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Production and Characterization of a Single-Chain Variable Fragment Linked Alkaline Phosphatase Fusion Protein for Detection of *O,O*-Diethyl Organophosphorus Pesticides in a One-Step Enzyme-Linked Immunosorbent Assay

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Supporting Information

ABSTRACT: A single-chain variable fragment (scFv) linked alkaline phosphatase (AP) fusion protein for detection of *O,O*-diethyl organophosphorus pesticides (*O,O*-diethyl OPs) was produced and characterized. The scFv gene was prepared by cloning V_L and V_H genes from hybridoma cells secreting monoclonal antibody with broad specificity for *O,O*-diethyl OPs. The amplified V_L and V_H regions were assembled using a linker $(Gly_4Ser)_3$ by means of splicing overlap extension polymerase chain reaction to obtain the scFv gene, which was cloned into the expression vector pLIP6/GN containing an AP gene to produce the scFv−AP fusion protein in *Escherichia coli* strain BL21. The protein was purified by antigen-conjugated immunoaffinity chromatography and characterized by sodium dodecyl sulfate−polyacrylamide gel electrophoresis, Western blotting, and competitive direct enzyme-linked immunosorbent assay (cdELISA). The fusion protein is bifunctional, retaining both antigen binding specificity and AP enzymatic activity. Analysis of spiked and blind river water and Chinese cabbage samples demonstrated that the fusion protein based $cdELISA_{FP}$ exhibited good sensitivity and reproducibility.

KEYWORDS: *single-chain variable fragment, alkaline phosphatase, fusion protein, broad specificity, organophosphorus pesticide*

INTRODUCTION

Immunoassays based on the specific binding of antibodies and antigens are now well-established techniques for the determination of trace amounts of small analytes. They are proven to be rapid, sensitive, and cost-effective analytical tools for routine monitoring of food contaminants.[1] Immunoassays are usually developed using a polyclonal antibody (PAb) or a monoclonal antibody (mAb) and standardized immunochemical methods. The most commonly used enzyme immunoassays for analyte detection are carried out with primary or secondary antibodies that are labeled with enzymes, such as horseradish peroxidase (HRP) or alkaline phosphatase (AP), which are used to generate the reporter signal. For traditional PAb's and mAb's, enzymes are conjugated to the antibodies by well-established chemical procedures,[2] which require several steps and may lead to randomly cross-linked molecules. With the rapid development of antibody engineering technology, the recombinant antibody (RAb) offers several advantages over the PAb and mAb. The production of RAb's can be simpler and less time-consuming once they are engineered and stably expressed in a large-scale bacterial or yeast expression system.[3] In addition, molecular cloning techniques have facilitated the modification of antibody genes to improve antibody affinity or specificity.[4] During the development of an enzyme immunoassay using RAb's, analyte binding is usually visualized by the detection of a peptide tag (e.g., c-myc tag or His tag) fused to the RAb.[5] This is achieved by an antitag antibody chemically or covalently linked to a reporter enzyme (e.g., anti-c-myc tag or immunoglobulin G−HRP (IgG−HRP)). As an attractive alternative, constructing genetically engineered fusion proteins of enzymes (e.g., AP) combined with the RAb can avoid the chemical coupling of enzymes with antibodies and the use of a second antibody.[4,6]

A broad-specificity immunoassay is an emerging technology that allows the simultaneous determination of a class of closely related small molecules.[7] It can detect positive samples among hundreds of negative samples in one simple test. Over the past few years, several immunoassays for the detection of a class of closely related food contaminants, such as pesticides,[8] veterinary drugs,[9] and mycotoxins,[10] based on PAb's or mAb's have been developed. The use of RAb's in the development of broad-specificity immunoassays for small molecules is an emerging trend because the broad specificity or affinity of antibodies can be improved through mutagenesis.[11,12] Previously, we produced a hybridoma cell line

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(12C2) that secrets an mAb with broad specificity to a class of *O*,*O*-diethyl organophosphorus pesticides (*O*,*O*-diethyl OPs).[13] This work was conducted with the aim to improve the immunoassay properties and lay the foundation for further improvement in broad specificity by developing an RAb through antibody engineering. Here a broad-specificity recombinant single-chain variable fragment (scFv) against *O*,*O*-diethyl OPs was produced by a simple method. The scFv gene was inserted into the expression vector pLIP6/GN containing the AP gene to prepare a bifunctional fusion protein (scFv−AP). The fusion protein was characterized by SDS−PAGE and Western blotting and used to develop a simple and rapid competitive direct enzyme-linked immunosorbent assay (cdELISA) for *O*,*O*-diethyl OPs in environmental and agricultural samples.

