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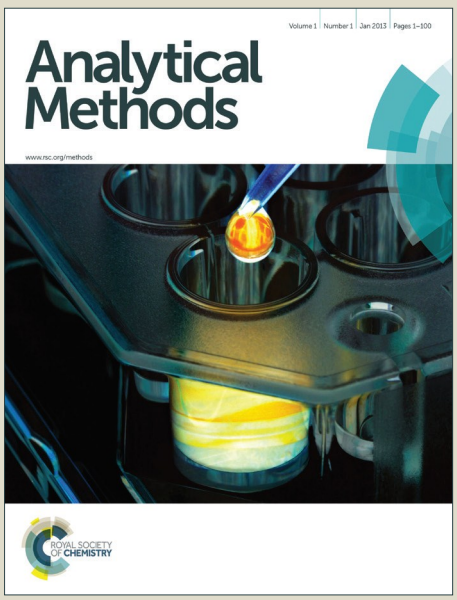

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Production and Characterization of a Single-Chain Fab Fragment for the Detection of O,O-Diethyl Organophosphorus Pesticides

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Abstract

A broad-specificity, single-chain antigen-binding fragment (scFab) for the detection of O,O-diethyl organophosphorus pesticides (DPPs) was produced and characterized. The recombinant plasmid encoding an Fab with broad specificity towards a specific class of DPPs was used as a template for amplification of the antibody heavy (Fd) and light (κ) chain genes, which were then cloned in two different orientations with the $(Gly_4Ser)_3$ linker. **SDS-PAGE and western blotting were used to identify expression of the scFabs. Indirect competitive ELISA (icELISA) was used to test the immunology activity of two different κ/Fd orientations.** The optimal scFab orientation was determined by characterizing the expression, specificity, and stability in comparison with their homologous scFv and Fab. The 50% inhibition of binding (IC_{50}) values of the *κ*-linker-Fd scFab for coumaphos and parathion determined with icELISA were ~1.5 ng/mL and 3.1 ng/mL, respectively, surpassing that of the reverse scFab orientation, Fd-linker-*κ* (3.7 μg/mL and 8.5 μg/mL, respectively). The IC_{50} of the *κ*-linker-Fd scFab was also similar to that for Fab and was relatively low in comparison with scFv. **Concentration of scFab in expression extract against the antigen** was consistent with that of scFv, while Fab displayed lower in comparison with both scFv and scFab. After incubation for 9 days, scFab and Fab still exhibited high antigen-binding activity while scFv showed almost no remaining affinity. Analysis of DPP-spiked vegetable samples demonstrated that scFab-based detection using icELISA and gas chromatography by the QuEChERS approach yielded good sensitivity and reproducibility for monitoring food safety.

1 Introduction

Organophosphorus pesticides (OPs) are a class of cholinesterase-inhibiting insecticides typified by effectiveness, broad-spectrum insecticidal activity, and lower cost, and are hence widely applied in both agricultural and domestic applications.[1,2] O,O-diethyl organophosphorus pesticides (DPPs), one of the principal sub-classes of the OPs, are widely used in agriculture. However, especially in developing countries, misuse and overuse of DPPs has frequently led to the pollution of the environment and the contamination of food and drugs with DPP residues, causing numerous poisonings of non-target species and significant numbers of human fatalities.[3] The sensitive detection of DPP residue in agricultural products and environmental samples is therefore critical. Over the years, immunoassays based on the highly specific antigen-antibody reaction have been used as a rapid screening method to detect residues of pesticides, veterinary drugs, and poisons in agricultural products; these applications have already proven the exceptional advantages of this approach.[4-7]

Recombinant antibodies are essential tools for detection, diagnostics, research, and therapy.[8, 9] This approach mainly involves the use of the single-chain variable fragment (scFv) or antigen-binding fragment (Fab) of the parent antibody. Many scFvs have demonstrated reduced affinity towards the antigen relative to their homologous Fab fragments.[10] In long-term storage, scFv fragments also display relatively low stability in comparison with the Fab fragments.[11] However, Fab expression in the periplasm of *Escherichia coli* is less efficient than for scFv. These observations are attributable to Fab being secreted into the periplasm of *E. coli*, whereas scFv remains in the cytoplasm. This is because Fab is assembled from two different polypeptides and is double the molecular size of scFv, resulting in its secretion into the periplasm where the correct inter-chain disulfide bond connections can be formed.[12] Furthermore, the Fab light chains tend to

form homodimers[13-16]. Therefore, both scFv and Fab recombinant antibodies face certain challenges in their application, which also pose major obstacles to their commercialization as diagnostic tools. Ideally, recombinant antibody fragments need to exhibit high affinity, be suitable for expression as a soluble protein in *E. coli*, and be stable in long-term storage. Recently, a novel recombinant antibody format was reported, namely the single-chain Fab fragment (scFab), which integrates properties of both Fab and scFv and with similar antigen affinities. scFab also has the potential for increased expression yield of functional antibody fragments in both *E. coli* and *Pichia pastoris* expression systems.[17-20] In addition, the linker length between the κ carboxy terminus and the amino terminal end of the Fd fragment can be manipulated to influence the yield of soluble antibody fragment production.[21, 22]

In the present study, a recombinant antibody comprising a flexible linker between the Fd and κ fragments in the format of a scFab was developed with improved affinity towards target antigens and with long-term stability in storage (Fig. 1). We tested the effect of two different κ/Fd orientations by comparing immunology activity of each with indirect competitive ELISA (icELISA). The optimal scFab orientation was determined by characterizing the expression level, immunological sensitivity and specificity in comparison with their homologous scFv and Fab. To evaluate the stability of optimal scFab, similar measurements were carried out following incubation at 37°C and compared with those for the homologous scFv and Fab. In addition, to validate the broad-specificity scFab-based icELISA, we challenged our assay with DPP-spiked real-world samples followed by QuEChERS analysis (a streamlined approach for the analysis of pesticide residues in food).[23, 24] These immunoassay results were confirmed by comparison with gas chromatography (GC) data.

