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# Established a new double antibodies sandwich enzyme-linked immunosorbent assay for detecting *Bacillus thuringiensis* (Bt) Cry1Ab toxin based single-chain variable fragments from a naïve mouse phage displayed library

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ABSTRACT

ScFvs are composed of the variable regions of the heavy and light chains via a short linker that maintain the specific antigen binding abilities of antibodies. In this study, we constructed a naïve mouse phage displayed library to generate scFvs against Cry1Ab toxin. After affinity panning, positive phage-scFvs were isolated, sequenced and characterized by ELISA. The best binding ability scFv-G9 was expressed and purified. SDS-PAGE indicated that the relative molecular mass of scFv was estimated at 28 kDa. The purified scFv-G9 was used to develop a new DAS-ELISA for detecting Cry1Ab toxin, within minimum detection limit of 0.008 μg $mL^{-1}$, a working range 0.018–6.23 μg $mL^{-1}$, and the linear curve displayed an acceptable correlation coefficient of 0.98. The cross-reactivity showed that scFv-G9 had strongly binding ability to Cry1Ac toxin, but not to Cry1B, Cry1C and Cry1F toxin. The average recoveries of Cry1Ab toxin from spiked leaf and rice samples were in the range 92.1–94.8%, and 91.6–98.6%, respectively, with a coefficient of variation (C.V) less than 5.0%. These results showed promising applications of scfv-G9 for detecting Cry1Ab toxin with new DAS-ELISA.



## 1. Introduction

*Bacillus thuringiensis* (Bt) are belong to Gram-positive spore-forming bacteria, they can produce parasporal crystal protein named Cry toxins, which are effective in killing various target insect pests by their specific pesticide activities (Bravo et al., 2007; Vachon et al., 2012). Cry toxins have their insect specificity and known as a safe insecticidal crystal protein for the control of disease-related insect vectors (Gómez et al., 2007; Frankenhuyzen, 2009). Despite the limitation of using Bt products as sprayed insecticides, many Cry toxins, such as Cry1Aa, Cry1Ab, Cry1Ac, Cry1C, Cry1F and Cry3A, have been introduced into transgenic crops providing a more targeted and effective way to control pests in agriculture (Bravo and Soberón, 2008; Bravo et al., 2011). The transgenic crops (including corn, cotton, and soybean) expressed Cry1Ab toxins, which produced by *B. thuringiensis* HD-1, are increasing exponentially worldwide and also very effective in controlling target insect pests (James, 2011). However, transgenic crops have not given worldwide acceptance because of concerns about the environmental and public health safety issues, such as potential Cry gene flow to other organisms, killing non-target insect pest, toxin or allergen production, and

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development of antibiotic resistance (Kaeppler, 2000). Aris and Leblanc (2011) even reported that they have detected the Cry1Ab toxins in maternal, fetal and non-pregnant women blood samples. The potential broad applications and risk of Cry toxins make it necessary to establish a sensitive method for detecting Cry toxins.

Currently, the detection of transgenic crops DNA/protein is routinely performed by polymerase chain reaction (PCR), real-time PCR and enzyme-linked immunosorbent assay (ELISA) (Asensio et al., 2008; Kamle and Ali, 2013). PCR (real-time PCR) is the preferred method for the identification and quantification of Cry genes because of its versatility, sensitivity and specificity (Kamle et al., 2011; Ballari et al., 2013). However, PCR techniques can not identify the level of Cry toxin protein expression, meanwhile they are time-consuming, require sophisticated instrumentation and complex to perform (Terzy et al., 2005; Randhawa et al., 2010). Thus, they are not adequate for the extensive high-throughput detecting of transgenic crops and environmental samples (Roda et al., 2006; Asensio et al., 2008). As an alternative, ELISA methods have a significant advantage of protein analysis in transgenic crops and their products. The double antibodies sandwich ELISA (DAS-ELISA) is the most commonly immunoassay used for the detection of Bt Cry toxins, where the analyte is sandwiched in between the two antibodies containing capture antibody and the detector antibody (Asensio et al., 2008). Many researches and commercially available immunoassay kits have all employed DAS-ELISA to established detection methods for various Cry toxins, such as Cry1Ac, Cry1Ab and Cry2Ab (Vázquez-Padrón et al., 2000; Roda et al., 2006; Shan et al., 2007; Giovannoli et al., 2008; Zhu et al., 2011; Kamle et al., 2013). Monoclonal antibodies (MAbs) usually as capture antibody firstly bind to analyte by high affinity and specificity. However, the conventional hybridoma technique with which it is difficult and tedious to obtain MAbs. The development of antibody engineering technology, such as phage antibody displayed technology, is an attractive alternative to hybridoma technique (Arap, 2005; Pande et al., 2010). Phage displayed technology is a system which makes it possible to generate monoclonal single chain variable fragments (scFvs) with desired binding affinity and specificities (Golchin and Aitken, 2008; Garet et al., 2010). Recently, there has been an increased interest in developing scFvs for molecular recognition, immunoassay detection, immunodiagnostics and immunotherapeutic applications (Weisser and Hall, 2009).