MATERIALS AND METHODS

Materials and Reagents. *O*,*O*-Diethyl OP standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The 12C2, immunizing hapten (4-(diethoxyphosphorothioyloxy)benzoic acid, hapten 1), and the coating antigen (4-(diethoxyphosphorothioylamino)butanoic acid−ovalbumin conjugate, hapten 2−OVA) were previously prepared and stored in our laboratory.[13] The reverse transcription polymerase chain reaction (PCR) kit was obtained from Promega Biotechnology Co. (Shanghai, China). TRIzol reagent, the agarose gel DNA purification kit, PCR product purification kit, and restriction enzymes *Sfi*I, *Not*I, and *Blg*I were purchased from Takara Biotechnology Co. (Dalian, China). The plasmid mini preparation kit was purchased from Tiangen Co. (Beijing, China). Primers for cloning the variable antibody genes and constructing the scFv genes were synthesized by Invitrogen Biotechnology Co. (Shanghai, China). The cloning vector pEASY-T3 was purchased from TransGen Biotechnology Co. (Beijing, China). The pLIP6/GN vector used for soluble protein expression was generously provided by Dr. Frédéric Ducancel (Pharmacology and Immunoanalysis Department, CEA/Saclay, Gif-sur-Yvette, France). *Escherichia coli* strains XL1-Blue and BL21 were stored in our laboratory. *Pfu* DNA polymerase and T4 DNA ligase were supplied by TransGen Biotechnology Co. (Beijing, China) and Fermentas GMBH (St. Leon-Rot, Germany), respectively. *N*-Hydroxysuccinimide (NHS)-activated Sepharose 4 Fast Flow medium was supplied by GE Healthcare Bio-Science (Uppsala, Sweden). The colorimetric substrate *p*-nitrophenyl phosphate was purchased from Aladdin-Reagent Co. (Shanghai, China). The antibacterial AP mAb, bovine serum albumin (BSA), OVA, and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Polystyrene microplates for the cdELISA were obtained from Guangzhou JET Bio-Filtration Products Co. (Guangzhou, China). Mixed cellulose ester microporous membrane was purchased from the Shanghai Xingya Purification Material Factory (Shanghai, China). Dual-layer (primary−secondary amine (PSA) in combination with graphitized carbon black) solid-phase extraction (SPE) columns (500 mg/500 mg/6 mL) were purchased from Shanghai ANPEL Scientific Instrument Co. Ltd. All other reagents were of analytical grade and were obtained from a local chemical supplier (Guangzhou Whiga Technology Co., Ltd., Guangzhou, China).

Instruments. PCR was performed in a DNA Engine thermal cycler, Western blotting and SDS−PAGE were performed with a Trans-Blot SD transfer cell and PowerPac Universal power supply, and the gels and film were imaged and analyzed in a Gel Doc XR system, which were all obtained from Bio-Rad (Hercules, CA). Ultrafiltration was performed in a Millipore Labscale ultrafiltration system (Millipore, Bedford, MA). All aqueous solutions and buffers were prepared with water purified with a Milli-Q system (Millipore, Bedford, MA). cdELISA values were recorded using a Wallac 1420 VICTOR^3 multilabel reader (PerkinElmer, Waltham, MA).

Cloning of V_L and V_H Genes. The total RNA was extracted from the 12C2 hybridoma cell line (about 1×10^7 cells) using TRIzol reagent according to the manufacturer's instructions. Following reverse transcription from mRNA (messenger ribonucleic acid) using a reverse transcription PCR kit, the first-strand cDNA (complementary deoxyribonucleic acid) was synthesized and used as a template for the amplification of the immunoglobulin genes for the antibody variable region light chains (V_L) and the variable region heavy chains (V_H) using primers V_L-back, V_L-forward, V_H-back, and V_H-forward (see also the Supporting Information).[14] The PCR protocol involved an initial denaturation at 94 °C for 5 min, followed by 25 cycles at 94 °C for 1 min, 56.7 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The amplification products (about 45 μL) were purified using the agarose gel DNA purification kit and cloned into the pEASY-T3 vector for plasmid production. The extracted plasmid was used for sequencing.

Production of Recombinant scFv Antibodies. The V_L gene fragment was used as a template for amplification of the V_L fragment containing the *Not*I site and a portion of the linker in the absence of V_L-forward-*Not*I and V_L-back-linker. The V_H fragment was used as a gene template to amplify the V_H fragment containing the *Sfi*I site and a portion of the linker in the absence of V_H-back-*Sfi*I and V_H-forward-linker. The PCR protocol used was the same as described above. The amplified modified V_H and V_L fragments were then overlapped to obtain the scFv gene using overlap extension PCR, resulting in the formation of the $(Gly_4Ser)_3$ linker from the overlapped gene portions residing between the V_H and V_L fragments. The PCR protocol for the overlap extension step involved an initial denaturation at 94 °C for 5 min, followed by 10 cycles at 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Finally, the gene for the scFv fragment containing the *Not*I and *Sfi*I sites was amplified using the primers V_L-forward-*Not*I and V_H-back-*Sfi* I with the same PCR protocol as used for cloning of V_L and V_H genes. The amplification products were purified with an agarose gel DNA purification kit and stored at −20 °C.

Construction of the Recombinant Plasmid pLIP6/GN−scFv. The recombinant plasmid encoding the scFv−AP fusion protein

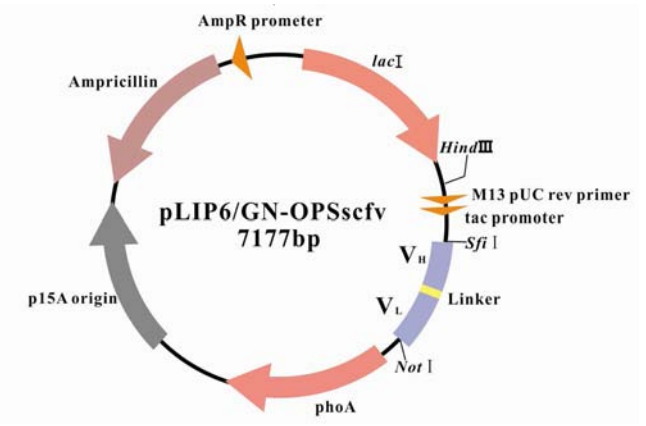

Figure 1. Expression vector pLIP6/GN−scFv.