2 Materials and methods

2.1 Materials and Reagents

In previous work, a Fab gene against O,O-diethyl organophosphorus was derived from a hybridoma cell line (12C2) secreting monoclonal antibodies (MAb). The Fd and *κ* chains of this Fab were then cloned into the pComb3XSS vector and evaluated with phage ELISA **(accession No.JQ692136 for light chain and No.JQ692137 for heavy chain)**. Functional scFv against O,O-diethyl organophosphorus were previously obtained and the coating antigen (4-(diethoxyphosphorothioyloxy)benzoic acid-ovalbumin conjugate, hapten 1-OVA) was also previously synthesized.[25] The pComb3XSS vector used for soluble protein expression and the TOP10F′ *E. coli* cells used for the production of scFv, Fab, and scFab were stored in our laboratory.

Pesticide standards were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The plasmid extraction kit and PCR product purification kit were supplied by Tiangen Biotech Co. (Beijing, China). The restriction enzymes, *Sac*I and *Spe*I, and the *Pfu* DNA polymerase were purchased from Thermo Fisher Scientific (Shanghai, China) and TransGen Biotech Co., Ltd. (Beijing, China), respectively. **Anti-His tag antibody** was purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse Fab antibody was obtained from Abcam (Shanghai, China). Primary-secondary amine (PSA), Pesticarb (PC), and C18 sorbents were purchased from Agela Technologies Co. (Guangzhou, China).

2.2 Optimization of various κ/Fd scFab orientations

2.2.1 Construction of the pComb3XSS-scFab vector

The recombinant plasmid encoding Fab (pComb3XSS-Fab) was used as a template for the amplification of the antibody Fd and *κ* chains using primers containing restriction enzyme sites and a portion of the $(Gly_4Ser)_3$ linker. scFab in two different Fd/*κ* orientations were assembled by overlap extension PCR using the $(Gly_4Ser)_3$ linker sequence. The assembled *κ*-linker-Fd and Fd-linker-*κ* scFabs were digested with restriction enzymes *Sac*I and *Spe*I, respectively, and ligated with pComb3XSS digested with the same *Sac*I or *Spe*I enzyme. The ligation products were separately transformed into *E. coli* TOP10F′ cells and plated onto LB-agar plates containing 100 μg/mL ampicillin, and incubated for 12 h at 37°C. The resulting colonies were confirmed with sequencing.

2.2.2 Expression of the two κ/Fd scFabs

The recombinant colonies were incubated for 12 h at 37°C, a single colony was picked for each variant and cultured at 37°C in 2× yeast extract and tryptone medium (containing 100 μg/mL ampicillin) until the OD_{600} reached 0.5–0.8. The protein expression of each recombinant was induced by the addition of IPTG to a final concentration of 0.1 mM and cultured at 28°C overnight with stirring (200 rpm). After centrifugation at 10,000 × *g* for 10 min at 4°C, the pellets were resuspended in a sucrose solution (20% sucrose and 1 mM ethylenediaminetetraacetic acid in 0.3 M Tris-HCl, pH 8.0) and the periplasmic scFab protein was extracted using cold osmotic shock. To confirm the target protein expression, the periplasmic extractions were subjected to SDS-PAGE and western blotting with antibodies against the His-tag.

2.2.3 Characterization of the scFabs with icELISA

The two different orientations of the expressed scFab (*κ*-linker-Fd and Fd-linker-*κ*) were compared with icELISA. To determine the sensitivity of each *κ*/Fd orientation, a 96-well

microplate was coated (100 μL/well) with 1 μg/mL coating antigen (H_1-OVA) in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) at 37°C overnight. The plate was washed four times with PBST (0.05% Tween-20 in 136.9 mM NaCl, 7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 2.7 mM KCl, pH 7.4) and blocked with 120 μL per well of blocking buffer (5% (w/v) skim milk powder in PBST) for 3 h at 37°C. Then, 50 μL of serially diluted organophosphorus pesticide standards or samples in PBS (136.9 mM NaCl, 7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 2.7 mM KCl, pH 7.4) were added to each well and incubated with **50 μL periplasmic extractions of scFabs (1.27 mg/mL) appropriately diluted with water.** After incubation for 1 h at 37°C, the plates were washed as described above. HRP-labeled anti-Fab mouse MAb diluted 1:5,000 in PBST (100 μL) was added to each well for 30 min at 37°C. The wells were then washed and **100 μL of HRP substrate solution (200 mg/mL TMB, 4 μL 30% H_2O_2) was added.** The reaction was stopped by the addition of 50 μL of 2% H_2SO_4. The absorbance of the solution in each well was measured at 450 nm using a multi-label microplate reader.

2.4 Characterization of the scFab

The expression, specificity, and stability of the optimal scFab orientation were compared with that of their homologous scFv and Fab.

