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Production of Recombinant ScFv Antibodies against Methamidophos from a Phage-Display Library of a Hyperimmunized Mouse

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A recombinant phage display library was generated using splenocyte mRNA isolated from a Balb/c mouse hyperimmunized with a hapten conjugate that mimicked the structure of methamidophos, one of the most acutely toxic organophosphate pesticides. Three recombinant single-chain variable fragment (scFv) antibodies with the highest specificity for methamidophos, termed 28D4, 29D0, and 36B2, were produced via a stringent selection protocol. In a competitive enzyme-linked immunosorbent assay, the IC_{50} values for 28D4, 29D0, and 36B2 were 46.25, 35.39, and 17.99 ng/mL, respectively. The cross-reactivity of the three scFv antibodies with other organophosphate pesticides was below 0.1% except for acephate (*O,S*-dimethyl acetylphosphoramidothioate). Nucleotide and deduced amino acid sequences indicated that the respective heavy chains and light chains of the three scFvs were involved in the distinctive VDJ segment rearrangements associated with somatic hypermutations during the process of several immunizations with higher dosages of immunogen. Taken together, these data constitute the first detailed description of an immunoassay that utilizes scFvs against the methamidophos, an analyte with a simple structure and low molecular mass (141 Da).

KEYWORDS: Methamidophos; scFv; phage display; panning; CI-ELISA; sequence analysis; hyperimmunization

INTRODUCTION

Methamidophos (*O,S*-dimethyl phosphoramidothioate) is an acutely toxic organophosphate that inhibits acetylcholinesterase (*1*). Acephate, an N-acetylated derivative of methamidophos with much lower mammalian toxicity, is metabolized to methamidophos by plants, animals, and soil bacteria (*2*−*4*). In the United States, the use of methamidophos is restricted to the control of aphids, leafhopper, Colorado potato beetle, whitefly, and stink bug in potatoes, cotton, and tomatoes; in 2007, countries of the European Union may restrict the use of this insecticide to potatoes (*5*). In other countries, however, methamidophos is used more widely and residues may pose a threat to human health, especially when sprayed vegetables are harvested too early, before the residues have fallen to safe levels (*6*).

Several excellent reviews report methods for extraction and subsequent analysis of methamidophos residues in fruits and vegetables (*7*−*10*). However, these traditional analytical methods require large amounts of solvent, time-consuming sample extraction and pretreatment, well-trained analysts, expensive instrumentation, and meticulous interpretation of the data generated in the analyses. Acetylcholinesterase inhibition assays are rapid and can be formatted for high-throughput analyses, but they detect all pesticides that inhibit the enzyme and thus lack specificity. Immunoassays provide a simple, powerful, and inexpensive method for pesticide analysis; however, high-quality antibodies are required to develop these assays (*11*).

Before the advent of recombinant DNA technology, antibodies (Abs) with predetermined affinity and specificity could be obtained only from animals (i.e., polyclonal antibodies) or the tissue culture supernatants of hybridoma cells (i.e., monoclonal antibodies). Development of polyclonal Abs with high affinity and specificity is time-consuming, and the results vary among animals. The isolation of monoclonal Abs requires large-scale screening strategies and expensive production costs. Recombinant approaches to Ab production can overcome these problems. Through phage-display technology and antibody engineering, the use of animals for antibody production can be minimized and eventually eliminated. More importantly, the affinity and specificity of an existing antibody or design of a new one can be achieved at the molecular level via site-directed mutagenesis, chain shuffling, or complementarity determining region (CDR)

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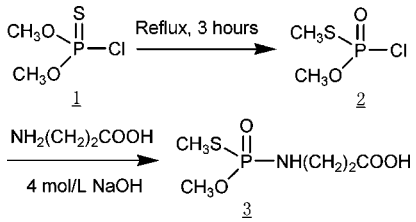

Figure 1. Procedure for synthesis of hapten used for immunogen and conjugate preparation.

grafting (*12*−*14*). These molecular techniques may also be employed to engineer antibodies for analysis of pollutants in complex environmental matrices. Recombinant antibodies can be produced cheaply and quickly, using a variety of expression systems (*15*−*18*).

While naïve recombinant antibody libraries have been used for a number of years to develop antibodies with medical applications, recombinant antibody libraries derived from immunized animals are only beginning to be exploited for environmental analysis. This approach can save the time and expense of repeated immunizations; once an adequate library is available, it can be screened to select the antibodies required for specific applications (*14*). Such immunized phage libraries were recently used for the production of antibody fragments against haptens such as atrazine (*19*), ampicillin (*20*), picloram (*21*), and 2,4-dichlorophenoxyacetic acid (2,4-D) (*22*). Here, we describe the isolation and characterization of scFvs against methamidophos. These antibody fragments were selected from a phage-displayed library prepared directly from splenocytes of a hyperimmunized mouse.