(pLIP6/GN−scFv) is shown in Figure 1. The scFv products and vector pLIP6/GN were both digested with *Sfi*I and *Not*I restriction enzymes. About 1 μg of the scFv or pLIP6/GN, 2.5 μL of the provided buffer, and 1 μL of *Sfi*I were added to an aseptic tube followed by addition of aseptic purified water to reach a final volume of 25 μL. Following mixing, the tube was incubated overnight at 50 °C. The digested products were purified with a PCR product purification kit. The purified PCR products were then further digested with 1 μL of *Not*I, 2.5 μL of the provided buffer, 2 μL of 0.1% BSA, and 2 μL of 0.1% Triton X-100, aseptic purified water was added to a final volume of 25 μL, and the mixture was incubated at 37 °C for 3 h. The digested products were purified with a PCR product purification kit and then ligated using the T4 DNA ligation enzyme.[15] The digested scFv (about 60 ng) was combined with the digested pLIP6/GN (about 100 ng),

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and the mix contained 2 μL of the provided buffer and 1 μL of T4 DNA ligation enzyme. Aseptic purified water was added to a final volume of 10 μL, and the mixture was incubated at 22 °C for 1 h. The ligation products were transformed into *E. coli* strain XL1-Blue competent cells.[14] Briefly, 10 μL of ligation products was added to the competent cells in an aseptic tube and placed in ice for 30 min after mixing. The tube was incubated at 42 °C for 90 s and immediately placed on ice for 2 min. A 900 μL volume of Luria−Bertani (LB) culture was added to the tube and cultured at 37 °C for 1 h with stirring (250 rpm). The cells were then seeded on LB−agar plates containing 100 μg/mL ampicillin, 100 μmol/L isopropyl β-D-thiogalactopyranoside (IPTG), and 40 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The ampicillin-resistant clones expressing the AP fusion protein hydrolyzed the BCIP substrate, resulting in blue colonies,[16] and the recombinant plasmid was extracted using a plasmid mini preparation kit and then used for both restriction enzyme analysis and sequence analysis. PhoA-back and PhoA-forward primers were used for sequencing.

Sequence Analysis. The V_L, V_H, and scFv DNA sequences were determined by a commercial facility (Invitrogen Biotechnology Co. Ltd., Shanghai, China). The determined sequences were aligned with immunoglobulin sequences in the international ImMunoGeneTics (IMGT) information system database,[17] and the amino acid sequences were deduced. The complementarity-determining regions (CDR1−CDR3) were deduced by online Web antibody modeling.[18] The sequences were submitted to the National Center for Biotechnology Information (NCBI) database.

Expression of Soluble scFv−AP Fusion Protein. The recombinant plasmid was transformed into *E. coli* BL21. The transformed colony was then cultured in 2× yeast extract and tryptone medium supplemented with 100 μg/mL ampicillin at 37 °C until the OD_{600} reached approximately 0.6−1.0. The promoter was then induced by adding 1 mmol/L of IPTG and incubated overnight on a shaker at 28 °C. Bacteria were collected from the culture by centrifugation at 10000*g* for 10 min at 4 °C. The pellet was resuspended with a saccharose solution (20% saccharose, 0.3 mol/L Tris−HCl (pH 8.0), 1 mmol/L ethylenediaminetetraacetic acid), and the periplasmic scFv protein was extracted using cold osmotic shock.[19] The suspension was centrifuged at 10000*g* for 10 min at 4 °C, and the supernatant containing soluble fusion protein was collected. The presence of the fusion protein was detected in both SDS−PAGE and Western blotting with antibodies against AP.[20]

Purification of the scFv−AP Fusion Protein. Purification of the fusion protein was performed on a self-prepared affinity-chromatography column by conjugating coating antigen to NHS-activated Sepharose 4 (Fast Flow) according to the manufacturer's instructions. For purification, 200 mL of supernatant was concentrated to 10−15 mL by ultrafiltration and loaded onto the coating antigen affinity-chromatography column. The column was washed with 50 mL of phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.4), and the scFv−AP fusion protein was eluted with glycine−HCl (0.1 mol/L, pH 3.0) and neutralized immediately with Tris−HCl (1 mol/L, pH 8.0). The pooled fractions were dialyzed against PBS (0.01 mol/L, pH 7.4) and concentrated using polyethylene glycol (PEG) 10 000. An aliquot was removed for confirmation of protein purity by SDS−PAGE and Western blotting, and the remainder was stored at −20 °C for further cdELISA analysis.

cdELISA Protocol. The cdELISA based on the bifunctional scFv−AP fusion protein ($cdELISA_{FP}$) was performed as follows. The 96-well microplate was coated with coating antigen (1 μg/mL, 100 μL/well) in carbonate buffer (0.05 mol/L, pH 9.6) overnight at 37 °C. The plate was washed two times with PBST (0.01 mol/L PBS containing 0.05% Tween-20, pH 7.4) and blocked with 5% skim milk in PBST (200 μL/well) for 3 h at 37 °C. After being washed two times with PBST, the plates were dried at 37 °C overnight. Organophosphorus pesticide standards or samples in PBS (0.02 mol/L, pH 6.2, 50 μL) containing 5% methanol were added to the wells followed by addition of the fusion protein diluted with PBS (1:100). After incubation for 1 h at 37 °C, the plates were washed five times with PBST. Then 100 μL/well of AP−substrate (1 mmol/L *p*-nitrophenyl phosphate, 1 mol/L Tris−HCl, 10 mmol/L $MgCl_2$, 50 mmol/L $ZnCl_2$; pH 8.0) was added, and the mixture was incubated at 37 °C for 20 min. The reaction was stopped by adding 50 μL of 3 M NaOH. The absorbance at 405 nm was measured with a multilabel reader. Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration as previously described.[13] A cdELISA based on the HRP-labeled mAb (mAb−HRP)[21] was also performed to compare the sensitivity and specificity of the fusion protein and its parent mAb.