2.4.1 Expression of the scFabs

To compare the relative expression yields of the optimal scFab, we maintained the same expression protocol for *κ*-linker-Fd scFab as well as for the scFv and Fab (see Section 2.2.2). The periplasmic recombinant antibody proteins (scFab, scFv, and Fab) were extracted with cold osmotic shock .The extracts of scFab, scFv, and Fab were subjected to western blotting with antibodies against the His-tag and **reactivity against coating antigen with icELISA** for

quantification of their expression levels.

2.4.2 Sensitivity and specificity of the scFabs

The sensitivity and specificity of the optimal scFab was evaluated with icELISA and compared with that of scFv and Fab using a series of O,O-diethyl organophosphorus pesticides according to the above icELISA steps (Section 2.2.3). The cross-reactivity (CR) values were determined with the following equation: CR (%) = [IC_{50} (hapten)/IC_{50} (cross-reactant)] × 100.

2.4.3 Stability of the scFabs

Stability measurements were carried out by incubating **the antibody extracts of scFab (6.34 mg/mL) and its homologous scFv (6.36 mg/mL) and concentrated Fab (6.47 mg/mL) in PBS at 37°C for 9 days. Concentrated of Fab was due to consistent reactivity against coating antigen of recombinant proteins.** The reactivity against coating antigen and sensitivity of the incubated antibodies was assayed once per day with icELISA (as described above).The maximal aborbance at 450 nm and IC_{50} of the incubated antibodies were detected.

2.5 Sample preparation

Three vegetable samples including Chinese cabbage, cucumber, and lettuce were acquired from a local market, and were confirmed to be OP free by GC analysis. The three samples were extracted following the QuEChERS protocol, and were subsequently spiked with three different concentrations of various OPs.[20, 21] Briefly, 15 g of a finely chopped vegetable sample was mixed with 1.5 g of sodium acetate and 6 g of anhydrous magnesium sulfate in 15 mL of acetonitrile containing 1% (v/v) acetic acid. The mixture was vigorously stirred and the organic phase was separated by centrifugation at 2,200 × *g* for 5 min. Aliquots (2 mL) of extract for each vegetable sample were placed into 15-mL tubes, to which 1,500 mg of anhydrous $MgSO_4$ and 200 mg each

of PSA, C_{18}, and PC were added for dSPE (dispersive solid-phase extraction) cleanup. After a second centrifugation step, clean extracts were evaporated with a stream of nitrogen gas at 40°C. The residues were washed with 1 mL of methanol, and the eluents were again evaporated with a stream of nitrogen gas at 40°C. The residues were dissolved with 5 mL of PBS (0.02 M, pH 6.2) containing 5% methanol and then subjected to analysis.

2.6 Evaluation of scFab-based icELISA assay with spiked samples

The dissolved solutions were analyzed directly with icELISA. The percentage of recovery was calculated as follows: Recovery (%) = (quantity measured/quantity spiked) × 100. It must be noted that the OP matrices effects this determination; hence each sample was run according to the procedure described above using pure extract to dilute the OP. For validation of this assay, the results from the icELISA were compared with the results obtained with a gas chromatograph with a flame photometric detector (GC-FPD; Agilent 6890N, Santa Clara, CA, USA), compiled by the Zhongshan Quality Supervision and Inspection Institute of Agricultural Products (Zhongshan, Guangdong, China). A 1-μL aliquot of each sample was injected into the GC system with an injector temperature of 250°C. Chromatographic separation was performed on an Agilent HP-5 column (30 m × 0.32 mm × 0.25 μm) at a constant helium flow rate of 3 mL/min. The column temperature started at 80°C, which was increased at a rate of 200°C/min until the temperature of 260°C was reached, at which point the temperature was maintained for an additional 3 min. The samples were determined with FPD with a detector temperature of 250°C.

3 Results and Discussion

3.1 Optimization of various κ/Fd scFab orientations

The two genes encoding Fd and κ were amplified by PCR. The Fd gene was ~675 bp in length while the κ gene was ~645 bp as measured with agarose gel electrophoresis (Fig. 2, lanes 1–4). The Fd and κ fragments including the linker overhangs were joined in two different orientations (*κ*-linker-Fd and Fd-linker-*κ*) with splicing overlap extension PCR to generate two scFab genes with a size of ~1365 bp each (Fig. 2, lanes 5 and 6).

After induction, the periplasmic proteins were extracted with cold osmotic shock and confirmed with SDS-PAGE and western blotting. The molecular weight of the recombinant scFabs was predicted to be ~50 kDa according to their amino acid sequences (Fig. 3A, lanes 1 and 2), which agreed with the western blot results (Fig. 3B, lanes 1and 2). This band was not observed in the induced periplasmic protein extract from *E. coli* TOP10F′ cells containing non-recombined pComb3XSS plasmid only. These results demonstrated that the scFabs were successfully expressed.

An icELISA based on anti-Fab-HRP antibody was developed to compare the sensitivities of the two different Fd/*κ* orientations towards the O,O-diethyl OPs. Several parameters for icELISA for O,O-diethyl OPs were optimized, including the concentration of the OPS-OVA coating, the concentration of scFab, and the competition time. The results suggested that the scFab in the Fd-linker-*κ* orientation had low affinity toward free coumaphos and parathion while the scFab in the *κ*-linker-Fd orientation exhibited much higher affinity (Fig. 4). Second, the 50% inhibition of binding (IC_{50}) values of the *κ*-linker-Fd scFab for coumaphos and parathion were determined by icELISA to be ~1.5 ng/mL and 3.1 ng/mL, respectively, clearly surpassing that of the Fd-linker-*κ* scFab (~3.7 μg /mL and 8.5 μg /mL, respectively). **Third, the IC_{50} values of the κ-linker-Fd scFab for other free DPPs were determined by icELISA to be shown in Table 1. Other free**

DPPs cannot bind the Fd-linker-κ scFab, even when additional DPPs were present at high concentrations of 1 mg/mL. The IC_{50} values of Fd-linker-κ scFab with other DPPs were not obtained. Therefore, the κ-linker-Fd scFab orientation showed greater sensitivity when compared with the Fd-linker-κ scFab. It is possible that antigen-facing Euclidean distances across the antibody complementarity-determining regions were affected by switching the orientation of κ and Fd. In other words, different orientations of scFab may distort the native conformation of these regions.[26,27] Although several previous reports have suggested that the domain orientation may have significant effects on antigen-binding activity, no systematic studies have thus far been performed test this hypothesis.

