column) equilibrated in distilled water and buffer A (20 mM Tris–HCl, pH 7.4, 500 mM NaCl, 10 mM imidazole). The column was washed sequentially with buffers B (20 mM Tris–HCl, pH 7.4, 500 mM NaCl, 20–500 mM imidazole). The

flow rate was 1 mL min^{-1}. The purity of the eluted fractions was checked by 12% SDS-PAGE.

2.7. Indirect competitive ELISA (ic-ELISA) for soluble and purified scFv

The 96-well plates were coated overnight at 4 °C with Cry1B toxin, and blocked in 200 μL of 3% BSA in PBS for 2 h at 37 °C. After washing three times with PBST, wells were incubated with 100 μL of a mixture of Cry1B toxin standard (0.05, 0.1, 0.2, 0.5, 1, 2 and 5 μg mL^{-1}) 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 15 min at room temperature and stopped with 50 μL/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-Cry1B 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 μL of scFv+50 μL of CBS), N is OD value of the negative control (100 μL of PBS), and S is the OD value of the standard (50 μL of scFv+50 μL of serial Cry1B toxin standard). The data was analyzed by ELISACalc (designed by Wange Chen) for establishing standard curves and calculating the IC_{50}.

2.8. Measurement of scFv affinity by non-competitive enzyme immunoassay

The affinity (K_{aff}) was determined using the method described by Beatty et al. (1987). The wells of a 96-well plate were coated with 100 μL of 4 different initial concentrations of Cry1B (5.0, 2.5, 0.125 and 0.0625 μg mL^{-1}) were incubated at 4 °C overnight. On the second day, the 96-well plate was emptied then washed 5 times with PBST, and blocked in 200 μL of 3% BSA in PBS for 2 h at 37 °C. After washing 5 times with PBST, modified the scFv concentration at 10^{-6} M, used the doubling dilution method (1:2–1:32), and added to the wells. Bound scFvs were probed by anti-His-HRP antibodies for 1 h at 25 °C and revealed by adding TMB substrate solution. OD_{450} was measured with an automatic microplate reader. According to a mono-valence of scFv, a mathematical processing of the Law of Mass Action leading to a simple expression of K_{aff} has been derived and validated: $K_{aff}=(n-1)/n[Ab_2]-[Ab_1]$, where $[Ab_1]$ and $[Ab_2]$ represent the respective scFv concentrations required to reach 50% of the maximum absorbencies obtained at two different concentrations of coated antigen ($[Ag]_1=n[Ag]_2$) and n is the dilution factor between the two concentrations of antigen used (Dai et al., 2001).

2.9. Cross-reactivity ELISA tests for soluble and purified scFv

Specificity is defined as the ability of the antibody to distinguish different Cry1 toxins from each other. In order to characterize the specificity of scFv-H9, we chose three kinds of different Cry1 toxins (Cry1Ab, Cry1Ac and Cry1C). Cross-reactivity was used to describe the specificity and calculated using the cross-reacting analyte and standard Cry1B toxin concentration toward 50% inhibition according to function: CR (%)=$IC_{50,\ Cry1B}/IC_{50,\ X}\times 100\%$, where CR is the cross-reactivity (%), $IC_{50,\ Cry1B}$ is the concentration of standard Cry1B toxin at 50% inhibition (μg mL^{-1}) and $IC_{50,\ X}$ is the concentration of cross-reacting analyte at 50% inhibition (μg mL^{-1}).

2.10. Assessed the ELISA by spiked samples

The ic-ELISA was used for Cry1B toxin determination in leaf samples and ground rice samples. 1 g of leaf samples or ground rice samples were spiked Cry1B toxin at three concentration levels (0.2, 0.5 and 1.0 mg kg^{-1}), and then shaken with 2 mL of protein extraction solution (0.1 M PBS pH 7.4, containing 0.1% BSA and 0.05% Tween-20). After gentle shaking at room temperature for 2 h, the suspension was centrifuged for 10 min at 10,000*g*. The extract was diluted by 10-fold PBS and then analyzed by ic-ELISA. Standards and blanks (free toxin samples) were used in all cases. Each concentration was replicated three times.