Analyses of Spiked Samples. For the spike-and-recovery test, river water and Chinese cabbage were chosen. River water samples were collected from the Zhujiang River, the largest drinking water source for the city of Guangzhou, China. Chinese cabbage was purchased from a local market. The samples were confirmed to be OP-free by high-performance liquid chromatography−electrospray ionization tandem mass spectrometry (HPLC−ESI-MS/MS) analysis. River water samples were filtered over a mixed cellulose ester microporous membrane to remove particles larger than 0.45 μm and spiked with three different concentrations of coumaphos, parathion, phoxim, quinalphos, and dichlofenthion. The final concentration of methanol was 5%. The spiked samples were diluted (1:1, v/v) with PBS (0.04 mol/L, pH 6.2) containing 5% methanol to eliminate the matrix effect and then used for $cdELISA_{FP}$ analysis. Chinese cabbage samples were washed, dried, and then finely chopped. Five gram samples of finely chopped cabbage were spiked with three different concentrations of coumaphos, parathion, phoxim, quinalphos, and dichlofenthion. Acetonitrile (10 mL) was added to the samples, and they were mixed in an oscillating mixer for 10 min. Then 1.5 g of sodium chloride was added to each sample, followed by mixing for 2 min. The mixtures were centrifuged at 3075*g* for 5 min at room temperature. The acetonitrile layer was collected and then dehydrated with anhydrous magnesium sulfate. The acetonitrile was evaporated to about 1 mL with a stream of nitrogen gas at 40 °C. The residues were passed through graphitized carbon black/PSA SPE columns (preconditioned with 5 mL of acetonitrile/methylbenzene (3:1, v/v)). The columns were washed with 15 mL of acetonitrile/methylbenzene (3:1, v/v), and the eluents were evaporated under vacuum at 40 °C. The residues were dissolved with 10 mL of PBS (0.02 mol/L, pH 6.2) containing 5% methanol and used for $cdELISA_{FP}$ analysis. The percentage of recovery was calculated as follows:

$$\text{recovery}(\%) = (\text{quantity measured}/\text{quantity spiked}) \times 100$$

Screening of Blind Samples. Ten blind samples (including five river water samples, samples 1−5, and five Chinese cabbage samples, samples 6−10) were collected and analyzed by the developed $cdELISA_{FP}$ method and confirmed by HPLC−ESI-MS/MS. Two blind Chinese cabbage samples, known to be positive, samples 7 and 8, were supplied by the China National Analytical Center, Guangzhou, China. Parathion was used to develop the standard curve for the $cdELISA_{FP}$. The average absorbance of each sample was used to calculate the percent inhibition (PI) using the following equation: $PI = [(A_0 - A_x)/A_0] \times 100$, where A_0 is the absorbance of the control (PBS containing 5% methanol) and A_x are the absorbances of the blind samples at 450 nm. Samples that demonstrated an inhibition lower than 10% were regarded as negative samples, and samples with a percent inhibition higher than 10% were considered positive.

HPLC−ESI-MS/MS Conditions. HPLC−ESI-MS/MS for simultaneous determination of coumaphos, parathion, phoxim, quinalphos, triazophos, dichlofenthion, azinphos-ethyl, phosalone, disulfoton, and phorate was completed by the China National Analytical Center, Guangzhou, China. Mobile phase A consisted of 0.2% acetic acid and 10 mM ammonium acetate in water, mobile phase B consisted of 0.2% acetic acid in acetonitrile, and they were used in the following gradient profile: 0 min, 55% A and 45% B; 8 min, 10% A and 90% B; then 8.1−14 min, 55% A and 45% B. The flow rate of the mobile phase was 0.2 mL/min, and an aliquot of 10 μL of each sample was injected into the HPLC system. The mass spectra were obtained with an Agilent 6410 Triple Quad mass spectrometer using the electrospray ionization technique. All pesticides were analyzed in the positive ionization mode.

RESULTS AND DISCUSSION

Cloning and Characterization of scFv Genes. Total RNA from the hybridoma cell line 12C2 was used to clone the V_L and V_H domain cDNAs. These two genes encoding the variable domains were amplified by PCR and sequenced. The amplification of V_L generated an expected 324-bp fragment, while V_H generated an expected 369-bp fragment. The DNA sequences of V_L and V_H were submitted to the NCBI database (GenBank accession numbers JF906063 and JF906064). The V_L and V_H genes were amplified to generate the enzyme site and linker overhang and then assembled by splicing overlap extension PCR to generate the scFv gene. PCR assembly and amplification of scFv produced an expected 735-bp fragment. The hybridoma cell line would be a good source of RNA, since it produces high-affinity mAb's that bind *O*,*O*-diethyl OPs. Therefore, there would be a good chance of cloning functional immunoglobulin genes from this cell line. We used an improved primer mix as suggested by Krebber et al.[14] to clone the V_L and V_H genes from the 12C2 cell line. The results indicated that the scFv gene was successfully generated.