3.2 Comparision of scFab, scFv, and Fab expression

To study the expression of scFab, periplasmic protein extracts of *E. coli*-expressed scFab, scFv, and scFab were obtained with cold osmotic shock and confirmed with western blotting and reactivity against coating antigen. **No bands were apparent for Fab in the periplasmic extract (Fig. 5, lane 1). To confirm whether Fab was expressed, albeit at a low level, the periplasmic extract was concentrated 4 times and subjected again to western blotting. A distinct band of ~23 kDa was then visible (Fig. 5, lane 2). However, a band of ~25kDa was detected for scFv of the periplasmic extract (Fig. 5, lane 3) and a band of ~50kDa was detected for scFab in the periplasmic extract (Fig. 5, lane 4) .For preparation of samples in western blotting, samples need to be heated 10min in the boiling water. In this condition, disulfide bond of Fab was cracked at high temperature and was changed into free Fd fragment and κ chain. Therefore, Fd fragments with His tag were a distinct band of ~23 kDa by western blotting, which presents the Fab.** The result suggested that the concentration of Fab in the periplasmic extract

was lower than for scFab and scFv. **To further compare different format of expressed recombinant antibodies, reactivity against coating antigen was analyzed by icELISA (Table S1). Absorbance at 450 nm of scFab is similar to scFv's. Fab shows lower performance to antigen in comparison with other. The result also suggests that concentration of scFab against coating antigen in the periplasmic extracts was consistent with that of scFv, while the expression of Fab was lower than the other two formats.**

3.3 Sensitivity and specificity of the scFabs

For the specificity test, the cross-reactivity and IC_{50} values of the *κ*-linker-Fd scFab for other O,O-diethyl OPs were determined, which showed that the IC_{50} and CR of the *κ*-linker-Fd scFab were similar to that of its homologous Fab derived from the same parent Mab, and were relatively low in comparison with scFv (Table 1). The results showed that the specificity of scFab was similar to that for Fab and was relatively low in comparison with scFv. This may be due to the tendency of scFvs to form aggregates, or to their lack of the CH1 and CL domains. The conformation of Fab and scFab is relatively similar to that of the parent Mab, in contrast with scFv. **The CR values of recombinant antibodies were higher than 100% for coumaphos, parathion, dichlofenthion, phoxim, and quinalphos, while the CR for other DPPs was between 2% and 100%. Computer-assisted molecular modeling studies were applied and the results showed that coupled to the carrier protein was changed the conformation of hapten and formed strcuture of two aromatic rings.[28] Therefore, antibodies exhibited the lower sensitivity for its immunizing hapten because of those DPPs with a simple substituent group and one benzene ring (such as parathion, phoxim, or dichlofenthion) or those DPPs with two aromatic rings (such as coumaphos, or quinalphos).**

3.4 Stability of the scFabs

To evaluate the stability of the scFab, each recombinant antibody was incubated at 37°C over 9 days. Binding activity against coated antigen and sensitivity determined once per day with icELISA. The scFab A_{max} and IC_{50} curves over time tended to be slightly lower compared with those for Fab, while scFv displayed even larger decreases over time in comparison with scFab and Fab (Fig. S1). After incubation for 9 days, scFab and Fab still exhibited relatively high antigen-binding activity while scFv showed almost no remaining affinity towards the antigen. These results indicated that the stability of Fab and scFab was superior to that of scFv. This is probably attributable to the known tendency of the scFv fragment to aggregate and form multimers, in contrast with the Fab fragment which is reported to stay monomeric.[29,30] In addition, scFvs lack the CH1 and CL domains, which are likely to increase hydrogen bonding and other stabilizing interactions (e.g., van der Waals force, π-π stacking) between the two chains. [31,32] It may be possible to prevent such degradation and improve the stability of scFab and Fab even further. The results also indicated that Fab was relatively stable in comparison with scFab, and this may be attributable to the interchain disulfide bonds of the Fab fragment playing an essential role in its stabilization, or the presence of the $(Gly_4Ser)_3$ linker in scFab having a negative impact on its stability.

3.5 Analyses of spiked samples

To evaluate the precision of the developed icELISA assay, a QuEChERS approach was applied. The interference of the OP matrix in the standard curve became insignificant after all the extracts were diluted 1：2 (Fig. S2).QuEChERS gave similar results to those obtained with the solid-phase extraction (SPE) method described in previous reports.[6] However, unlike traditional SPE methods

that require hours of sample passage through the SPE columns, our QuEChERS-based extraction method was much less time consuming even though it involved further clean-up steps using $MgSO_4$ and sorbents such as PC, C_{18}, and PSA to remove the interfering matrix. Using our method, the preparation was completed in less than 2 h, while the SPE treatment required at least 3–4 h. Our method also reduces the amount of sample used and provides significant savings in terms of reagents and other analytical materials.[33,34]

In this study, the QuEChERS treatment was also applied to the analysis of Chinese cabbage, cucumber, and lettuce samples spiked with three different concentrations of four different DPPs. The mean recovery values of the four selected DPPs were in the desirable range of 81.2–121.8% for Chinese cabbage, 90.8–116.1% for cucumber, and 82.7–123.4% for lettuce (Table 2). Coefficients of variation (CV) ranging from 6.8 to 20.1% were obtained, demonstrating that the icELISA scFab-based assay can be used to accurately assay DPP residues in agricultural samples.