3. Results

3.1. Isolation of scFv phages against Cry1B

Specifically binding clones were determined by the fraction of input phage that binds the Cry1B toxin. In these experiments, the concentration input phage used for each round of selection was kept constant at $\sim 10^8$ particles/mL. Input and output titers from each round are shown in Table 1. Approximately 100-fold increase in output was obtained after four rounds of panning. There was clear enrichment of the positive clones during successful bio-panning experiments.

Table 1
Recovery of phages during selection against Cry1B.

Selected round	Input [a]	Output[a, b]	% Phage Bound[c]
1	2.0×10^8	1.4×10^4	0.007
2	2.5×10^8	1.6×10^4	0.006
3	4.0×10^8	5.5×10^6	1.38
4	7.1×10^8	7.2×10^6	1.01

[a] Phage inputs and outputs were calculated by transduction of *E. coli* TG1 to ampicillin resistance.
[b] Number of eluted scFv phages expressed as colony forming unit per milliliter (cfu mL^{-1}).
[c] %Phage bound=(output/input)×100.

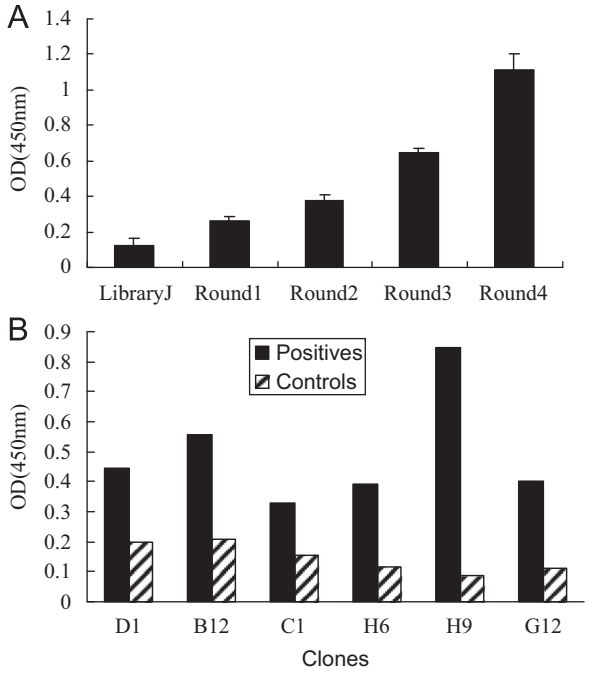

Fig. 1. Polyclonal phages ELISA and monoclonal phage ELISA against Cry1B. (A) Culture supernatants containing approximately 10^8 phage particles per milliliter were analyzed by Polyclonal phages ELISA. Absorbance values are the mean of three independent determinations (after subtraction of the unspecific binding). Error bars show the mean ± standard deviation from triplicate measurements. (B) The binding to Cry1B of clones expressing scFv fragments was determined by monoclonal phage ELISA. Phage-infected *E. coli* TG1 culture supernatants containing approximately 10^8 phage particles per milliliter were analyzed in the assay. Absorbance values are the means of three independent determinations.

3.2. Specificity of scFv phages against Cry1B toxin by polyclonal phage ELISA

The ability of the scFv phages selected from the Tomlinson J library to bind to Cry1B toxin was firstly assessed by polyclonal phage ELISA. As shown in Fig. 1(A), few scFv phages from the unselected J library are able to recognize Cry1B toxin. However, clones selected in rounds one through three displayed binding for Cry1B toxin with OD_{450} ranging from 0.27 to 0.65 for the Tomlinson J library. After round 4 of panning, ELISA results showed an increase in binding was observed (OD_{450} 1.11).

3.3. Monoclonal scFv-phage ELISA

From the Tomlinson J library, 300 individual clones were picked at random from TYE plates used for the titration of recoveries from the fourth round. The clones were screened in phage ELISA against immobilized Cry1B toxin. As appeared in

Fig. 1(B), six clones (D1, B12, C1, H6, H9, and G12) were selected by monoclonal phage ELISA as positive binders to Cry1B toxin. These clones gave OD_{450} readings ranging from 0.328 to 0.849 in ELISA. Two clones (H9 and B12) were selected from the Tomlinson J library based on an OD_{450} reading of 0.849 and 0.557, showed a high binding ability to the Cry1B toxin. Clones were expressed in *E. coli* HB2151 for further study.