Gómez et al. (2001, 2006) have employed the phage displayed libraries to successfully obtain scFvs against Cry1Ab toxin involved in interaction with toxin receptors. Fernández et al. (2008) have exploited the phage-displayed peptides mimicking receptors of Cry toxin and identified the epitopes that mediate binding of Cry toxins. Our previous studies have also obtained the scFvs against Cry toxins and established the indirect competition-ELISA methods for detecting Cry toxins (Zhang et al., 2012; Wang et al., 2012). In this study, we firstly reported the selection of anti-Cry1Ab scFvs instead of MAbs as capture antibodies from a naïve mouse phage displayed library. The expressed scFvs were purified and applied in DAS-ELISA, and showed promising application in detection of Cry1Ab toxin from agricultural and environmental samples.

## 2. Material and methods

### 2.1. Chemicals

Cry toxins used in this study were all purchased from You Long Bio. Co. Ltd (Shanghai, China) and dissolved in sodium carbonate buffer (CBS, pH 9.6), stored at −20 °C。 Phagemid pCANTAB5E ($Amp^+$, SfiI, NotI restriction sites, E-tag sequence, Amber stop codon) and Anti-E-Tag affinity chromatography were purchased from Pharmacia. *Escherichia coli* strains TG1 and HB2151 for selection and production of soluble scFvs, Helper phage M13K07 ($Kana^+$) were obtained from MRC (Cambridge, England). Restriction endonucleases, T4 DNA ligase, kits for total RNA isolation system, reverse transcription system and mRNA isolation system were purchased from Promega, HRP-anti-M13 and HRP-goat-anti-rabbit antibodies were purchased from GE Healthcare (Beijing, China). Trypsin and tetramethylbenzidine (TMB) were purchased from Sigma. All other reagents used were of analytical grade.

### 2.2. Construction of naïve mouse scFvs displayed library

Total RNA was prepared from spleen tissue of the 6-week-old female Balb/c mouse using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The quality of RNA was assessed by gel electrophoresis; quantity and purity of RNA were determined by UV absorbance at 260 and 280 nm. The first strand cDNA was generated using oligo $(dT)_{15}$ and SuperScript™ III kit (Invitrogen, USA) according to the manufacturer's instructions. Primers for PCR amplification of construction scFv were designed according to the Table 1. Firstly, $V_H$ and $V_L$ were amplified by PCR reaction containing 1 μL of cDNA and 2 pmol each primer set. The samples were cycled 30 times at 94 °C 60 s, 55 °C 60 s, and 72° C 60 s. The PCR products were then purified using a gel extraction kit (QIAGEN, Valencia, CA, USA).

A sequence encoding flexible linker of 15 amino acids $(Gly4Ser)_3$ was containing the mouse $V_L$ reverse primer (MJL-R1) in the 3′ ends for generation $V_L$-linker and complementary sequence of mouse $V_H$ forward primers for construction of scFvs. These $V_L$-linker and $V_H$ fragments were combined with a $(Gly_4Ser)_3$ linker to generate scFvs by overlap extension PCR (SOE-PCR), The purified amplification scFvs were double digested by restriction enzymes SfiI and NotI, and the purified scFv products were ligated with the linearized phagemid vector pCANTAB5E which have been digested by the same restriction endonucleases. The recombinant phagemids were introduced into competent *E. coli* TG1 cells by electroporation to express phage-displayed scFv.

### 2.3. Validation of the naïve mouse scFvs displayed library

Phages antibodies for panning were prepared as described by Garet et al. (2010) with some modification. Briefly, Phages ($10^9$ cfu/mL) of the mouse library with