MATERIALS AND METHODS

Chemicals, Reagents, and Instrumentation. Bovine serum albumin (BSA), ovalbumin (OVA), *N*-hydroxysuccinimide (NHS), *N*,*N*-dicyclohexylcarbodiimide (DCC), goat anti-mouse IgG peroxidase conjugate, 3,3′,5,5′-tetramethylbenzidine (TMB), and Freund's complete and incomplete adjuvant were purchased from Sigma Chemical Co. (St. Louis, MO). β-Alanine was purchased from Shanghai Chemicals Co. (Shanghai, China). Organic starting materials for hapten synthesis, methamidophos, and the organophosphorous pesticides used for cross-reactivity studies (acephate, dischlorvos, dimethoate, phorate, parathionmethyl, and isocarbophos) were supplied by Jiangsu Pesticide Research Institute (Jiangsu, China). Enzyme-linked immunosorbent assay (ELISA) high-binding microplates were obtained from Corning Co. (Cambridge, MA). The Recombinant Phage Antibody System (Mouse ScFv Module, Expression Module, Anti-E-Tag Antibody) was purchased from Amersham Pharmacia (Amersham Pharmacia, United States). A UV−vis spectrophotometer (Beckman 640, United States) was used for analysis of hapten−protein conjugates. Mass spectra were obtained on a GCMS-QP2010 spectrometer (Shimadzu, Japan). NMR spectra were obtained using a General Electric ACF-300 MHz spectrometer (Bruker, Germany). A microplate washer from Prolong New Technology Co. (Beijing, China) was used to wash ELISA plates. Absorbance (*A*) was measured using a microtiter plate reader (Thermo Electron Co., United States); this device was controlled by a personal computer containing the standard software package EasySoftware.

Hapten Synthesis and Verification. The methamidophos hapten used in this work was synthesized from compound **1** (*O*,*O*-dimethyl phosphorochloridothioate), as shown in **Figure 1**. Compound **1** (50 mL) was refluxed for 4 h to obtain **2** (*O*,*S*-dimethyl phosphorochloridothioate), which was used subsequently without further purification. The resulting product (2.5 mL, 13.1 mmol) of **2** was dissolved in 4 mL of 4 M sodium hydroxide, and the solution was cooled to 4 °C. β-Alanine (2.905 g, 32 mmol) was dissolved in 4 mL of 4 M NaOH and cooled to 4 °C, and this solution, along with 3 mL of cold 4 M sodium hydroxide, was added to compound **2** in five equal portions, with at least 5−10 min between additions. The reaction mixture was stirred in an ice bath for 2 h. After acidification to pH 4.0 with concentrated hydrochloric acid, the carboxylic derivative, which separated as an oil, was extracted with ethyl acetate (three 50 mL portions). The ethyl acetate phase was washed several times with diluted hydrochloric acid and extracted with 1 M bicarbonate solution (two 50 mL portions). The aqueous layer was acidified with concentrated hydrochloric acid and extracted with ethyl acetate again. The organic phase was dried over anhydrous sodium sulfate and evaporated at reduced pressure to remove organic solvent. The resulting thick white liquid was lyophilized to yield 1.472 g (yield: 78.3%) of pure white solid **3** {3-[methoxy(methylthio)phosphorylamino]propanoic acid}, the hapten (HM3) of methamidophos. ^{1}H NMR ($CDCl_3$): δ 2.06 (s, 3H, CH_3S), 2.45−2.49 (t, J = 6.0 Hz, 2H, CH_2−COOH), 3.12−3.20 (t, 2H, J = 6.0 Hz, CH_2−CH_2COOH), 3.56 (s, 3H, CH_3O), 4.62 (s, 1H, NH). ^{13}C NMR (aceton-d_6): δ 176.93 (COOH), 52.62 (CH_3O), 37.81 (CH_2), 37.02 (CH_2), 15.45 (CH_3S); GC-MS molecular ion peak (m/z = 213) was the base peak.