3.4. PCR amplification and DNA sequencing of the positive clones

The six selected clones using monoclonal phage ELISA were checked for the presence of full length V_H and V_L insert with PCR. The selected clones were all identified for full length single chain fragments (935 bp) [Fig. 2(A)]. Then, the six phage clones picked for sequencing, five clones from the libraries J had distinct nucleotide sequences (C1 clone and D1 clone had the same nucleotide sequences and renamed C1). Fig. 2(B) showed amino acid sequences deduced from the obtained nucleotide data. All five sequences had different amino acid substitutions that were expected and mostly were in complementarity determining regions (CDR). In the CDR area, the amino acid sequences shared relatively low sequence homology. Apparently, the degree of homology was observed higher in the variable region of the light chain (V_L) domain compared with the variable region of the heavy chain (V_H) domain. These regions indicated probably the most important contact-associated epitopes.

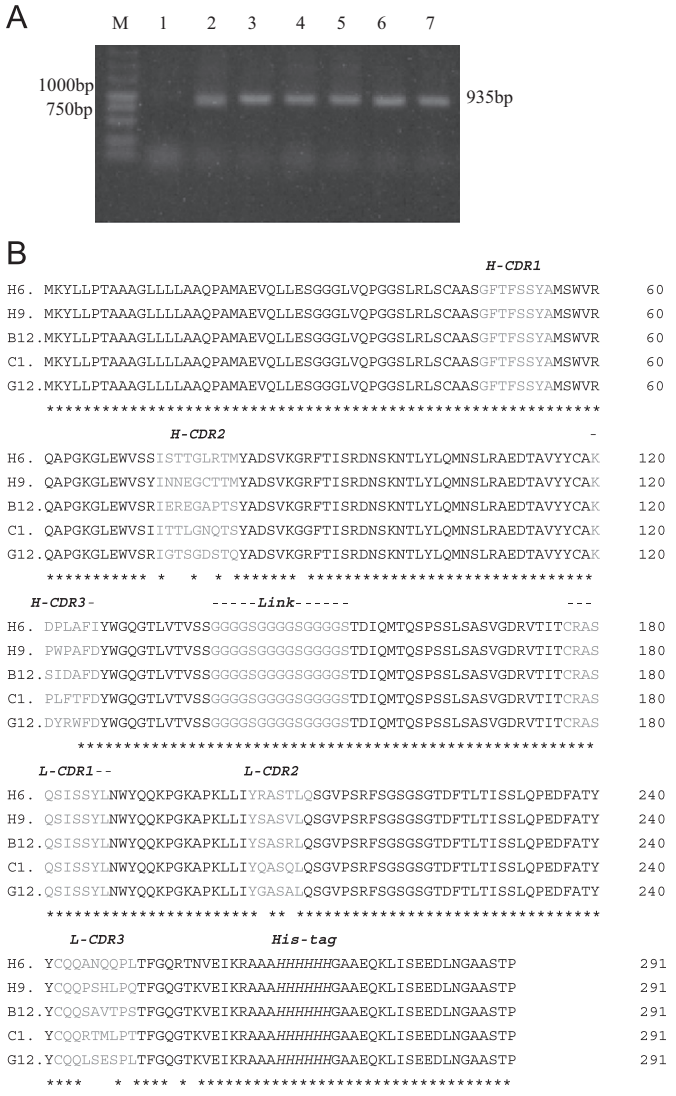

Fig. 2. PCR amplification and sequencing of the chosen clones. (A) Clones with full length scFv gene had bands at 935bp. Lane M: DNA marker. Lanes 1–7: empty TG1, H10, E3, C8, B12, C12 and G1. (B) Amino acid sequences were deduced from the nucleotide sequences. Positions of the respective complementarity determining regions for the variable domains of heavy chain (H-CDR 1–3) and light chain (L-CDR 1–3) were indicated by gray symbols.