Characterization of Recombinant Plasmid pLIP6/GN−scFv. The scFv amplification product was digested and then inserted between the *Sfi*I/*Not*I cloning site at positions +6 and +7 of the bacterial AP in the pLIP6/GN vector. The ligation product was transformed into *E. coli* strain XL1-Blue, and the extracted plasmid was characterized by restriction enzyme digestion and sequence analysis. As shown in Figure 2,

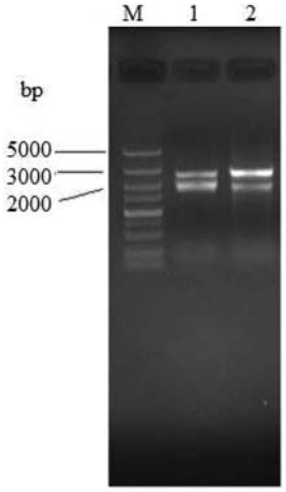

Figure 2. Agarose gel electrophoresis of the digested recombinant plasmid pLIP6/GN−scFv: lane M, DL 5000 marker; lane 1, empty vector pLIP6/GN digested with *Bgl*I; lane 2, recombinant vector pLIP6/GN−scFv digested with *Bgl*I.

digestion of the empty vector pLIP6/GN with *Bgl*I resulted in two bands of 2000 bp and one band of 3000 bp (lane 1), while digestion of the vector pLIP6/GN−scFv with *Bgl*I resulted in one band of 2000 bp and two bands of 3000 bp (lane 2). The nucleotide and deduced amino acid sequences of the scFv are shown in Figure 3. As expected, insertion of the 0.7 kb DNA encoding the single-chain variable domains between codons +6 and +7 of the AP gene restored the initial frame of the AP gene in the vector. These data indicated that recombinant plasmid pLIP6/GN−scFv had been successfully constructed.

atg ccg **gcc cag ccg gcc** atg gaa gtg aaa gtt gag gag tct gga gga ggc ttg gtg caa cct gga gga tcc
M P A Q P A M E V K V E E S G G G L V Q P G G S

atg aaa ctc tcc tgt gtt gcc tct gga atc act ttc agt cac tac tgg atg aat tgg gtc cgc cag tct cca gag
M K L S C V A S G I T F S H Y W M N W V R Q S P E
H-CDR1

aag ggg ctt gag tgg gtt gct gaa att aga ttg aga ttt agt aat cat gta aca caa tat gcg gag tct gtg aaa
K G L E W V A E I R L R F S N H V T Q Y A E S V K
H-CDR2

ggg agg ttc acc atg tca aga gac gat tcc aaa agt agt gtc tac ctg caa atg aac aac tta aga gct gaa
G R F T M S R D D S K S S V Y L Q M N N L R A E

gac act ggc att tat tac tgt acc agc atc tac tat gat aac ctg tac tac gct atg gac tac tgg ggt caa gga
D T G I Y Y C T S I Y Y D N L Y Y A M D Y W G Q G
H-CDR3

acc tca gtc acc gtc tcc tcg *ggt gga ggc ggt tca ggc gga ggt ggc tct ggc ggt ggc gga tcg* gat att
T S V T V S S *G G G S G G G S G G G S* D I

ctg atg acc cag tct cca gcc tcc cta tct gca tct gtg gga gaa act gtc acc atc aca tgt cga gca agt gaa
L M T Q S P A S L S A S V G E T V T I T C R A S E

aat att cac aat tat tta gca tgg tat cag cag aaa cag gga aaa tct cct caa ctc ctg gtc tat tat gca aaa
N I H N Y L A W Y Q Q K Q G K S P Q L L V Y Y A K
L-CDR1

acc tta gca gat ggt gtg cca tca agg ttc agt ggc agt gga tca gga aca caa tat tct ctc aag atc aac agc
T L A D G V P S R F S G S G S G T Q Y S L K I N S
L-CDR2

ctg cgg cct gaa gat ttt ggg act tat tac tgt caa cat ttt tgg act act cct cgg acg ttc ggt gga ggc acc
L R P E D F G T Y Y C Q H F W T T P R T F G G G T
L-CDR3

aag ctg gaa atc aaa cgt **gcg gcc gca** gtt
K L E I K R A A A V

Figure 3. Nucleotide and deduced amino acid sequences of scFv for OPs. Nucleotide sequences corresponding to restriction sites *Sfi*I and *Not*I are indicated in bold type. The linker fragment $(Gly_4Ser)_3$ is shown in italic type. The complementarity-determining regions of the V_H (H-CDR) and V_L (L-CDR) domains are underlined.

Several recombinant fusion proteins with both AP enzymatic activity and antigen binding capacity have been reported. Rau et al.[4] inserted the *E. coli* AP coding region and the scFv cloning cassette in the vector pASK75 for scFv−AP expression. Suzuki et al.[6] inserted an scFv−AP fusion gene into an expression vector incorporating the T7 promoter for expression, the *pelB* leader for translocation, and the His tag for purification. In our work, the scFv gene was inserted into the vector pLIP6/GN for expression of the fusion protein. The pLIP6/GN vector presents unique restriction sites, *Sfi*I and *Not*I, between codons coding for residues +6 and +7 of mature AP, thus allowing the periplasmic exportation of the fusion protein and correct processing of the signal peptide, following induction of the *tac* promoter with IPTG.[22] This facilitates disulfide bond formation, solubility, extraction, and purification of proteins.[23] In the empty pLIP6/GN vector, the AP gene is out of frame but is restored upon cloning of the foreign DNA insert, permitting a visual selection of blue colonies on BCIP agar plates.[24] Moreover, the AP produced by this system has two mutations resulting in a variant enzyme with enhanced enzymatic activity.[25]