Conjugation of Haptens with Carrier Proteins. The hapten of HM3 was conjugated with proteins (BSA and OVA) using the active ester method (*23*). One micromole of hapten was incubated for 5 h with stirring at room temperature with stoichiometric amounts of NHS and DCC in 1500 μL of DMF. The mixture was cooled at 5 °C for 2 h. After centrifugation, 500 μL of the clear supernatant containing the active ester was slowly added to 2 mL of a 10 mg/mL BSA solution in 0.2 M phosphate buffer, pH 8.0 (another 500 μL to OVA). The mixture was stirred at 10 °C for 5 h to complete the conjugation and then dialyzed against 0.2 M phosphate buffer (pH 6.8), which was replaced with fresh buffer six times a day for 64 h. Conjugate formation was confirmed spectrophotometrically. UV−vis spectra showed qualitative differences between the carrier protein and conjugates in the region of maximum absorbance of haptens. The molar ratio of hapten to protein in the conjugates was then estimated from spectral data obtained from the hapten, the protein, and the corresponding conjugate.

Immunization. Six 8-week-old Balb/c mice were immunized with the immunogen HM3-BSA. Each mouse was injected intraperitoneally with a 1:1 (V/V) mixture of 150 μg of immunogen dissolved in sterile phosphate-buffered saline (PBS: 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, and 0.2 g of KH_2PO_4 per liter of water, pH adjusted to 7.5) and Freund's complete adjuvant in a total volume of 250 μL in the first injection. Subsequent boost injections were performed at 3 week intervals with the mixture of 150 μg of immunogen dissolved in sterile PBS and Freund's incomplete adjuvant in a volume of 250 μL. Blood samples were collected a week after each boost to monitor the immune response against methamidophos using a competitive indirect enzyme-linked immunosorbent assay (CI-ELISA, described below). After the eighth immunization, the mouse whose serum possessed the lowest IC_{50} value was sacrificed for splenocyte extraction 3 days after a final intraperitoneal injection of 200 μg of HM3-BSA diluted in sterile PBS.

Cloning and Phage-Display Library Construction. Mouse ScFv Module and Expression Module were used to construct a mouse scFv phage display library according to the protocols supplied by the manufacturer. Briefly, DNA fragments encoding V_H and V_L were amplified from reverse-transcribed mRNA by polymerase chain reaction (PCR) and fused by a DNA fragment encoding a linker peptide. The assembled scFv fragments were ligated into the phagemid vector pCANTAB5E and subsequently transformed into *Escherichia coli TG1* cells. The size of the library was evaluated by titer. The scFv genes of 50 randomly picked clones were checked by PCR, and the BstN I digestion of the PCR products was used to determine the diversity of the library by looking for identical BstN I fingerprints after electrophoresis in a 4% agarose gel (*24*). The library was rescued by infection with the helper phage M13K07 for the next round of panning.

Panning. Three wells of a 96-well microplate were each filled with 100 μL of HM3-OVA diluted to 50 μg/mL in sterile PBS. The microplate was incubated at 4 °C overnight and washed three times with sterile PBS. Each microwell was blocked with 200 μL of 10% MPBS [1 × PBS containing 10% (w/v) of nonfat dry milk solids] at 37 °C for 1 h. Wells were washed three times with sterile 1 × PBS, and 100 μL of preblocked phage suspension was added to each well. The microplate was incubated at 37 °C for 2 h with mild shaking. Wells were washed 20 times with sterile PBST [1 × PBS containing 0.05%

(v/v) Tween 20, pH 7.5] followed by 10 washes with sterile 1 × PBS. Each well was eluted with 50 μL of 0.1 M triethylamine at 37 °C for 10 min and neutralized with 25 μL of 1 M Tris-HCl, pH 7.4. Neutralized phages were used immediately to infect log phase *E. coli TG1* cells ($OD_{600\ nm}$ = 0.5) for 20 min at 37 °C. The cells were then plated on SOB (20 g of bacto-tryptone, 5 g of bacto-yeast extract, 0.5 g of NaCl, 0.95 g of $MgCl_2$, and 15 g of bacto-agar per liter of medium) agar plates and incubated at 37 °C for 16 h. Colonies were scraped from the plate and resuspended in 2 × YT (17 g of bacto-tryptone, 10 g of bacto-yeast extract, and 5 g of NaCl per liter of water) medium containing 100 μg/mL ampicillin and 2% glucose to express phage with the helper phage M13K07. Aliquots of concentrated phage were used for another round of panning.