3.5. Soluble anti-Cry1B scFv antibody obtained and purified

The scFvs were expressed in *E. coli* HB2151 and the proteins from the medium, and periplasm whole cells were each extracted to verify the location and functionality of each soluble scFv. Single bands of approximately 30 kDa were detected for each clone [Fig. 3(A)]. Since all scFv contain a C-terminal six residue His-tag, the soluble scFv could be purified using Ni metal ion affinity chromatography. We used the gradient elution method (the concentration of imidazole containing 20–500 mM) and confirmed the optimized elution concentration of imidazole was 400 mM [Fig. 3(B)].

3.6. Indirect competitive ELISA (ic-ELISA)

In order to evaluate the sensitivity of scFv-H9 for detecting Cry1B, an immunoassay of ic-ELISA was carried out. A standard curve was established in a range 0.05–5 $\mu g\ mL^{-1}$. Competition was observed with increasing concentrations of Cry1B, which indicated that scFv-H9 recognized free Cry1B. The linear range of standard ic-ELISA was 0.19–1.1 $\mu g\ mL^{-1}$ and 50% inhibition of control (IC_{50}) was 0.84 $\mu g\ mL^{-1}$ for Cry1B, the largest inhibition of scFv-H9 was 61.29%, as shown in Fig. 4.

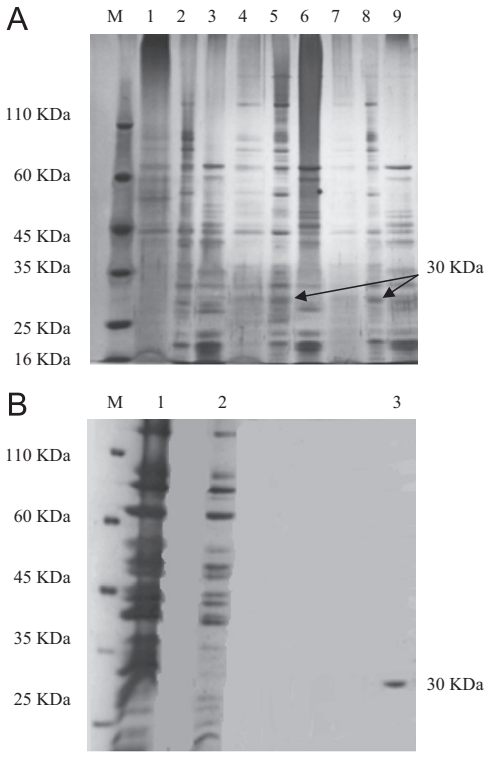

Fig. 3. SDS-PAGE analysis of soluble expression and purified of the scFv. (A) Soluble scFv induced by IPTG. M: A protein mass marker served as a standard, lanes 1–3: supernatant, PBS treatment, TES treatment of non-induced HB2151 as control. lanes 4–6: H9 postive clone; lanes 7–9: B12 postive clone. The treatment method of two positive clones was all same as the control. (B) SDS-PAGE analysis of anti-Cry1B scFv after affinity purification from periplasmic extract. M: A protein mass marker served as a standard, lane 1: Crude periplasmic extract, lane 2: washing with binding buffer (10 mM imidazole), lane 3: purified recombinant antibody by elution buffer (400 mM imidazole).

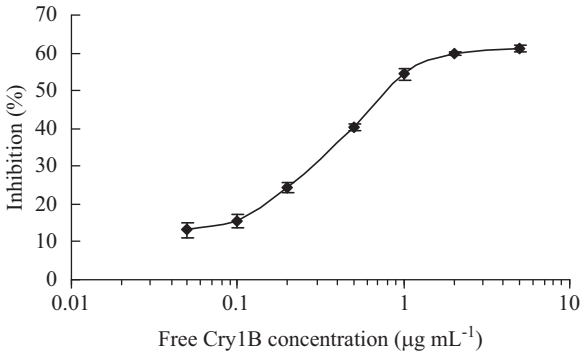

Fig. 4. ScFvH9-based standard inhibition curve by ic-ELISA. Cry1B standards were diluted in CBS into different concentrations between 0.05 and 5 μg mL^{-1}. The linear range of detection was between 0.19 and 1.1 μg mL^{-1} and the calculated 50% inhibition of control (IC_{50}) valued 0.84 μg mL^{-1}. The data represent mean ± standard deviation from triplicate measurements.