3.6 Comparison of icELISA and GC-FPD analyses

To study the sensitivity of the developed icELISA assay, 27 lettuce samples were spiked with parathion at 0, 2, 5 and 10 ng/mL. After sample extraction and clean-up by QuEChERS, the purified extract was analyzed with both icELISA and GC-FPD. The relationship between the data from icELISA and GC is shown in Fig. 8. Good correlation ($R^2 = 0.9887$, n = 11) between the icELISA and GC-FPD results was obtained. Overall, the results indicate that the developed icELISA method can be used as a rapid screening tool for the analysis of DPPs in food samples.

Conclusions

In this study, recombinant anti-DPP scFab genes in two different Fd/κ orientations with broad

specificity against a class of DPPs were successfully cloned. Indirect competitive ELISA (icELISA) was used to determine the optimum κ/Fd orientation of the scFab. The κ-linker-Fd scFab orientation showed greater sensitivity when compared with the reverse orientation. The optimal scFab was further characterized in terms of expression, specificity, and stability with comparison to its homologous scFv and Fab fragments. The expression extract of scFab displayed high activity towards the antigen that was consistent with that of scFv, while the expression extract of Fab displayed lower activity towards the antigen under the same conditions. The IC_{50} of the *κ*-linker-Fd scFab was also similar to that of Fab and was relatively low in comparison with scFv. scFab and Fab also exhibited higher stability over time while scFv showed almost no remaining affinity towards the antigen following long-term incubation at 37°C. Analysis of DPP-spiked Chinese cabbage, cucumber, and lettuce samples demonstrated that scFab-based icELISA and GC-FPD using QuEChERS revealed good sensitivity and reproducibility for monitoring food safety. Therefore, the optimal recombinant scFab produced in this study could be used to develop a rapid and sensitive immunoassay that is particularly suited for commercial ELISA for the detection of DPPs.

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References

1 C. Lu, D. B. Barr, M. A. Pearson and L. A. Waller, *Environmental Health Perspectives*, 2008, **116**, 537-542.

2 J. M. Van Emon, *Journal of Aoac International,* 2010, **93**(6): 1681-1691.

3 M. Eddleston, A. H. Dawson and N. A. Buckley, *Lancet*, 2008, **371**, 2170-2171.

4 Z. L. Xu, J. X. Dong, H. Wang, Z. F. Li, R. C. Beier, Y. M. Jiang, H. T. Lei, Y. D. Shen, J. Y. Yang and Y. M. Sun, *Journal of agricultural and food chemistry*, 2012, **60**, 5076-5083.

5 Z. L. Xu, Y. D. Shen, W. X. Zheng, R. C. Beier, G. M. Xie, J. X. Dong, J. Y. Yang, H. Wang, H. T. Lei, Z. G. She and Y. M. Sun, *Analytical chemistry*, 2010, **82**, 9314-9321.

6 Z.-L. Xu, H. Deng, X.-F. Deng, J.-Y. Yang, Y.-M. Jiang, D.-P. Zeng, F. Huang, Y.-D. Shen, H.-T. Lei, H. Wang and Y.-M. Sun, *Food Chemistry*, 2012, **131**, 1569-1576.

7 Z. L. Xu, G. M. Xie, Y. X. Li, B. F. Wang, R. C. Beier, H. T. Lei, H. Wang, Y. D. Shen and Y. M. Sun, *Analytica chimica acta*, 2009, **647**, 90-96.

8 M. Hust and S. Dubel, *Trends in Biotechnology*, 2004, **22**, 8-14.

9 M. J. Taussig, O. Stoevesandt, C. A. K. Borrebaeck, A. R. Bradbury, D. Cahill, C. Cambillau, A. de Daruvar, S. Duebel, J. Eichler, R. Frank, T. J. Gibson, D. Gloriam, L. Gold, F. W. Herberg, H. Hermjakob, J. D Hoheisel, T. O Joos, O. Kallioniemi, M. Koegll, Z. Konthur, B. Korn, E. Kremmer, S. Krobitsch, U. Landegren, S. van der Maarel, J. McCafferty, S. Muyldermans, P.-A. Nygren, S. Palcy, A. Plueckthun, B. Polic, M. Przybylski, P. Saviranta, A. Sawyer, D. J. Sherman, A. Skerra, M. Templin, M. Ueffing and M. Uhlen, *Nature Methods*, 2007, **4**, 13-17.

10 R. E. Bird and B. W. Walker, *Trends in biotechnology*, 1991, **9**, 132-137.

11 K. Kramer, M. Fiedler, A. Skerra and B. Hock, *Biosensors & bioelectronics*, 2002, **17**, 305-313.

12 A. Skerra, I. Pfitzinger and A. Pluckthun, *Bio/technology*, 1991, **9**, 273-278.

13 J. D. Marks, H. R. Hoogenboom, A. D. Griffiths and G. Winter, *The Journal of biological chemistry*, 1992, **267**, 16007-16010.