Expression, Purification, and Characterization of the scFv−AP Fusion Protein. The confirmed positive plasmid was used to transform *E. coli* BL21, and one clone was picked and cultured. Following induction, the periplasmic proteins were extracted by cold osmotic shock and characterized by SDS−PAGE and Western blotting. A band of approximately 75 kDa was detected from the induced cell culture with SDS−PAGE (Figure 4A, lane 2). This band was absent in the noninduced cell culture (Figure 4A, lane 1). The Western blotting results indicated that the recombinant fusion protein was present as a single band with a molecular mass of about 75

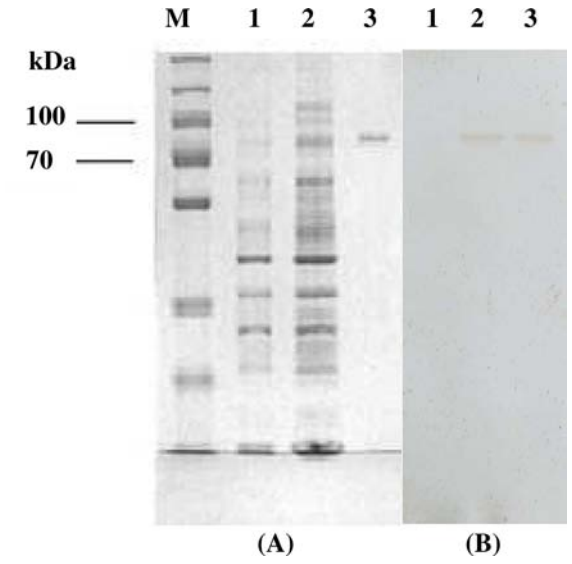

Figure 4. Characterization of the scFv–AP fusion protein by SDS–PAGE (A) and Western blotting (B): lane M, low molecular mass protein standards; lane 1, noninduced periplasmic protein; lane 2, induced periplasmic protein; lane 3, purified scFv–AP fusion protein.

kDa (Figure 4B, lane 2). The antibacterial AP mAb specifically recognized the AP enzyme, and no degradation products were revealed by Western blotting, showing that the scFv–AP fusion protein was stable and no endogenous AP was detected in the expression system. After characterization of the fusion protein by SDS–PAGE and Western blotting, noncompetitive direct ELISAs were conducted using plate-coating antigens BSA, OVA, and hapten 2–OVA. The results showed that the specific binding characteristics to hapten 2–OVA and the functionality of the AP enzyme were retained in the constructed fusion protein. Consequently, the fusion protein was purified using immunoaffinity chromatography (hapten 2–OVA coupled to NHS-activated Sepharose 4). The SDS–PAGE (Figure 4A, lane 3) and Western blotting (Figure 4B, lane 3) results indicated that immunoaffinity chromatography produced a good purification of the fusion protein.

cdELISA$_{FP}$. A cdELISA based on the bifunctional scFv–AP fusion protein and a cdELISA based on mAb–HRP were both developed to compare their specificities and sensitivities to the *O*,*O*-diethyl OPs. As shown in Figure 5, the dose-dependent curves for parathion and triazophos from the cdELISA$_{FP}$ were similar to those based on mAb–HRP. The cross-reactivity and IC$_{50}$ values for other *O*,*O*-diethyl OPs were also similar (Table 1). These data showed that characteristics of the fusion protein were similar to those of its parent mAb. A one-step cdELISA$_{FP}$ can be developed by omitting the use of enzyme-labeled secondary antibodies.

Most RAb's that have been synthesized from hybridoma cell lines were reported to show characteristics similar to those of their parent mAb's,[3,15] while some reported RAb's had altered analytical properties.[26,27] The possible reasons for differences between the binding characteristics of the mAb secreted by a hybridoma cell line and those of the corresponding derived RAb were discussed by Kramer et al.[3] Using the fusion protein can reduce the time required for immunochemical detection, since the bifunctional fusion protein used in the cdELISA$_{FP}$ omits the use of an enzyme-labeled secondary antibody. The entire incubation time for the cdELISA$_{FP}$ was 80 min, while the incubation time would be 130 min or longer[8,9] for a

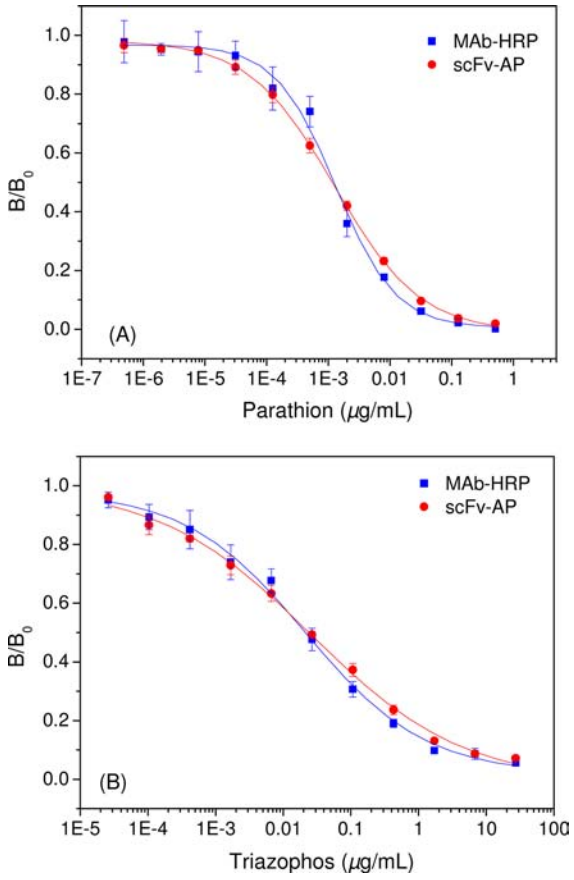

Figure 5. Dose–response curves for two selected OPs (A, parathion; B, triazophos) based on the fusion protein scFv–AP and mAb–HRP. Each point represents the average of three replicates and the standard deviation of the mean.

competitive indirect ELISA based on using a traditional PAb or mAb. Moreover, the approach using gene fusion to prepare immunoconjugates is simple and reproducible (with the ability to store bacterial cells indefinitely), and it produces a homogeneous population of compounds in contrast to traditional antibody production procedures.[28,29] The production of a fusion protein can be adapted to industrial scale and be rapidly purified in one step. Therefore, the genetic approach is an advancement in the preparation of AP-labeled reagents, resulting in products which could replace conventional immunoconjugates commercially available for use as secondary antibodies.