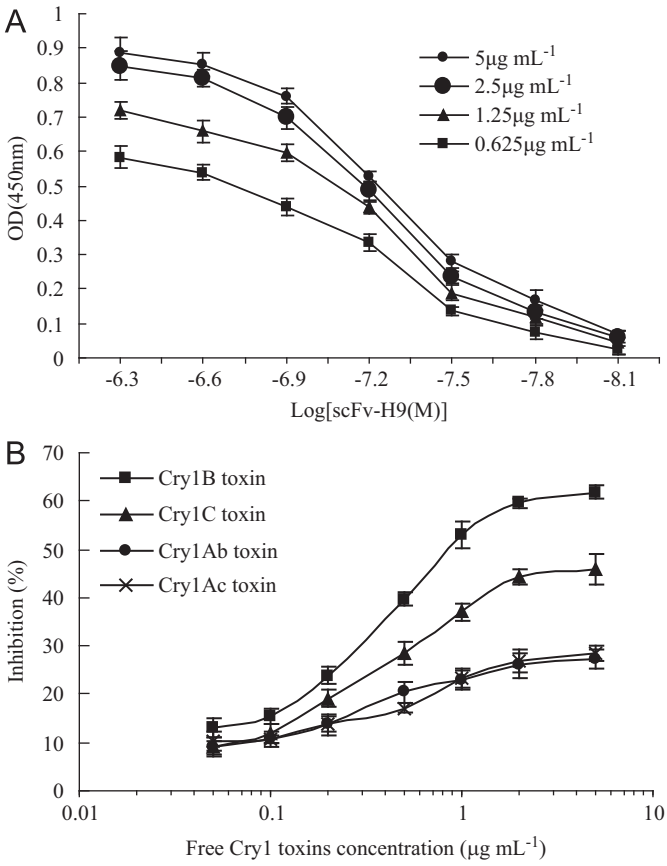

Fig. 5. ELISA for affinity and cross-reactivity of scFv-H9. (A) The concentrations of Cry1B in the coating solution were: 5, 2.5, 1.25 and 0.625 μg mL^{-1}. The data represent mean ± standard deviation from three duplicate measurements. Calculated scFv-H9 concentration (M) of every curve at OD-50, According to the formula, the average of six numbers of affinities was acquired. (B) Competitive ELISA of scFv-H9 with various structurally related Cry1 toxins (Cry1B, Cry1C, Cry1Ab and Cry1Ac) is demonstrated. Results were plotted as the mean percent inhibition vs. concentration of soluble Cry1 toxins. Percent inhibition of scFv-H9 was calculated from this type of competitive ELISA.

3.7. Affinity of the scFv-H9

The relative affinity constant of scFv-H9 for Cry1B was calculated using a method described by Beatty et al., which allows the measurement of K_{aff} by solid-phase EIA using different antigen concentrations for the coating. Based on 4 concentrations of Cry1B (5, 2.5, 1.25 and 0.625 μg mL^{-1}) used for coating [Fig. 5(A)], 6 affinity constants (3 for $n=2$, 2 for $n=4$ and 1 for $n=8$) were obtained for scFv-H9. The mean K_{aff} value of scFv-H9 for Cry1B is approximately $(1.95 \pm 0.12) \times 10^7$ M^{-1} (Table 2).

3.8. Cross-reactivity of scFv-H9

The cross-reactivity of scFv-H9 to other Cry1 toxins was examined, four standard curves obtained with the scFv-H9 to Cry1B, Cry1Ab, Cry1Ac, and Cry1C by ic-ELISA, and the cross-reactivity obtained by division of these midpoints [Fig. 5(B)]. When the cross-reactivity of scFv-H9 to Cry1B was defined to be 100%, whereas the cross-reactivity of scFv-H9 to the other Cry1 toxins such as Cry1Ab and Cry1Ac was 8.53% and 7.58%, respectively, and the other Cry1 toxin such as Cry1C was showed higher cross-reactivity (12.8%) (Table 3).