14 P. Holliger, T. Prospero and G. Winter, *Proceedings of the National Academy of Sciences of the United States of America*, 1993, **90**, 6444-6448.

15 J. D. Marks, W. H. Ouwehand, J. M. Bye, R. Finnern, B. D. Gorick, D. Voak, S. J. Thorpe, N. C. Hughes-Jones and G. Winter, *Bio/technology*, 1993, **11**, 1145-1149.

16 R. Rauchenberger, E. Borges, E. Thomassen-Wolf, E. Rom, R. Adar, Y. Yaniv, M. Malka, I. Chumakov, S. Kotzer, D. Resnitzky, A. Knappik, S. Reiffert, J. Prassler, K. Jury, D. Waldherr, S. Bauer, T. Kretzschmar, A. Yayon and C. Rothe, *The Journal of biological chemistry*, 2003, **278**, 38194-38205.

17 H. Thie, S. Binius, T. Schirrmann, M. Hust and S. Dubel, *New biotechnology*, 2009, **26**, 314-321.

18 L. M. Walker, D. R. Bowley and D. R. Burton, *Journal of molecular biology*, 2009, **389**, 365-375.

19 K. Zhang, M. L. Geddie, N. Kohli, T. Kornaga, D. B. Kirpotin, Y. Jiao, R. Rennard, D. C. Drummond, U. B. Nielsen, L. Xu and A. A. Lugovskoy, *mAbs*, 2015, **7**, 42-52.

20 E. Jordan, L. Al-Halabi, T. Schirrmann, M. Hust and S. Dubel, *Microbial cell factories*, 2007, **6**, 38.

21 M. Hust, T. Jostock, C. Menzel, B. Voedisch, A. Mohr, M. Brenneis, M. I. Kirsch, D. Meier and S. Dubel, *BMC biotechnology*, 2007, **7**, 14.

22 J. T. Koerber, M. J. Hornsby and J. A. Wells, *Journal of molecular biology*, 2015, **427**, 576-586.

23 S. J. Lehotay, *J. AOAC Int.*, 2007, **90**, 485-520.

24 Z.L. Xu, W.J. Sun, J.Y. Yang, Y.M. Jiang, K. Campbell, Y.D. Shen, H.-T. Lei, D.-P. Zeng, H. Wang and Y.-M. Sun, *Journal of agricultural and food chemistry*, 2012, **60**, 2069-2075.

25 L.J. Zhou, Q.X. Huang, H.T. Lei, Y.X. Wang, Q. Wang, Z.L. Xu, H. Wang and Y.M. Sun, *Chemical Journal of Chinese Universities-Chinese*, 2012, **33**, 2269-2274.

26 D. Lu, X. Jimenez, L. Witte and Z. Zhu, *Biochemical and biophysical research communications*, 2004, **318**, 507-513.

27 A. Liu, Y. Ye, W. Chen, X. Wang and F. Chen, *Journal of industrial microbiology & biotechnology*, 2015, **42**, 255-262.

28 Z. L. Xu, H. Wang, Y. D. Shen, M. Nichkova, H. T. Lei, R. C. Beier, W. X. Zheng, J. Y. Yang, Z. G. She and Y. M. Sun, *Analyst*, 2011, **136**, 2512-2520.

29 J. F. Rippmann, M. Klein, C. Hoischen, B. Brocks, W. J. Rettig, J. Gumpert, K. Pfizenmaier, R. Mattes and D. Moosmayer, *Applied and Environmental Microbiology*, 1998, **64**, 4862-4869.

30 J. D. Marks, H. R. Hoogenboom, A. D. Griffiths and G. Winter, *The Journal of biological chemistry*, 1992, **267**, 16007-16010.

31 S. Ewert, A. Honegger and A. Pluckthun, *Methods*, 2004, **34**, 184-199.

32 D. Rothlisberger, A. Honegger and A. Pluckthun, *Journal of molecular biology*, 2005, **347**, 773-789.

33 S. G. Dmitrienko, E. V. Kochuk, V. V. Apyari, V. V. Tolmacheva and Y. A. Zolotov, *Analytica chimica acta*, 2014, **850**, 6-25.

34 M. Eddleston, A. H. Dawson and N. A. Buckley, *Lancet*, 2008, **371**, 2170-2171.

Figure captions

Fig.1 Recombinant antibodies: single-chain variable fragment (scFv)(A)was composed of VL and VH with the $(Gly_4Ser)_3$ linker; antigen-binding fragment (Fab)(B)was composed of Fd and κ with disulfide bond. scFab in different Fd/*κ* orientations were assembled by overlap extension PCR with the $(Gly_4Ser)_3$ linker:(C) Fd/*κ* were assembled to scFab κ-linker-Fd with the $(Gly_4Ser)_3$ linker. (D) Fd/*κ* were assembled to scFab Fd-linker-κ with the $(Gly_4Ser)_3$ linker;

Fig.2 PCR products of Fd, κ and scFab: Lane 1, Fd gene of Fd-linker-κ orientation scFab; Lane 2, κ gene of Fd-linker-κ orientation scFab; lane 3, Fd gene of κ-linker-Fd orientation scFab; lane 4, κ gene of κ-linker-Fd orientation scFab; lane M, DNA molecular weight standards (DL-2000); lane 5, splicing overlap extension PCR to generate Fd-linker-κ orientation scFab gene; lane 6, splicing overlap extension PCR to generate κ-linker-Fd orientation scFab gene.