Recovery Tests. The cdELISA$_{FP}$ was applied to analyze river water and Chinese cabbage samples spiked with three concentrations of five different OPs. As shown in Table 2, recoveries of the five selected OPs were in the desirable range of 78.0–116.5% for river water samples and 74.0–125.7% for Chinese cabbage samples. Similar recoveries were obtained with the mAb-based ELISA for agricultural samples[21] and environmental water samples.[30] Better reproducibility for river water samples was observed than for Chinese cabbage samples, which might be attributed to the complicated pretreatment required for Chinese cabbage. These results demonstrate that the cdELISA$_{FP}$ can be used in the determination of OP residues in environmental and agricultural samples.

Screening of Blind Samples. The quantitative analysis of individual OPs is not possible using a broad-specificity immunoassay, but it can be developed as a semiquantitative

Table 1. Cross-Reactivity of scFv−AP and MAb−HRP for *O,O*-Diethyl OPs

	scFv−AP		mAb−HRP	
analyte	IC_{50} (nmol/mL)	CR[a] (%)	IC_{50} (nmol/mL)	CR[a] (%)
hapten 1	56.1	100.0	42.7	100.0
coumaphos	0.5	14315.7	0.3	18405.7
parathion	1.3	4203.1	1.3	3246.7
phoxim	10.3	559.5	9.5	462.5
quinalphos	17.5	329.4	21.2	206.8
dichlofenthion	23.2	263.0	32.1	144.3
triazophos	25.3	239.7	23.6	195.4
azinphos-ethyl	46.2	144.5	38.4	132.3
phosalone	65.9	107.9	79.5	68.1
disulfoton	227.2	23.4	217.6	18.6
phorate	291.3	17.3	232.0	16.5
bromophos-ethyl	1241.6	6.1	1055.5	5.5
sulfotep	1046.0	6.0	1189.9	4.0
chlorpyrifos	1925.3	3.5	1537.8	3.4
isazophos	2614.2	2.3	1469.6	3.1
diazinon	2921.7	2.0	1453.2	3.1
pirimiphos-ethyl	3172.2	2.0	1066.2	4.6
terbufos	6125.7	0.9	5230.9	0.8
ethion	>10000	<0.7	7489.5	0.8

[a]CR (%) was calculated according to the following equation: CR = [(IC_{50}(hapten 1) (nmol/mL))/(IC_{50}(cross-reactant) (nmol/mL))] × 100.

screening method.[21] The value of PI was used to determine whether a sample contained OPs. When samples showed a PI lower than 10% (LOD of the developed cdELISA$_{FP}$ in river water and Chinese cabbage samples, 0.05, 0.2, 1.8, 2.7, and 4.6 ng/mL for coumaphos, parathion, phoxim, quinalphos, and dichlofenthion, respectively), they were regarded as negative, and they were considered positive when the PI was higher than 10%. Five river water samples (samples 1−5) were collected from small rivers in Guangzhou city, and five Chinese cabbage samples (samples 6−10, which included two positive samples, blind samples 7 and 8, that were kindly provided by the China National Analytical Center, Guangzhou, China) were collected from local food markets, Guangzhou, China. They were analyzed by the developed cdELISA$_{FP}$ and further confirmed by HPLC−ESI-MS/MS. As shown in Table 3, the collected river water samples and Chinese cabbage samples were negative both by cdELISA$_{FP}$ and by HPLC−ESI-MS/MS analysis. Remarkable inhibition of the antibody−antigen interaction was found for the two positive samples 7 and 8, which were confirmed to be positive by HPLC−ESI-MS/MS. The results indicated that the developed cdELISA$_{FP}$ is an ideal screening method for OP residues in water and cabbage samples prior to chromatographic analysis. Compared with analysis of all samples only by HPLC−ESI-MS/MS, the proposed ELISA screening method to identify the positive samples from a large

Table 3. Results of 10 Blind Samples Analyzed by the Developed cdELISA$_{FP}$ and Confirmed by HPLC−ESI-MS/MS

sample[a]	inhibition[b] (%)	result[c]	HPLC−ESI-MS/MS (concn, ng/mL or ng/g)
1	6.6	−	ND[d]
2	−2.4	−	ND
3	−3.5	−	ND
4	7.9	−	ND
5	8.1	−	ND
6	6.7	−	ND
7[e]	75.3	+++	parathion (26.3)[e]
8[e]	35.4	++	triazophos (11.9)[e]
9	8.7	−	ND
10	9.1	−	ND

[a]Key: samples 1−5, river water; samples 6−10, Chinese cabbage. [b]Percent inhibition was calculated using the equation [($A_0 - A_x$)/A_0] × 100, where A_0 is the absorbance of the control at 450 nm and A_x are the absorbances of the samples. [c]Key: +++, strongly positive; ++, medium positive; +, weakly positive; −, negative. [d]ND = not detected (out of the LODs). [e]Positive samples and data from HPLC−ESI-MS/MS analysis were kindly provided by the China National Analytical Center, Guangzhou, China. The LODs for HPLC−ESI-MS/MS were 0.15 ng/mL or ng/g for coumaphos and parathion, 0.06 ng/mL or ng/g for phoxim, quinalphos, triazophos, azinphos-ethyl, and phosalone, 0.3 ng/mL or ng/g for dichlofenthion and phorate, and 0.7 ng/mL or ng/g for disulfoton for river water and Chinese cabbage samples, respectively.