To select for phage-displaying scFv with high specificity and affinity to methamidophos, the concentration of coating conjugate HM3-OVA used in panning was decreased to increase the stringency of selection. Wells were coated with 100 μL of HM3-OVA in PBS at concentrations of 50, 5, 3, 1, and 0.5 μg/mL for panning rounds one to five, respectively. Simultaneously, the stringency was further enhanced by increasing the number of washings from 20 to 40 times for the third to the fifth panning rounds. Individual colonies from the fifth round of panning were inoculated into a 96-well microplate for monoclonal ELISA as described in detail in the instructions for the Expression Module of the Recombinant Phage Antibody System.

Expression and Purification of Soluble scFv. Production of soluble scFv fragments specific for the methamidophos was achieved by transfecting phage from the positive clones identified by monoclonal ELISA into *E. coli HB2151*. This strain does not carry the suppressor that is needed for production of scFv-gene III fusion proteins, resulting in the production of only scFv polypeptides. The procedures for infecting *E. coli HB2151* cells and production of soluble antibodies were described in detail in the instructions for the Expression Module of the Recombinant Phage Antibody System. Expressed products from whole cell extracts, supernatants, and periplasmic extracts of the specific colonies were analyzed to determine the location of accumulation of the soluble antibodies.

CI-ELISA. CI-ELISA was performed to assess the specificity of the antibody to free methamidophos and the cross-reactivities of structurally related compounds to the antibody. The coating antigen HM3-OVA (0.1 μg in 100 μL) diluted in carbonate buffer was added to wells of microplates and incubated at 4 °C overnight. Wells were emptied and washed five times with PBST. PBS (200 μL) containing 3% (w/v) nonfat dry milk solids was added to each well to block any unoccupied sites on the plate. Plates were incubated at 37 °C for 1 h. At the same time, serial dilutions of antiserum or soluble scFvs in PBS according to checkerboard titration by indirect ELISA were preincubated for 1 h at 37 °C with the same volume of standard solutions containing serial dilutions (10^{-4}−10^5 ng/mL per well) of free methamidophos or structurally related compounds to methamidophos in PBS. This mixture (100 μL) was added to individual wells of the microtiter plate after the plate had been blocked and washed five times with PBST. The plate was incubated at 37 °C for 2 h, followed by six washings with PBST. Goat anti-mouse IgG conjugated with horseradish peroxidase (diluted 1:1000 with PBST, 100 μL/well) was added to plates containing mouse antiserum, and these samples were incubated for 1 h at 37 °C. The plates with scFv fragments were incubated with an anti-E-tag monoclonal antibody diluted 1:1000 in PBST containing 0.1% (w/v) gelatin for 1 h at 37 °C. The scFv plates were washed six times with PBST, and 100 μL/well of goat anti-mouse IgG (1:1000 dilution in PBST) conjugated with horseradish peroxidase was added and incubated for 1 h at 37 °C. All wells were washed six times with PBST after the anti-mouse IgG was removed, and 100 μL/well of TMB solution (3.3 μL of 30% H_2O_2, 400 μL of 0.6% TMB in DMSO per 25 mL of acetate buffer, pH 5.5) was added. The color development was stopped after 10−15 min with 2 M H_2SO_4 (100 μL/well). The absorbance was measured at 450 nm with a DNM-9602 microplate reader. The signal detected was inversely proportional to the concentration of free methamidophos or compounds structurally related to methamidophos used in the assay. Standard calibration curves were plotted with binding (B/B_0, where B_0 and B are corrected absorbance in the absence and presence of methamidophos, respectively) against the concentrations of methamidophos used in individual treatments. The affinity of the Ab in terms of IC_{50} (the concentration that inhibits 50% of the binding) was determined. The cross-reactivity values were calculated as the ratio of the IC_{50} of the methamidophos standard to the IC_{50} of the test compounds and expressed as a percentage.

Determination of Antibody−Antigen Affinities. The affinity constants of selected scFv antibodies against antigen were determined by using competition ELISA (*25*, *26*). In brief, scFv antibodies at 5 nM (which was within the range of values that gave a linear relationship between antibody concentration and ELISA signal in titration experiments) were equilibrated with increasing concentrations of HM3-OVA (ranging from 1 nM to 10 μM) in 100 μL volume MPBST (PBS containing 2% skim milk and 0.05% Tween 20) for 1 h at room temperature. The pre-equilibrated scFv antibodies were then transferred to a microtiter plate coated with HM3-OVA and processed by standard ELISA assay as described above. The concentration of HM3-OVA at which the half-maximal ELISA signal was detected corresponding to the dissociation constant (K_D) for antibody−antigen affinity (*27*).