3.9. Recovery studies

Samples matrix effects were examined by sample dilution with buffers. Leaf and rice samples were spiked with Cry1B at different concentrations (0.2–1.0 mg kg^{-1}) and assayed by ic-ELISA. The measured recoveries of Cry1B from the spiked samples are given in Table 4. The average recoveries ranged from 88.5% to 96.4%, with a coefficient of variation (C.V) less than 6.0%, indicating good accuracy for the quantitative detection of Cry1B toxin in agricultural and environmental samples.

Table 2
Affinity calculated from the non-competitive ELISA curves of scFv-H9 against Cry1B.

Antibody	$K_{n=2}$		$K_{n=4}$			$K_{n=8}$	Mean ± SD
scFv affinity (10^7 M^{-1})							
scFv-H9	2.13	1.89	2.06	1.93	1.82	1.85	1.95 ± 0.12

$K_{n=2}$ is the collection of affinity calculated from every 2 curves differed in amount of coated Cry1B by 2-fold. Analogized from this, $K_{n=4}$ and $K_{n=8}$ were calculated and listed above.

Table 3
Percent cross-reaction of soluble and purified scFv-H9 in ELISA with selected Cry1 toxins.

Toxins	scFv-H9	
	IC_{50} (μg mL^{-1})	CR (%)
Cry1B	0.92	100
Cry1C	7.19	12.80
Cry1Ab	11.73	8.53
Cry1Ac	12.13	7.58

The cross-reactivity potential was approximated at the IC_{50} value, which was estimated at 50% inhibition.

Table 4
Recoveries of Cry1B toxin from spiked samples by ic-ELISA.

Sample	Spiked Cry1B (mg kg^{-1})	Mean recovery ± SD (%)[a]	C.V (%)
Leaf	0.2	89.5 ± 4.2	4.7
	0.5	92.1 ± 4.9	5.3
	1.0	96.4 ± 2.1	2.2
Rice	0.2	94.5 ± 3.6	3.8
	0.5	88.5 ± 3.8	4.3
	1.0	95.6 ± 2.5	2.6

[a] Data from ELISA are the means of triplicate measurements.

4. Discussion

The development of antibody engineering technology provided the tools necessary to generate novel antibodies (such as scFvs) for the detection (Christine et al., 2007; Wen et al., 2010). 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 (Dai et al., 2003; Weisser and Hall, 2009). Phage display is a reliable means for the preparation of scFvs from both immune and non-immune sources, without the restraints of the conventional hybridoma approach (Zhao et al., 2010; Deckers et al., 2009). We employed the Human single-fold scFv Libraries J (Tomlinson J libraries) to generated antibody specifically binding the antigen. After four rounds of selection, the phage bound from the first to the fourth round was 7.0×10^{-5} and 1.0×10^{-2}, respectively. The results showed a progressive enrichment of phages-scFv binding Cry1B, during the successive rounds of selection. In monoclonal phage immunoassay, 300 individual clones from the fourth round were picked at random; six positive clones were obtained and named B12, H6, H9, H11, C1 and D1. After PCR amplification and DNA sequencing, the selected clones were all identified for full length scFvs, C1 and D1 clones showed the same DNA sequence, and both were renamed as C1. The translation of the DNA sequences into amino acid sequences and subsequent protein alignment were accomplished using readily available free web based tools: (www.bioinformatics.Org/SMS/revcomp.html;ca.expasy.org/tools/dna.htmlandwww.ebi.ac.uk/Tools/clustalw2/index.html). The results showed we succeeded in selecting clones incorporating complete sequences of scFvs. Only scFv clones with complete nucleotide sequences or amino acid inserts were used for further studies.