Table 2. Recoveries of Five Selected OPs from Spiked River Water and Chinese Cabbage Samples (n = 3)

	river water			Chinese cabbage	
analyte	concn added (ng/mL)	concn found (ng/mL) (mean ± SD)	recovery (%) (mean ± SD)	concn found (ng/g) (mean ± SD)	recovery (%) (mean ± SD)
coumaphos	0.5	0.6 ± 0.03	110.0 ± 6.0	0.4 ± 0.04	74.0 ± 8.0
	1.0	0.8 ± 0.1	78.0 ± 11.0	0.8 ± 0.2	77.0 ± 15.0
	2.5	2.1 ± 0.2	85.6 ± 8.4	2.1 ± 0.3	84.8 ± 12.0
parathion	1.5	1.4 ± 0.2	91.3 ± 12.0	1.7 ± 0.3	112.7 ± 16.7
	3.0	2.9 ± 0.3	97.3 ± 10.3	3.2 ± 0.3	107.0 ± 9.3
	7.5	6.8 ± 0.6	90.4 ± 7.6	6.9 ± 0.7	91.6 ± 9.2
phoxim	10	11.7 ± 0.9	116.5 ± 9.4	11.1 ± 1.0	111.0 ± 9.9
	20	18.9 ± 1.1	94.6 ± 5.7	19.7 ± 4.1	98.3 ± 20.4
	50	46.2 ± 3.6	92.3 ± 7.3	61.8 ± 6.5	123.6 ± 13.0
quinalphos	20	22.4 ± 2.5	112.1 ± 12.7	25.1 ± 3.1	125.7 ± 15.8
	40	41.9 ± 3.1	104.8 ± 7.8	46.5 ± 3.6	116.3 ± 8.9
	100	79.5 ± 10.2	79.5 ± 10.2	91.3 ± 14.5	91.3 ± 14.5
dichlofenthion	20	18.3 ± 1.8	91.3 ± 8.9	18.7 ± 2.1	93.3 ± 10.7
	40	39.5 ± 4.2	98.6 ± 10.5	31.7 ± 6.5	79.2 ± 16.4
	100	87.3 ± 6.6	87.2 ± 6.6	105.6 ± 11.7	105.6 ± 11.7

number of samples followed by confirmation by HPLC−ESI-MS/MS would be a cost-effective process.

In summary, we cloned the V_L and V_H genes from hybridoma cells secreting an mAb with broad specificity for a class of *O*,*O*-diethyl OPs and used sequencing and comparison to gene databases to eliminate the aberrant genes. This procedure was useful for rapidly and simply obtaining functional V_L and V_H genes from a hybridoma cell line. The functional V_L and V_H genes were assembled with a $(Gly_4Ser)_3$ linker to obtain a functional scFv gene. The scFv gene was then cloned into the expression vector pLIP6/GN containing an AP gene to produce the bifunctional (antigen binding specificity and AP enzymatic activity) scFv−AP fusion protein upon expression. The use of a fusion protein can accelerate the immunochemical detection of OPs by omitting the use of secondary antibodies or the use of an enzyme-labeled primary antibody. Further efforts will be made to study the interaction mechanism of the scFv and OPs by molecular modeling, which can lead to improved antibody affinity and broad specificity of the scFv by site-directed mutagenesis.

■ ASSOCIATED CONTENT

Supporting Information

Table S1 showing the primers used for cloning variable region genes and generating the synthetic gene encoding the scFv and Figure S1 showing the agarose gel electrophoresis of V_L, V_H, and scFv genes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

AP, alkaline phosphatase; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; *Blg*I, restriction enzyme; BSA, bovine serum albumin; CDR, complementarity-determining region; cdELISA, competitive direct enzyme-linked immunosorbent assay; $cdELISA_{FP}$, cdELISA based on the bifunctional scFv−AP fusion protein; cDNA, complementary deoxyribonucleic acid; HPLC−ESI-MS/MS, high-performance liquid chromatography−electrospray ionization tandem mass spectrometry; HRP, horseradish peroxidase; IgG, immunoglobulin G; IMGT, international ImMunoGeneTics information system database; IPTG, isopropyl β-D-thiogalactoside; mAb, monoclonal antibody; mAb−HRP, HRP-labeled mAb; mRNA, messenger ribonucleic acid; *Not*I, restriction enzyme; OPs, organophosphorus pesticides; PAb, polyclonal antibody; PBST, 0.01 mol/L phosphate-buffered saline containing 0.05% Tween-20; pEASY-T3, cloning vector; *Pfu*, DNA polymerase; pLIP6/GN, protein expression vector; PI, percent inhibition; PSA, primary−secondary amine; RAb, recombinant antibody; scFv, single-chain variable fragment; scFv−AP, bifunctional fusion protein; *Sfi*I, restriction enzyme; V_H, gene for antibody variable region heavy chains; V_L, gene for antibody variable region light chains; 12C2, anti-OP broad-specificity mAb hybridoma cell line.

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