Analyses of Methamidophos-Fortified Samples. Water samples were collected from an industrial tap. Rice and cabbage samples were obtained from a field where the insecticide was not applied. For the spike-and-recovery test of tap water, three final concentrations (10, 50, and 200 ng/mL) of methamidophos in tap water were prepared without any sample cleanup procedures. For the spike-and-recovery test in rice and cabbage samples, stock solutions of methamidophos were prepared in distilled water at the concentrations of 25, 125, and 500 ng/mL. One milliliter each of these stock solutions was added to 1 g of pesticide-free leaves of finely chopped cabbage or finely ground grains of rice. After setting the samples aside for 24 h, the leaves or grains were incubated in 5 mL of methanol for 15 min with five vigorous shakes and then filtered through a filter paper. The container and the residues were rinsed with 5 mL of methanol, the rinse solutions were also filtered, and the filtrates were combined. Methanol was evaporated under reduced pressure, and the residue was dissolved in 1 mL of PBS. These simple extracts of rice and cabbage samples and tap water were analyzed by the ELISA. The recovery (%) was calculated as follows:

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

ScFv Sequence Determination. The scFv DNA of the specific clones that bound to methamidophos was sequenced by a commercial facility (Takara Co., Ltd., Dalian, China). All sequences were searched in the Kabat database using a BLAST search to compare them with previously sequenced V_H and V_L chains (*28*). The sequence analysis working (SAW) program and the international immunogenetics (IMGT) database were used to propose assignment of scFvs to potential germline genes and to assess V and J segment usage. The IMGT tool DNA plot was accessed via its website (*29*).

RESULTS AND DISCUSSION

Synthesis of Haptens and Their Conjugation to Carrier Proteins. The insecticide methamidophos in itself cannot be used as an immunogen because of its low molecular mass (141 Da) and simple structure. The amine group in methamidophos is a phosphoramide and thus very stable, so it does not have the character of α-NH_2 group, which is required for many of the chemistries that link haptens to carrier proteins (*30*). Therefore, haptens mimicking the analyte insecticide and containing reactive groups for conjugation to carrier proteins must be synthesized to develop antibodies and conjugates for ELISA. Other workers have reported the synthesis of an artificial antigen for methamidophos via both EDC [1-ethyl-3-(3-dimethyl-aminopropyl) carbodimide hydrochloride] (*31*) and phosphorylation conjugation methods (*30*, *32*); however, the antibodies obtained using these conjugates were of low affinity and specificity. This is probably because methamidophos was covalently linked to the carrier protein without any spacer arms and this conjugation procedure may have masked hapten's determinant groups. In the present study, the hapten of metha-

Table 1. Phage Recovery during Screening of the Phage Display Library[a]

round of panning	coating concn (μg/mL)	input phage (p.f.u.)	output[b] phage (p.f.u.)	phage recovery
1	50	7.5×10^{11}	8.5×10^{5}	1.1×10^{-4}
2	5	7.5×10^{11}	9.0×10^{4}	1.2×10^{-5}
3	3	3.0×10^{11}	8.2×10^{5}	2.7×10^{-4}
4	1	1.0×10^{11}	7.5×10^{5}	7.5×10^{-4}
5	0.5	1.0×10^{11}	7.2×10^{5}	7.2×10^{-4}

[a] Phage-displayed scFvs were selected against HM3-OVA and reinfected into *E. coli TG1*. On the basis of the number of colonies formed after reinfection of eluted phage particles to host bacteria.

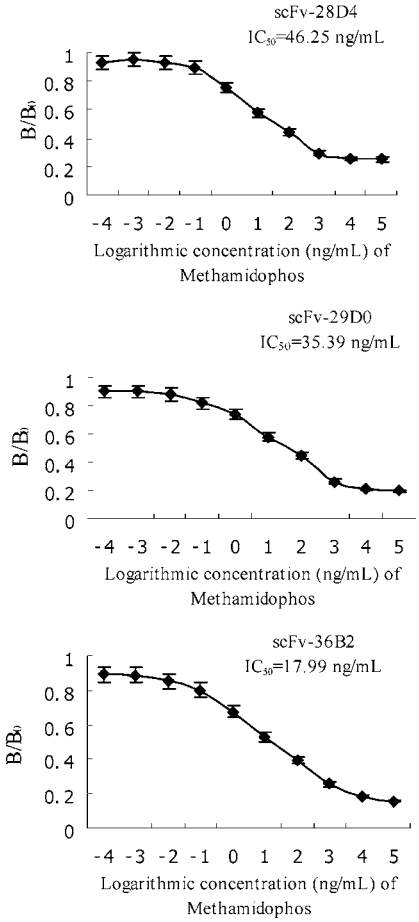

Figure 2. Competitive ELISAs for methamidophos using three scFvs selected from a recombinant library prepared from a hyperimmunized mouse. CI-ELISAs were constructed as described in the Materials and Methods using purified scFv-28D4, 29D0, and 36B2. B_0 and B are corrected absorbances in the absence and presence of methamidophos, respectively. The error bars represent the standard deviation calculated from replicate ($n = 4$) calibration curves, which were obtained with the same set of standards.