**Table 1**
The use of the primers for the construction of the naïve mouse phage displayed library.

| | |
|---|---|
| **Mouse $V_L$ forward primers** | |
| MJL-F1 | 5′-CCGTTTGATTTCCAGCTTGGTGCC-3′ |
| MJL-F2 | 5′-CCGTTTTATTTCCAGCTTGGTCCC-3′ |
| MJL-F4 | 5′-CCGTTTTATTTCCAACTTTGTCCC-3′ |
| MJL-F5 | 5′-CCGTTTCAGCTCCAGCTTGGTCCC-3′ |
| **Mouse $V_L$ reverse primers** | |
| MJL-R1 | 5′-GACATTGAGCTCACCCAGTCTCCA-3′ |
| **Mouse $V_H$ forward primers** | |
| MJH-F2 | 5′-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCC-3′ |
| **Mouse $V_H$ reverse primers** | |
| MJH-R2 | 5′-AGGTSMARCTGCAGSAGTCWGG-3′ |
| **Primers for Linker** | |
| 5′-GGGGCCAGGGACCACGGTCACCGTCTCCTCAGGTGGAGGCGGTTCAGGCGGAGG | |
| TGGCGGAGG GGCTCT GGCGGTGGCGGATCGGACATTGAGCTCACCCAGTCTCCA-3′ | |
| **Primers for generation of scFv genes in SOE-PCR** | |
| MJL-F1 (NotI) | |
| 5′-GAGTCATTCTGCGGCCGCCCGTTTGATTTCCAGCTTGGTGCC-3′ | |
| MJL-F2 (NotI) | |
| 5′-GAGTCATTCTGCGGCCGCCCGTTTTATTTCCAGCTTGGTCCC-3′ | |
| MJL-F4 (NotI) | |
| 5′-GAGTCATTCTGCGGCCGCCCGTTTTATTTCCAACTTTGTCCC-3′ | |
| MJL-F5 (NotI) | |
| 5′-GAGTCATTCTGCGGCCGCCCGTTTCAGCTCCAGCTTGGTCCC-3′ | |
| MJH-R2 (Sfi I) | |
| 5′-GTCCTCGCAACTGCGGCCCAGCCGGCCATGGCCCAGGTSMARCTGCAGSAGTCWGG-3′ | |
| **Primers for identified the scFv genes were cloned into the phagemid vector pCANTAB5E** | |
| R1: 5′-CCATGATTACGCCAAGCTTTGGAGCC-3′ | |
| R2: 5′- CGATCTAAAGTTTTGTCGTCTTTCC-3′ | |

S = G/C, R = G/A, M = A/C, W = A/T.

4 mL MPBS (2% (w/v) skimmed milk-PBS) were added into plastic cell culturing flasks coated with Cry1Ab toxin (the first round was 100 μg $mL^{-1}$, the remaining three rounds were 75, 50 and 25 μg $mL^{-1}$, respectively) for shaking 1 h and standing 1 h at room temperature. The binding phages were eluted with Trypsin (1 mg $mL^{-1}$) for 30 min with end-over-end mixing at room temperature. The eluted phages were used to infect fresh exponentially growing culture of *E. coli* TG1 cells ($OD_{600}$ = 0.4) and incubated at 37 °C in a water bath (without shaking) for 30 min to allow optimal infection. Following M13K07 helper phage rescue, phage particles were amplified for further rounds of panning.

We picked randomly individual colonies from the fourth round and inoculated in 100 μL of 2 × TY containing 100 μg $mL^{-1}$ ampicillin and 1% glucose in a 96-well culture plate, grown shaking (250 rpm) overnight at 37 °C. 2 μL each culture from plates was grown in a new plate of 2 × TY (200 μL) with ampicillin and 1% glucose shaking until O.D. 600 of 0.4. Each culture was infected with helper phage. The plate was centrifuged for 15 min at 1800 *g* and 4 °C, and the binding of individual phage supernatant (100 μL) to Cry1Ab and to MPBS alone was then determined by monoclonal phage-ELISA as described (Zhang et al., 2012).

### 2.4. Colony PCR and sequencing

The selected phage particles were infected into *E. coli* HB2151 non-suppressor strain and grown with shaking for 2 h at 37 °C. Colony PCR (94 °C 10 min, then 94 °C 1 min, 55 °C 1 min, 72 °C 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 (about 1000 bp). The PCR products were examined by electrophoresis on 1% agarose gel. The single-stranded DNAs of the chosen monoclonal phages were sequenced by Invitrogen (Shanghai, China). Two sequencing primers were used by pCANTAB5E-R1 and pCANTAB5E-R2. Readily available web based tools were used for translation and alignment of DNA sequences.

### 2.5. Expression and purification of soluble scFv-G9

The full length and well sequenced clones (G9) were picked into 50 mL 2 × TY with 100 μg $mL^{-1}$ ampicillin and 1% glucose to an optical density (OD) of 1.0 at 600 nm. Expression of the scFvs was induced by adding of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and cultures were further grown with 12 h at 30 °C. After centrifuged at 3300 *g* for 30 min, the supernatant was clarified by filtration through disposable 0.22 μm filter and stored in 4 °C. To obtain soluble protein from the periplasm, the 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 soluble scFvs were collected. The control group was empty HB2151 as the same treatments (Rouet et al., 2012; Zhang et al., 2012). The products were analyzed by 12% SDS-PAGE with Silver Straining.

Periplasmic soluble scFv-G9 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 supernatant was collected and applied to Anti-E-Tag affinity chromatography according to the manufacturer instructions. The purity of the eluted fractions was checked by 12% SDS–PAGE.

### 2.6. Optimization of the antibodies and establishment the immunoassay

The DAS-ELISA conditions, including the concentration of scFv and PAbs, were optimized according to the checkerboard titration method (Lorenzen et al., 1990). After optimized the concentrations of the scFv-G9 and anti-Cry1Ab rabbit PAbs, the new DAS-ELISA for detecting Cry1Ab toxin was established using the method described previously (Roda et al., 2006). The limit of detection for the assay was calculated as the mean of the blank control values plus five times its standard deviations (Kumar 2011), and the working range was calculated also by Roda et al. (2006). The data represent mean ± standard deviation from triplicate measurements.