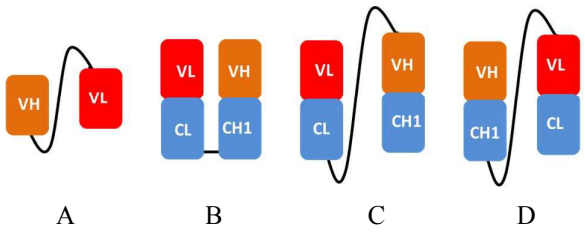
Fig.3 SDS–PAGE (A) and western blotting (B) analyses of anti-DPPs scFab expression: lane M, protein molecular weight standards; Lane 1, periplasmic protein from *E. coli* TOP10F′ containing plasmid pComb3XSS after induction; Lane 2, periplasmic protein from *E. coli* TOP10F′ containing plasmid pComb3XSS scFab *κ*-linker-Fd after induction; Lane 3, periplasmic protein from *E. coli* TOP10F′ containing plasmid pComb3XSS scFab Fd-linker-*κ* after induction. (B) Western blot analysis of scFv expression. The samples were loaded as in (A).

Fig.4 The sensitivities of two different orientations scFabs (*κ*-linker-Fd and Fd-linker-*κ*) were compared with icELISA. Dose-response curves of scFab *κ*-linker-Fd (A) and scFab Fd-linker-*κ* (B) for two selected DPPs coumaphos and parathion. Every point represents the average of three repeated tests and the standard deviation of the tests.

Fig.5 Western blotting analyses of anti-DPP scFv, Fab and scFab expression: lane M, protein molecular weight standards; lane 1, periplasmic protein from *E. coli* TOP10F′ containing plasmid pComb3XSS Fab after induction; lane 2, induced periplasmic protein from *E. coli* TOP10F′ containing plasmid pComb3XSS Fab were concentrated

after induction. lane 3, periplasmic protein from *E. coli* TOP10F′ containing plasmid pComb3XSS scFv after induction; lane 4, periplasmic protein from *E. coli* TOP10F′ containing plasmid pComb3XSS scFab after induction;

Fig.6 Comparison of icELISA and GC-FPD for parathion determination in lettuce samples: Three lettuce samples were spiked with parathion at 2, 5 and 10 ng/mL for three times. After sample extraction and clean-up by QuEChERS, the purified extract was analyzed with both icELISA and a gas chromatograph with a flame photometric detector. The results from the icELISA were compared with the results obtained with GC-FPD.



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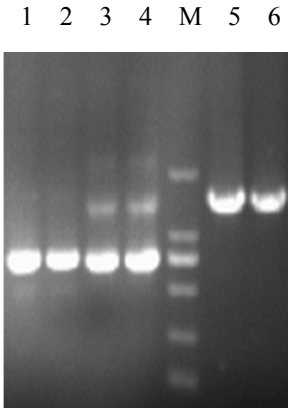

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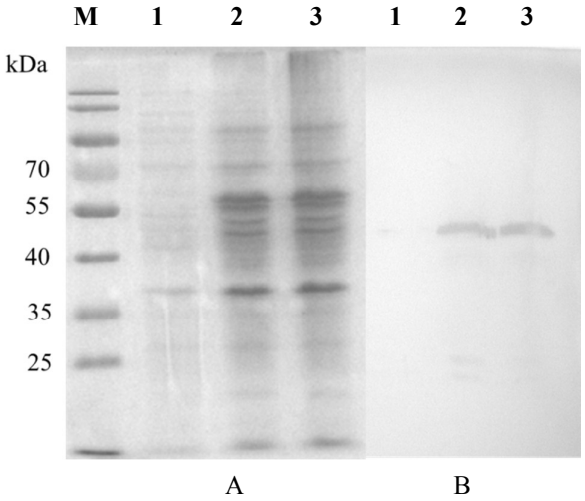

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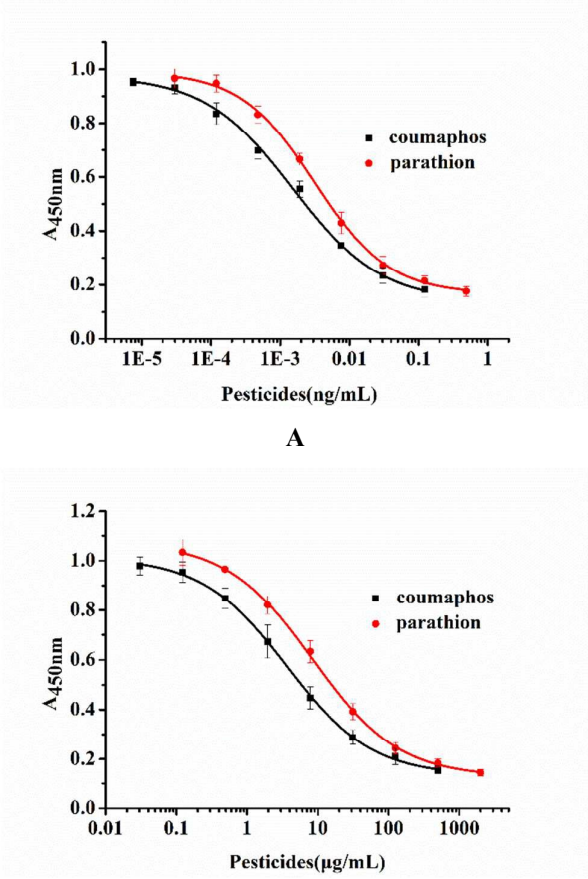