midophos was synthesized with a three carbon spacer arm derived from β-alanine. The use of a three carbon spacer arm was hypothesized to improve exposure of the hapten's determinant groups by extending the structure farther out in space and farther out from the protein mass (*33*, *34*).

UV−vis spectra obtained from continuous wavelength scanning showed significant differences between the spectra of the conjugate and that of the unreacted carrier protein (data not shown). The molar ratio of hapten to protein (as assessed by this spectrophotometric method) was 42:1 and 26:1 for HM3-BSA and HM3-OVA, respectively (primary data not shown).

Table 2. Cross-Reactivity of the scFv Antibodies for Compounds Related to Methamidophos

compound	cross-reactivity (%)[a] scFv-28D4	scFv-29D0	scFv-36B2
methamidophos	100	100	100
acephate	5.3	5.1	4.9
dischlorvos	<0.1	<0.1	<0.1
dimethoate	<0.1	<0.1	<0.1
phorate	<0.01	<0.01	<0.01
parathionmethyl	<0.01	<0.01	<0.01
isocarbophos	<0.01	<0.01	<0.01

[a] Cross-reactivity (%) = (IC_{50} of methamidophos/IC_{50} of test compound) × 100.

Table 3. Recovery of Methamidophos from Fortified Samples Using a CI-ELISA and scFv-36B2

sample	theoretical (ng/mL)	detected (ng/mL) (mean ± 1 SD, $n = 4$)	recovery (%) (mean ± 1 SD)
tap water	10	10.7 ± 0.8	107.0 ± 8.3
	50	51.7 ± 2.8	103.5 ± 5.6
	200	206.5 ± 7.1	103.2 ± 3.6
rice	25	19.5 ± 1.9	78.0 ± 7.6
	125	102.5 ± 7.6	81.9 ± 6.1
	500	412.7 ± 8.0	82.5 ± 1.6
cabbage	25	20.4 ± 0.8	81.7 ± 3.2
	125	108.7 ± 6.9	86.9 ± 5.6
	500	423.1 ± 9.7	84.6 ±1.9

Construction of Phage-Display scFv Library. After the eighth immunization, the mouse that possessed the lowest IC_{50} value for methamidophos in a CI-ELISA was sacrificed for splenocyte extraction 3 days after a final injection of HM3-BSA. Following first-strand cDNA and amplification of the V_H and V_L chains, the predominant PCR products were of the expected sizes for V_H (340bp) and V_L (320 bp) fragments. These V_H and V_L chains were purified, assembled, cloned into pCANTAB5E expression vector, and used to transform TG1 cells. The phage-display library consisted of 10^6 clones, with a final diversity of 9.8×10^5 separate clones. Of 50 randomly picked clones tested, only two restriction patterns were indistinguishable in a 4% agarose gel electrophoresis and all contained inserts of the expected size (750 bp) fragments (data not shown).

Selection for Clones that Bound Methamidophos. The selection of phage with specificity for methamidophos was based on increasing the stringency of the selection at each round of panning. Phage recovery after each round is shown in **Table 1**. Phage recovery decreased nearly 10-fold from rounds one to two, when equivalent numbers of phage were panned against a 10-fold lower concentration of coating antigen. In the subsequent rounds of selection, phage recovery gradually increased and then remained nearly constant from rounds four to five. The efficiencies of recovery during panning indicate that phage with specificity for methamidophos were enriched during the selection procedure. The enrichment factor was not as impressive as those previously reported from experiments with antigen-unbiased repertoires (*35*). Enrichments observed in the present study are similar to those reported by Tout et al. (*21*), who rationalized that an immune library is already biased toward antibodies with specificities and affinities for the antigen used in the original immunization; therefore, a lower enrichment factor is anticipated.