### 2.7. Assessed the ELISA by spiked samples

The DAS-ELISA method was used for Cry1Ab toxins determination in leaf samples and ground rice samples. 1 g of leaf samples or ground rice samples were spiked Cry1Ab toxins at three concentration levels (0.05, 0.1 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 DAS-ELISA. Standards and blanks (free toxin samples) were used in all cases. The data represent mean ± standard deviation from triplicate measurements.

### 2.8. Cross-reactivity ELISA

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-G9, 2 μg $mL^{-1}$ different Cry1 toxins (Cry1Ab, Cry1Ac, Cry1B, Cry1C and Cry1F) in CBS buffer were coated, blocked with 200 μL per well of 3% MPBS. After washing with PBST, the purified scFv-G9 (10 μg $mL^{-1}$) was incubated 2 h at room temperature. The plates were added HRP-anti-E tag MAbs and incubated at room temperature for 1 h. After added tetramethylbenzidine (TMB) substrate solution, color development was performed for

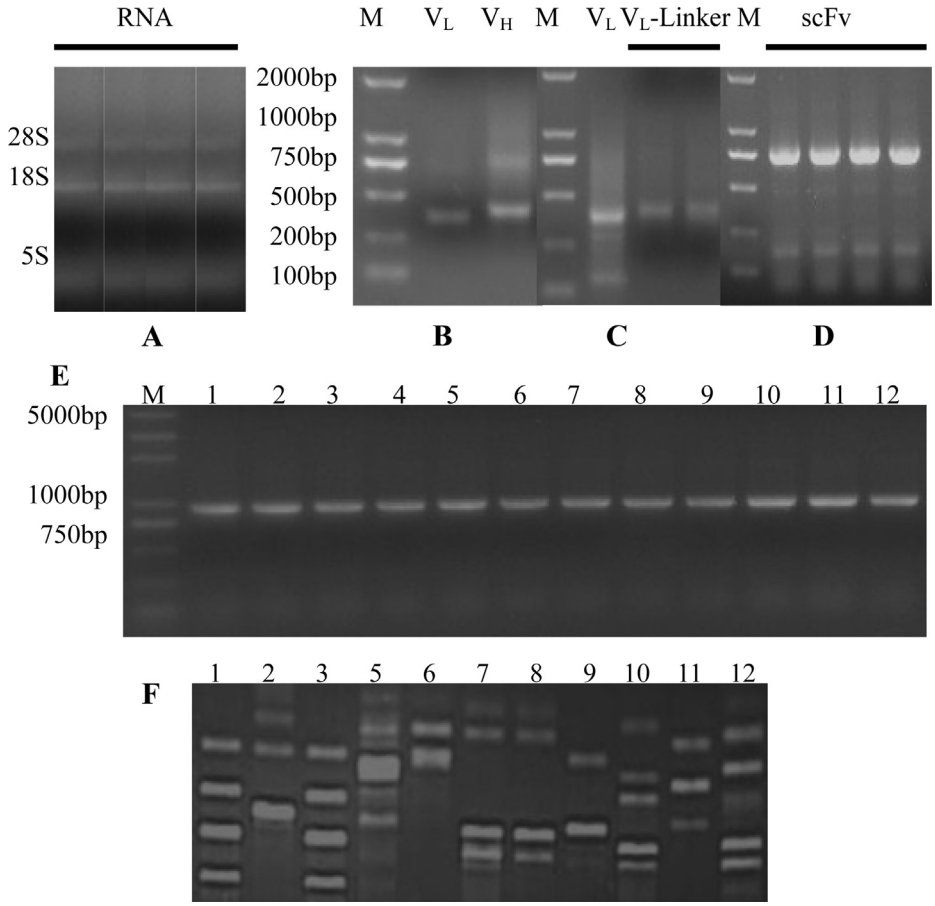


**Fig. 1.** Construction and identification of naïve mouse scFvs displayed library. (A). Total RNA extraction from spleen tissue of mouse using TRIzol Reagent. (B). The amplified variable regions of heavy ($V_H$) and light-chains ($V_L$) from mouse spleen cells. (C). The primers for linker and $V_L$ forward primers were used to generate $V_L$-linker by the templates of $V_L$ (D) These $V_L$-linker and $V_H$ fragments were combined with a $(Gly_4Ser)_3$ linker to generate scFvs by overlap extension PCR (SOE-PCR). (E). Randomly picked 12 phage clones from the plate were amplified by PCR using pCANTAB5-R1 and pCANTAB5-R2 primers. (F). The amplified products were digested with *Bst*NI at 60 °C for 4 h. The restriction patterns of samples were analyzed by agarose gels.

10 min at room temperature and stopped with 50 mL/well 2 M sulfuric acid. $OD_{450}$ was measured by automatic microplate reader (Thermo, USA). All analyses were represented mean ± standard deviation from triplicate measurements.