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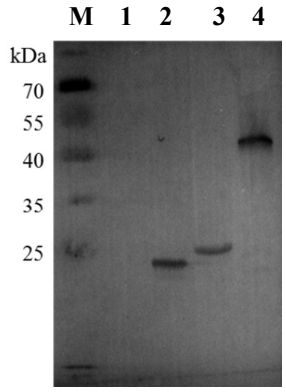

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Table 1 Cross-Reactivity of scFab, scFv and Fab for O,O-Diethyl OP

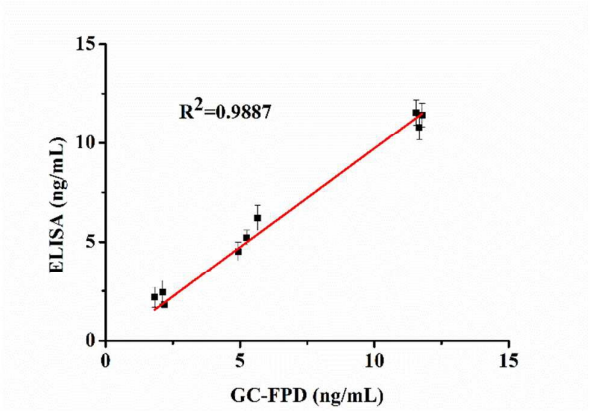
Analyte	scFv[25]		Fab		scFab	
	IC_{50}[a] (ng/mL)	CR[b] (%)	IC_{50} (ng/mL)	CR (%)	IC_{50} (ng/mL)	CR (%)
Hapten 1	337.5	100.0	135.7	100.0	139.6	100.0
Coumaphos	1.9	22792.5	1.0	17747.0	1.5	9306.7
Parathion	6.2	5441.2	3.1	4422.1	3.1	4503.2
Dichlofenthion	89.3	410.1	41.2	357.7	42.6	327.7
Phoxim	93.3	371.7	44.9	310.9	46.9	285.5
Quinalphos	171.3	202.5	75.3	185.1	120.5	115.9
Triazophos	214.7	169.7	106.6	137.4	155.2	89.9
Phosalone	525.7	81.4	263.0	65.4	278.3	50.2
Phorate	462.4	65.5	325.8	37.4	303.0	46.1
Chlorpyrifos	1863.4	21.9	895.3	18.3	1254.8	11.1
Bromophos-ethyl	2834.9	16.2	1064.8	17.3	1801.7	7.7
Sulfotep	2903.2	12.9	1547.8	9.7	2147.2	6.5
Isazophos	3794.4	9.6	2289.5	6.4	2564.3	5.4
Diazinon	11456.4	3.1	4515.6	3.2	4924.6	2.8
Pirimiphos-ethyl	17208.5	2.1	6333.0	2.5	6523.1	2.1

[a] IC_{50}, the 50% inhibition of binding; [b] CR, cross-reactivity values.

Table 2 Recoveries of Four Selected OP from vegetable Samples (n = 3)[a]

Analyte	Added (ng/mL)	Chinese cabbage		cucumber		Lettuce	
		Found	%R[b] ±%CV[c]	Found	%R ±%CV	Found	%R ±%CV
Parathion	2	1.6	81.2±19.8	2.3	116.1±16.0	2.1	107.3±15.8
	5	6.1	121.8±6.8	5.4	107.1±4.5	5.3	107.0±16.7
	10	8.8	87.7±7.7	10.6	106.1±7.6	11.1	110.8±2.8
Dichlofenthion	10	10.9	109.3±10.3	9.1	91.2±10.1	10.6	106.1±6.8
	20	18.8	94.2±20.1	20.3	101.4±18.7	23.1	115.6±13.5
	50	57.2	114.4±10.6	55.2	110.3±12.9	61.7	123.4±10.7
Phoxim	20	24.3	121.5±16.9	26.1	115.6±13.9	14.0	70.1±16.8
	40	44.5	111.3±11.8	38.7	96.8±8.0	39.3	98.3±14.1
	100	93.2	93.2±11.9	94.1	94.1±14.5	108.3	108.3±13.6
Quinalphos	20	23.1	115.5±18.5	21.1	105.4±16.2	24.1	120.6±14.0
	40	37.3	93.3±14.9	42.4	105.9±13.4	40.7	101.7±17.7
	100	104.4	104.4±11.6	94.8	90.8±17.8	82.7	82.7±15.9

[a] For one concentration, three samples were spiked and determined by icELISA. [b] R, recovery values. [c] CV, coefficient of variance, which was obtained from intra-assay.



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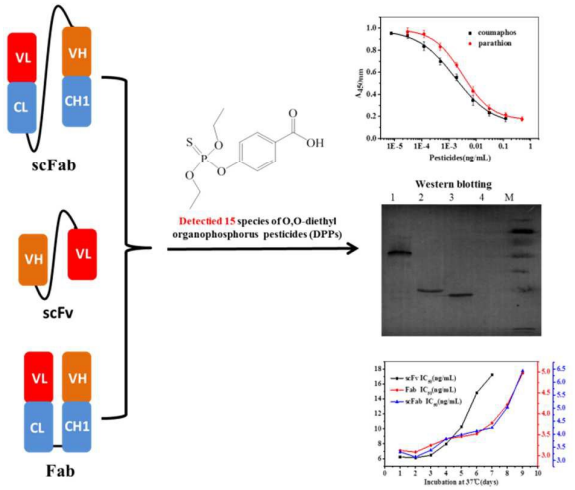

Recombinant antibody of scFab was developed with improved affinity towards target antigens and with long-term stability in storage.