Table 4. Amino Acid Sequence Alignment of scFv-28D4, 29D0, and 36B2 Antibody Fragments[a] against Methamidophos

clone	heavy chain	light chain
	FR1	
scFv-28D4	QVQLQESGPELVKPGASVKLSCKASGYNFT	DIELTQSPAIMSASPGEKVTITC
scFv-29D0	QVQLQESGPELVRPGVSVMISCKGSGYTFT	DIELTQSPAIMSASYGEKVTMSC
scFv-36B2	QVQLQESGPSLVKPGASVKMSCKASGYNFT	DIELTQSPAIMSASPGEKVTTTC
	CDR1	
scFv-28D4	TYYMH	SASSSVSYMH
scFv-29D0	DYAMH	RASSSVNYMY
scFv-36B2	SYYMH	SASSSVSYMH
	FR2	
scFv-28D4	WVKPRPGQGLEWIG	WFQQKSGSSPKIWIY
scFv-29D0	WVKQSHTKSLEWIG	WYQQKSDASPKLWIY
scFv-36B2	WVKQRPGQGLEWIG	WYQQKSGTSPKRWIY
	CDR2	
scFv-28D4	GIYPGNGVTNLNAKFKT	GISNLAS
scFv-29D0	VISTYAGNTIYNQKFTG	YTSNLAP
scFv-36B2	GIYPGDGSTNLNEKFKT	DTSKLAS
	FR3	
scFv-28D4	KVTLTVDKSSTTAYMHLSSLTSEDSAVYYCQT	GVPARFSGSGSGTSFSFTINSMEAEDVATYYC
scFv-29D0	KATMTVDKSSSTAYMELAGLTSDDSAVYYCVR	GAPARFSGSGSGNSFSLTISSMEGEDAATYYC
scFv-36B2	KTTLTADKSSSTAYMLLSSLTSEDSAIYFCQT	GVPARFSGGGSGTSFSFTINSMEAEDVATYYC
	CDR3	
scFv-28D4	ESF	QQRSIYPPTWT
scFv-29D0	LVRPGAMDF	QQFTSSPSIWT
scFv-36B2	EGAMDF	QQRSIYPPTWS
	FR4	
scFv-28D4	WGQGTTVTVSS	FGGGTKLEIKR
scFv-29D0	WGQGTTVTVSS	SGGGTKLEIKR
scFv-36B2	WGQGTTVTVSS	FGGGTKLEIKR

[a] Sequences of anti-methamidophos scFvs, using Kabat residue numbering and CDR definitions.

To verify that decreasing the concentration of coating conjugate in each round did indeed improve the selection process, 20 phage clones were randomly picked from each round. These phages were rescued and tested for binding to immobilized HM3-OVA by monoclonal ELISA. Seven clones obtained from the third round (35% of the total), 12 clones from the fourth round (60% of the total), and 16 clones from fifth round (80% of the total) showed binding activity to immobilized HM3-OVA (positive binding activity was defined as an absorbance for HM3-OVA that was 3-fold above background, coupled with no detectable binding to OVA, primary data not shown). Then, these data confirmed that the percentage of scFv-phage that bound to immobilized HM3-OVA increased as the coating concentration of HM3-OVA used in the panning experiments decreased.

Production and Characterization of Methamidophos-Specific scFv. After screening, three clones, designated scFv-28D4, scFv-29D0, and scFv-36B2, were chosen for further study, based on specificity studies using CI-ELISA. Soluble scFv antibodies were obtained and purified to homogeneity using an anti-E tag/protein G affinity column. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed a single band of 31 kDa (data not shown). The results from a CI-ELISA using soluble methamidophos and scFv-28D4, scFv-29D0, and scFv-36B2 are shown in **Figure 2**. The IC_{50} values for methamidophos in assays constructed with scFv-28D4, scFv-29D0, and scFv-36B2 were 46.25, 35.39, and 17.99 ng/mL, respectively. The limits of detection (defined as two standard deviations above the minimal detectable level) were 0.26, 0.06, and 0.04 ng/mL for scFv-28D4, scFv-29D0, and scFv 36B2, respectively.

CI-ELISAs were also constructed for organophosphate pesticides structurally related to methamidophos, and the IC_{50} values were used to determine the % cross-reactivity, as shown in **Table 2**. Acephate has a small cross-reactivity in the immunoassay, most likely because the structures of methamidophos and acephate differ by a single N-acetyl group. For all other pesticides tested, cross-reactivities were below 0.1%. The lower IC_{50} values and the negligible cross-reactivity indicate that soluble scFv fragments antibodies of the three positive clones have high specificity to methamidophos.