## 3. Results

### 3.1. Amplification of the $V_L$ and $V_H$ fragments for generation of scFvs

The total RNA was extracted successfully from mouse spleen cells (Fig. 1(A)). In this study, the $V_L$ and $V_H$ fragments were amplified clearly at about 340 bp and 400 bp by PCR employing the cDNA as the template (Fig. 1(B)). Furthermore the amplified products of the $V_L$-linker and $V_H$ were combined by the 15 amino acid flexible linker, using splicing, by SOE-PCR, resulting in the scFv gene repertoire (Fig. 1(C, D)).

### 3.2. Construction of mouse phage displayed library

Transformation of the *E. coli* TG1 with the recombinant phagemid yielded a library of $4 \times 10^8$ clones. PCR results showed that all the picked 12 clones were all about 1000 bp as expected of the scFv gene together with a part of the pCANTAB5E backbone (Fig. 1(E)). ScFv genes were amplified and digested with *Bst*NI, the results indicated that we succeeded in establishing a naïve mouse phage displayed library with excellent diversity (Fig. 1(F)).

### 3.3. Validation of the mouse scFv library

In these experiments, input and output titers from each round are shown in (Fig. 2(A)). Approximately 10000-fold increase in output was obtained after four rounds of panning. 200 phage clones randomly picked from the 4th round for monoclonal ELISA, 9 clones (named D4, B6, E8, G8, G9, H9, C10, B11 and H11) showed positive binding to Cry1Ab (Positive clones' absorptive values were more than 2.1 times of the negative ones, P/N > 2.1) (Fig. 2(B)). ScFv-G9 given the great binding ability to Cry1Ab, therefore, was selected for further analysis.

### 3.4. Colony PCR and sequencing analysis

The selected clones were all identified for full length scFv (Fig. 3(A)). Then, phage clones picked for sequencing,