Based on the above results, we selected the H9 and B12 positive clones for small scale production of soluble scFv antibody fragments in the non-suppressor *E. coli* HB2151 strain according to the Tomlinson library protocol. After the selected clones infected into *E. coli* HB2151 strain, each soluble scFv was expressed and characterized. SDS-PAGE analysis for these two clones show bands corresponding approximately 30 kDa molecular weight, the molecular weights of scFvs are generally indicated to be between 25 and 35 kDa. Thus, the results would imply that we succeeded in expressing the soluble scFv fragments for the Tomlinson J library clones in the *E. coli* HB2151strain. Although all six phage-scFvs bound to Cry1B, only scFv-H9 showed binding to Cry1B toxin in soluble format. It suggested this variation may be due to differences in the folding of the antigen binding sites of the phage-bound and the format of antibodies versus the soluble scFv (Tout et al., 2001; Shepelyakovskaya et al., 2011). Another explanation was the concentration was different from expressed scFv on the surface of the phages to expressed soluble scFvs (Scott et al., 2008). Following successful soluble expression and purification, we then proceeded to further test scFv-H9 in ic-ELISA, affinity and cross-reactivity ELISA tests.

Competitive immunoassay is the most common immunoassay configuration used for analysis of toxins. In ic-ELISA using the scFv-H9 showed that this antibody was able to specifically detect Cry1B, within a working range 0.05–5 $\mu g\,mL^{-1}$ Cry1B (Fig. 4). Though the IC_{50} or detection limit of the antibody in this study is less sensitive compared to commercial ELISA kit of transgenic Bt (such as Envirologix, USA). However, it is important to note that this antibody is from phage displayed library and it is not possible to directly immunize animals with toxins for obtaining the antibodies. These results indicate that the antigen-binding capacity of scFv fragments is not affected when are expressed as soluble fragment. Although scFv fragments can appear high affinities to their intended targets (Griffiths and Alexander, 1998; Griffiths et al., 1993, 1994), some studies have reported affinities lower than their corresponding monoclonal antibodies (Boulter-Bitzer et al., 2010). In this study, the scFv generally exhibited lower binding activity (about $10^7\,M^{-1}$). The apparent reason for this lower affinity of scFvs may be related to avidity in that monoclonal antibodies are divalent, while scFvs are monovalent (Yuan and Parrish, 2000), and the library we used is non-immunized (Neri et al., 2011). Cross-reactivity ELISA results indicated that the soluble and purified scFv-H9 had cross-reactivity with other Cry1 toxins (Cry1Ab, Cry1Ac and Cry1C), and showed higher cross-reactivity with Cry1C, However, these results are not completely surprising—in consideration of the high level of homology in amino acid sequences (generally 45–78%) within the subfamilies, such as Cry1A, Cry1B and Cry1C (Crickmore et al., 1998). Based on binding and competition of Bt toxins (Rang et al., 2004), Cry1B and Cry1C compete for a binding site and do not compete with Cry1Ab and Cry1Ac toxins. Moreover, some research appears that 90% of all monoclonal antibodies can recognize other antigens when screened across several antigens (Eteshola, 2010). Thus, the results obtained here would indicate the necessity to affinity maturation of scFv molecules used in error-prone PCR, DNA shuffling or other directed-evolutionary method (Crameri et al., 1996; Boulter-Bitze et al., 2010). The deduced amino acid sequences of CDRs might also help us to create other scFv fragments with even higher affinity and specificity by further mutagenesis study (Enamul et al., 2009). Matrix effect is one of the common issues of immunoassay when performed in real sample analysis. Many studies have been reported this can be reduced by sample dilution with buffers (Guertler et al., 2009; Liu et al., 2011; Wang et al., 2011). In this study, the recovery results suggested that when sample was processed by 10-fold dilution with PBS, the matrix effects were almost negligible.

In summary, phage display has been shown to be a cost-effective and valid technique for the production of antibody fragments such as scFvs (Arap, 2005). We have successfully isolated a number of novel scFv clone variants from the phage libraries with specific binding affinity to actual epitopes of Cry1B toxin. Phage display and successful application of scFvs with ic-ELISA could be a powerful approach for the detection Cry1B toxin in agricultural and environmental samples.

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