The affinity constants of selected scFv antibodies against antigen were estimated by indirect ELISA (*27*). The values for K_D of the scFv 28D4, 29D0, and 36B2 were 3.35×10^{-8}, 2.26×10^{-8}, and 1.24×10^{-8} M, respectively, which showed that these antibodies appear to have high affinity to methamidophos.

In this study, IC_{50} values of the three positive clones were lower than could be achieved in previous work using pAbs (*31*) and mAbs (*32*) raised against methamidophos. The assay sensitivity for the scFv antibodies is similar to that of scFvs obtained by Tout et al. (*21*) and to the sensitive antibody against 2,4-D produced by Brichta et al. (*22*) using a phage library.

Recovery of Methamidophos from Fortified Environmental and Agricultural Samples. In this study, scFv antibody from scFv-36B2 clone, which possessed the lowest IC_{50}, was used to analyze methamidophos-fortified samples. Recoveries of methamidophos from tap water, rice, and cabbage samples were in the desirable range of 78−107% (**Table 3**). The recovery data of rice and cabbage samples indicate that there was no significant matrix effect in the determination of methamidophos in food samples. The tolerances for methamidophos residues on fruits and vegetables in the United States range from 0.5 to 1.0 mg/kg (*36*). These results demonstrate that soluble scFv fragments from these positive clones should have the sensitivity to be used in CI-ELISA to determine the methamidophos residues in environmental and agricultural samples.

Sequence Analysis. The amplified DNA fragments coding for the scFv-28D4, 29D0, and 36B2 were characterized by

sequence analysis. As shown in **Table 4**, all three sequences had different amino acid substitutions, and as expected, most were in the complementarity-determining regions (CDRs) (*28*). The light chains of all three clones were slightly different from each other, but all belonged to the same Vk4 family joined to the same Jk2 segment (*29*). The heavy chains of the three clones all belonged to the same J558 family. They were all joined to the J_H2 joining segments. The major variation of the three clones was found in the CDR3 of the heavy chain, with differences in both the sequence and the length (*29*).

As reported, J558 gene expression is higher in the peripheral lymphocytes, where B cells continuously encounter exogenous antigens (*37*). These differentiated B cells become progressively affinity-matured by somatic hypermutation throughout a prolonged immunization. Hapten antibody libraries frequently suffer from the low percentage of functional hapten-specific V_H-V_L pairings at the beginning of the immunization, and functional hapten antibody genes are further diluted in the course of combinatorial cloning in the subsequent library synthesis (*38*). We postulated that hyperimmunization would be required to enrich splenic B cells with hapten-selective Ab genes and, therefore, immunized each mouse eight times with a relatively large quantity (150 μg) of immunogen. This immunization protocol may have increased the percentage of hapten-specific scFvs in the final library and thus allowed us to select scFvs with increased affinity and specificity of for the methamidophos ligand.

In summary, we have successfully obtained recombinant antibodies with high specificity against organophosphate pesticide methamidophos (MW = 141 Da) directly from the phage-display scFv library of the hyperimmunized mouse. These antibodies appear to have binding properties superior to previously reported monoclonal antibodies (*30*, *32*). These antibody fragments bind with relatively high affinity to methamidophos, and preliminary data indicate that immunoassays constructed using these scFvs should have the sensitivity and specificity to detect residues of this pesticide in fruit and vegetable samples. The structure of hapten used to synthesize the immunogen and the hyperimmunization protocol most likely contributed to an increased percentage of specific scFvs for methamidophose in the final library. Selection of scFvs from the library by limiting the concentration of coating antigen and eliminating high off-rate binders with the same specificity was also used to improve the binding affinity to the small hapten under study herein. DNA sequence analysis indicated that affinity maturation of antibodies specific to methamidophos was the result of somatic hypermutation. The deduced amino acid sequences of CDRs may also help us to create other scFv fragments with even higher affinity and specificity for methamidophos via site-directed mutagenesis. The recombinant scFv fragments generated in this study may also prove invaluable in developing rapid and affordable immunoassays for quantification of methamidophos in environmental samples.

ABBREVIATIONS USED

HM3-BSA, hapten of methamidophos conjugated to bovine serum albumin; HM3-OVA, hapten of methamidophos conjugated to ovalbumin; scFv, single variable chain fragment; V_H, variable region on heavy chain; V_L, variable region on light chain; gene III, minor coat protein of filamentous phage M13; CI-ELISA, competitive inhibition enzyme-linked immunosorbent assay; CDR, complementarity determining region.

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