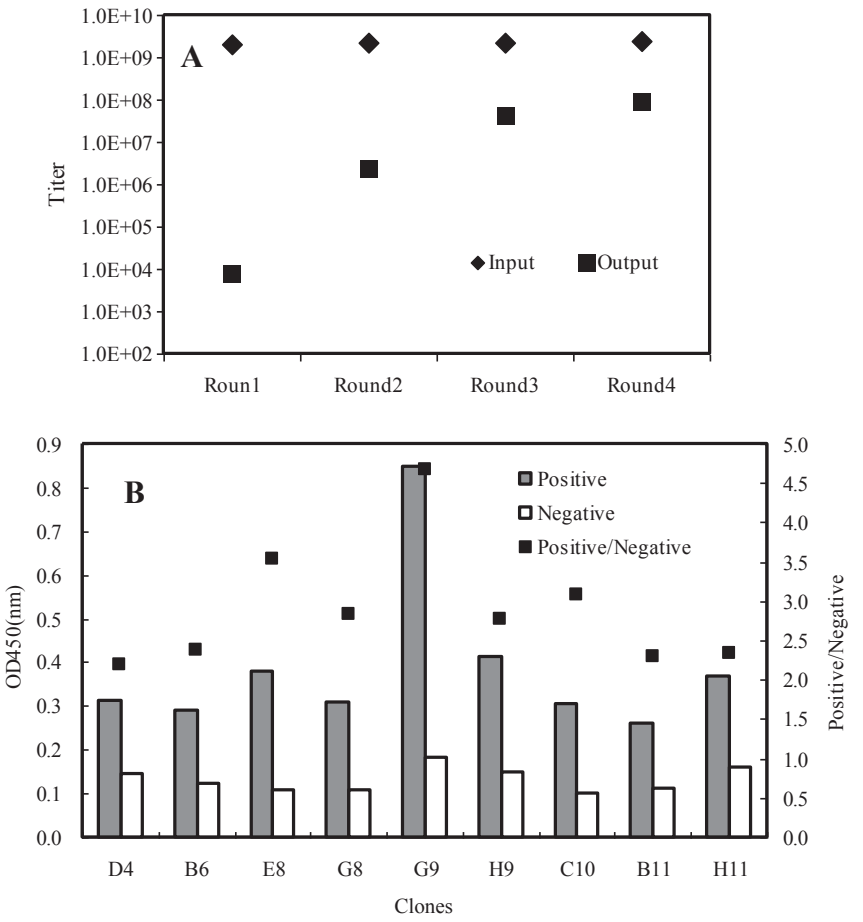


**Fig. 2.** Recovery of phages during selection against Cry1Ab (A) and monoclonal phage ELISA against Cry1Ab (B).

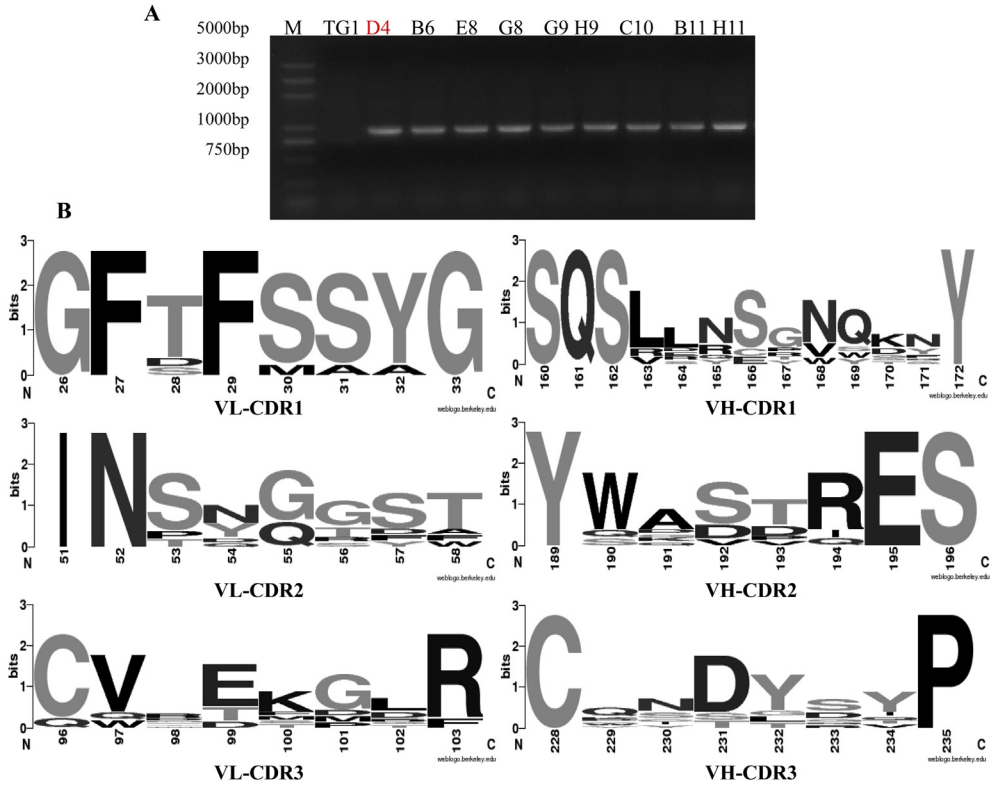


**Fig. 3.** PCR identification (A) and sequences analysis of the chosen clones (B). The sequence Logo of 9 positive clones were selected from CDR1-3 region of $V_L$ and $V_H$, respectively are shown in this figure.

translation and analysis by WebLogo (Crooks et al., 2004). Fig. 3(B) showed amino acid sequences deduced from the obtained nucleotide data. In the CDR area, all sequences had different amino acid substitutions that were expected and mostly were in complementarity determining regions (CDR). In the $V_H$ and $V_L$ domain, amino acid sequences of CDR-2 and CDR-3 areas showed greatest differences as the hypervariable regions.

### 3.5. Expression and purification of scFv-G9

We chose the scFv-G9 to produce the soluble scFv antibody fragments in *E. coli* HB2151 strain under the optimized condition. After expression and purification, SDS-PAGE analysis for scFv-G9 showed we successfully expressed and purified a band corresponding approximately to 28 kD molecular weight (Fig. 4).

### 3.6. Optimization of the antibodies and establishment the immunoassay method

The optimal concentration of the scFv-G9 and anti-Cry1Ab rabbit PAbs was found to be 10 μg $mL^{-1}$ and 2 μg $mL^{-1}$, respectively (Fig. 5(A, B)). The established sandwich antibodies immunoassays method showed that scFv-G9 was able to specifically capture Cry1Ab, within minimum detection limit of 0.008 μg $mL^{-1}$, a working range 0.018–6.23 μg $mL^{-1}$ for Cry1Ab, and the linear curve displayed an acceptable correlation coefficient of 0.98 (Fig. 5(C)).

### 3.7. Assessed the ELISA by spiked samples

The measured recoveries of Cry1Ab from the spiked samples are given in Table 2. The average recoveries ranged

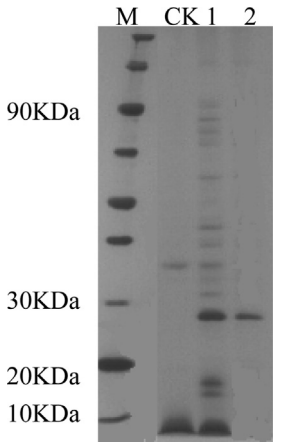


**Fig. 4.** Analysis of scFv-G9 soluble expression and purification in *E. coli* HB2151 by SDS–PAGE. (M: A protein mass marker served as a standard; CK: non-induced HB2151 as control; 1: Crude periplasmic extract; 2: purified scFv-G9 by Anti-E-Tag affinity chromatography).

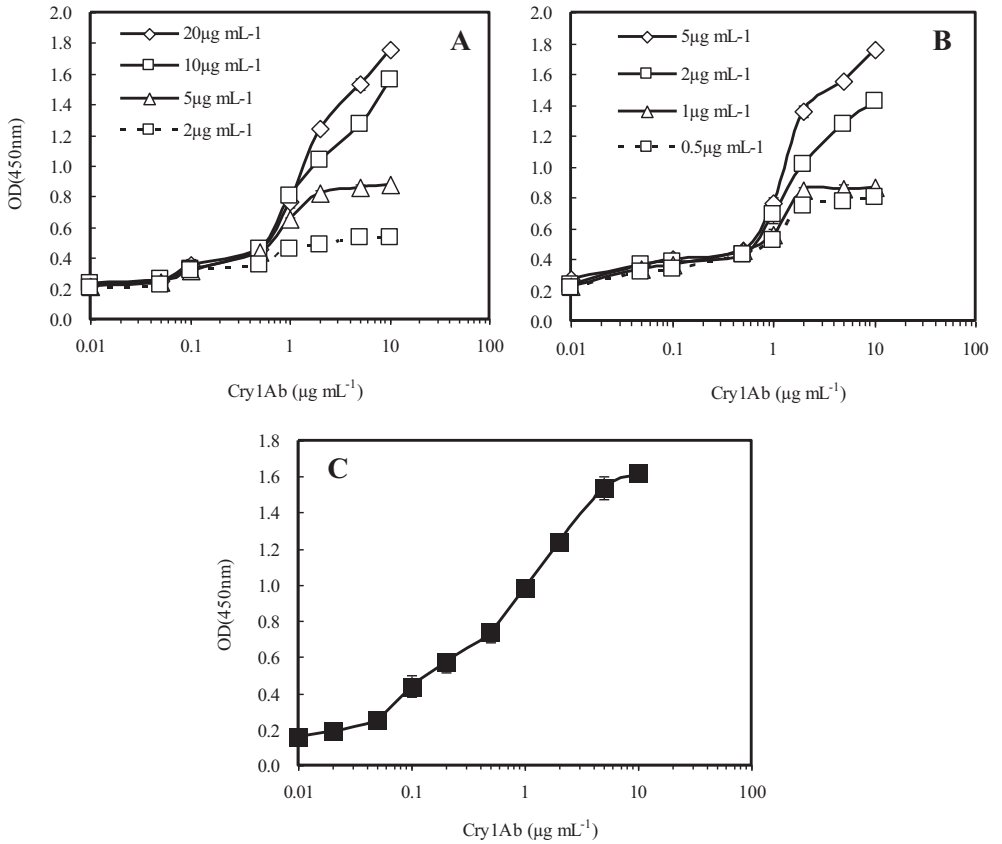


**Fig. 5.** Optimization of the concentration antibodies (including A: capture antibody: scFv-G9 and B: detection antibody: anti-Cry1Ab PAbs) and establishment double sandwich ELISA based on scFv for detection of Cry1Ab toxin (C). The data represent mean ± standard deviation from triplicate measurements.

from 91.6% to 99.2%, with a coefficient of variation (C.V) less than 5.0%, showing good accuracy for the quantitative detection of Cry1Ab toxin in agricultural and environmental samples.

### 3.8. Cross-reactivity ELISA

The cross-reactivity of scFv-G9 to other Cry1 toxins was examined, five OD values obtained with the scFv-G9 to Cry1Ab, Cry1Ac, Cry1B, Cry1C and Cry1F by sandwich antibodies ELISA (Fig. 6). The cross-reactivity showed that scFv-G9 had strongly binding ability to Cry1Ac toxin, but not to Cry1B, Cry1C and Cry1F toxin.

**Table 2**
Recoveries of Cry1Ab toxin from spiked samples by DAS-ELISA.

| Sample | Spiked Cry1Ab (mg kg$^{-1}$) | Mean recovery ± SD (%)[a] | C.V (%) |
|---|---|---|---|
| Leaf | 0.05 | 99.2 ± 3.2 | 3.2 |
| | 0.1 | 92.1 ± 2.5 | 2.7 |
| | 1.0 | 94.5 ± 3.2 | 3.4 |
| Rice | 0.05 | 98.6 ± 2.7 | 2.8 |
| | 0.1 | 91.6 ± 4.1 | 4.5 |
| | 1.0 | 92.4 ±3.4 | 3.7 |

[a] The data are the means of triplicate measurements.

## 4. Discussion

Phage displayed library commonly consisted by scFvs, fused to the phage minor coat protein gene III and displayed on the surface of filamentous phage, without requiring the immunization animals and classic hybridoma techniques (Smith, 1985; Better et al., 1988; McCafferty

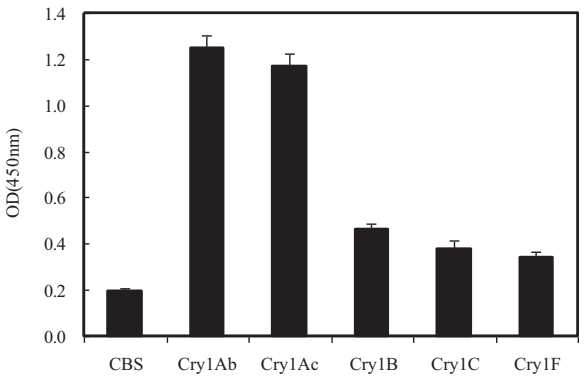


**Fig. 6.** Cross-reaction of soluble and purified scFv-G9 in DAS-ELISA with selected Cry1 toxins. The data represent mean ± standard deviation from triplicate measurements.

et al., 1990; Vaughan et al., 1996). Recently, construction of naïve mouse libraries was reported as described elsewhere (Okamoto et al., 2004; Imai et al., 2006; Sepulveda and Shoemaker, 2008; Sommavilla et al., 2010; Sun et al., 2012). However, naïve mouse libraries usually face the problems of the low library capacity and efficiency of scFv gene diversities (Kawamura et al., 2006; Sepulveda and Shoemaker, 2008). In this study, we described the design, construction and characterization of naïve mouse antibody phage display libraries, the results showed that we have successfully constructed a naïve mouse library with about $10^8$ clones and indicated excellent scFv gene diversities in this library.

To ascertain the quality of the naïve mouse library, we employed the library to generated antibody specifically binding to the Cry1Ab toxins. After four rounds of selection, the phage bound (output/input) was increased by $10^4$ times from the first to the fourth round. The results showed a progressive enrichment of phages-scFv binding Cry1Ab, during the successive rounds of selection. In monoclonal phage immunoassay, we randomly picked 200 individual clones from the fourth round and finally obtained nine positive clones, named D4, B6, E8, G8, G9, H9, C10, B11 and H11, on of them, G9 has the relative high binding ability. After PCR amplification and DNA sequencing, the selected clones were all identified for full length scFvs with about 1000 bp. The translation of the DNA sequences into amino acid sequences and subsequent amino acid sequences alignment by WebLogo were accomplished using readily available free web based tools: (http://web.expasy.org/translate/; http://weblogo. berkeley.edu/logo.cgi). The results showed we succeeded in selecting clones incorporating complete sequences of scFvs. Amino acid sequences of CDR-2 and CDR-3 areas showed greatest differences as the hypervariable regions which indicated probably the most important to involved binding to Cry1Ab toxins.

We used the best binding ability clone scFv-G9 for small scale production of soluble scFv in the non-suppressor *E. coli* HB2151 strain as the same method reported in our previous study. Customarily, three factors including induction times, IPTG concentration and temperature, had the greatest effects on antibodies production (Shi et al., 2003; Choi and Lee, 2004; Hust et al., 2009). Under the optimizing expression condition, the results indicated that we succeeded in expressing the soluble scFv fragments from the naïve mouse library clones in the *E. coli* HB2151 strain. Following successful soluble expression and purification, we then preceded scFv-G9 to further test in establishment the DAS-ELISA, cross-reactivity tests and spiked assay.

The optimal concentrations of the anti-Cry1Ab capture antibody (scFv-G9) and anti-Cry1Ab detection antibody (PAbs) were determined. The scFv-G9 concentration (10 μg $mL^{-1}$) giving a good linear calibration was selected as the optimal concentration. The same procedure was employed for the optimization of the anti-Cry1Ab detection antibody and determined the optimal concentrations of PAbs was 2 μg $mL^{-1}$. Though the detection limit and working range of the established DAS-ELISA method in this study are less sensitive compared to standard DAS-ELISA and commercial ELISA kit of transgenic Bt (such as EnviroLogix Quantiplate® Kit, USA) (Roda et al., 2006; Paul et al., 2008; Ramirez-Romero et al., 2008). However, it is important to note that scFv from phage displayed library without hybridoma technique, maintains the specific affinity to the antigen and can be produced in large scale. Compared with our previous studies about using the scFv to establish indirect-competitive immunoassay method (Zhang et al., 2012; Wang et al., 2012), DAS-ELISA is known to be superior to competitive methods in terms of sensitivity and working range (Schlick et al., 1999; Zhang et al., 2010). Cross-reactivity ELISA results indicated that the soluble and purified scFv-G9 had strongly binding to Cry1Ac toxins, it was the same report in other studies and indeed, Cry1Ab immunoassay kits are usually commercialized as Cry1Ab and Cry1Ac simultaneous assays (Roda et al., 2006). It suggested that Cry1Ab and Cry1Ac have highly homologous sequences and significant similar three-dimensional structure (Crickmore et al., 1998). When performed in real sample analysis by immunoassay, matrix effect that may cause false positive or negative results should be eliminated (Guertler et al., 2009). For this reason, we processed samples by 10-fold dilution with PBS; the matrix effects were almost negligible.

Although, we successfully selected scFv against Cry1Ab toxin from the naïve mouse display library and firstly established a novel DAS-ELISA method based scFv insteading of MAbs as capture antibody. However, the poor stability and lower affinity of scFv may be an important factor of restricting its in practical application. We can reasonably improve the stability and affinity in further study with specificity sequences of scFvs by using antibody engineering and library technology (Woern and Pluecthun, 2001; Wark and Hudson, 2006). In conclusion, it was demonstrated that novel DAS-ELISA method based scFv can be useful for the detection and quantification of Cry1Ab protein.

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## Conflict of interest

We declared that we have no conflict of interest.

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