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ARTICLE

Development of a Mab-Based Heterologous Immunoassay for the Broad-Selective Determination of Organophosphorus Pesticides

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A broad-selective monoclonal antibody (Mab) for organophosphorus (OP) pesticides was raised using heterologous indirect enzyme-linked immunosorbent assay (ELISA) to screen hybridomas. On the basis of this Mab, five coating antigens were used to develop homologous and heterologous indirect competitive ELISAs. With the most suitable competitor, a sensitive and broad-selective ELISA was developed. The IC_{50} values were estimated to be 20.32 ng/mL for parathion, 21.44 ng/mL for methyl-parathion, 42.15 ng/mL for fenitrothion, and 58.85 ng/mL for isocarbophos. Spike recoveries were between 70.52 and 103.27% for the detection of single pesticide residues of the four OP pesticides in purple-clayed paddy soil. Moreover, the chosen ELISA was then applied to the detection of mixtures of parathion and methyl-parathion in soil samples. The average recovery and coefficient of variation were 80.91 and 4.82%, respectively. Results proved that this broad-selective ELISA would be useful for the multiresidue determination of OP pesticides.

KEYWORDS: Heterologous; monoclonal antibody (Mab); organophosphorus (OP) pesticides; broad-selective determination; enzyme-linked immunosorbent assay (ELISA)

INTRODUCTION

There is growing public concern over environmental pollution from pesticides. Organophosphorus (OP) pesticides are considered hazardous substances due to their high toxicity to nontarget species and their persistence in the environment (*1*). They can be detected in many compartments of the ecosystem and in organisms. Nowadays, chromatographic methods have been successfully used for the detection of OP pesticides (*2*). However, these applications are relatively time-consuming and usable only in a laboratory scale. On the other hand, immunoassays prove to be simple, highly sensitive, and cost-effective, and they do not need sophisticated instrumentation (*3*). Particularly, enzyme-linked immunosorbent assay (ELISA) is demonstrated as a powerful tool for high sample throughput and on-site screening analysis in pesticide monitoring programs (*4*).

Broad-selective (class-selective) detection of pesticides prior to chromatographic determination may be an attractive approach for multiresidue monitoring. If the total quantity of two or more pesticides detected in a sample is less than the maximum residue limit (MRL), the sample needs no further inspection (*5*). To date, a broad-selective immunoassay has been developed by combining more than one monospecific antibody (*6*) or using a bispecific monoclonal antibody (Mab) (*7*), and it has also been established by employing a broad-selective antibody that originated from a multihapten antigen (*8*) or a class-selective antibody derived from a generic hapten (*9*), the latter being the most common way. Up to now, numerous broad-selective immunoassays have been developed for the determination of OP pesticides using polyclonal antibodies (Pabs) (*9*−*13*), but none of them have been applied to the determination of the total quantity of mixed pesticides because of the vast differences in assay sensitivity among various pesticides.

Because Mabs offered a more definite selectivity than Pabs and no limitation in production, some investigators tried to develop ELISAs with class-level selectivity using Mabs against the common functional group of OP pesticides. Jang et al. (*14*) developed a Mab-based ELISA for four OP pesticides with the sensitivity (IC_{50}) ranging from 100 to 300 ng/mL. Liu et al. (*15*) described a heterologous ELISA for the determination of six OP pesticides, but the sensitivity was not satisfactory (IC_{50} > 580 ng/mL). Recently, a Mab-based ELISA for class-selective detection of OP pesticides was reported by Piao et al. (*5*), showing better sensitivity (IC_{50} values ranging from 2 to 282 ng/mL).

To develop broad-selective ELISAs for a total assay of two or more pesticides, the differences in sensitivities to various pesticides would be low (*16*). In this study, a broad-selective Mab against OP pesticides was selected using heterologous indirect ELISA (i.e., the immunizing hapten and the coating hapten differ in their molecular structures). On the basis of the Mab and a proper coating hapten, a heterologous immunoassay was developed that has a similar sensitivity between parathion and methyl-parathion as well as fenitrothion and isocarbophos. Results indicated that the established ELISA could be applied to detection for the total quantity of an unknown mixture of related OP pesticides.

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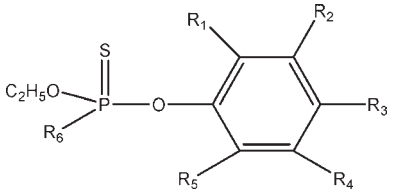
Table 1. Chemical Structures of Parathion and Its Haptens



compound	R_1	R_2	R_3	R_4	R_5	R_6
parathion	H	H	NO_2	H	H	C_2H_5O
hapten **1**	H	H	NO_2	H	H	$NH(CH_2)_4COOH$
hapten **2**	H	H	C_2H_5	H	H	$NH(CH_2)_3COOH$
hapten **3**	CH3	H	H	H	H	$NH(CH_2)_3COOH$
hapten **4**	H	CH3	H	H	H	$NH(CH_2)_3COOH$

MATERIALS AND METHODS

Reagents. Parathion and the other sorts of pesticide standards were obtained from the National Standards Co. (Beijing, China). Ovalbumin (OVA; MW, 45000), bovine serum albumin (BSA; MW, 67000), peroxidase-labeled goat antimouse immunoglobulins ($G\alpha M^{HRP}$), culture media RPMI-1640, hypoxanthine-aminopterin-thymidine, hypoxanthine-thymidine medium supplements, pristane, complete and incomplete Freund's adjuvants, dimethyl sulfoxide (DMSO), and polyethylene glycol 3350 were purchased from Sigma-Aldrich (Madrid, Spain). The SP2/0 mouse plasmacytoma line was from Shanghai Institute of Cell Biology (Shanghai, China). *O*-Phenylenediamine (OPD) and Tween-20 were purchased from Shanghai Chemical Reagents Co. (Shanghai, China). Thin-layer chromatography (TLC) was carried out on 0.2 mm precoated silica gel F_{254} (100−200 mesh, particle size) on glass sheets. Phosphate-buffered saline (PBS, 10 mM, pH 7.4), carbonate-buffered saline (CBS, 50 mM, pH 9.5), and phosphate-citrate buffer (pH 5.6) were self-prepared. All of the other chemicals and organic solvents were of analytical grade.

Instrumentation. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained from an AVANCE DMX 500 spectrometer (Bruke, Berlin, Germany) operating at 400 MHz for solutions in $CDCl_3$. Chemical shifts were given relative to tetramethylsilane. Electrospray ion mass spectra were measured using an Esquire-LC00075 mass spectrometer (Bruke). Electron ion mass spectra were obtained with a HP 5890/5973 mass spectrometer (Agilent, Wilmington, DE). Ultraviolet−visible (UV−vis) spectra were recorded on a spectrophotometer (Xinmao, Shanghai, China). Ninety-six-well and 24-well cell culture plates were purchased from Corning (Cambridge, MA). The ELISA was carried out in 96-well polystyrene microplates (JET BIOFIL, High Binding Plates, Canada). The plates were washed with a DEM plate washer (Beijing Tuopu Analytical Instruments Co. Ltd., China), and absorbencies were read with a 550 plate reader (Bio-Rad, Hercules, United States).

Hapten Synthesis. Five haptens were used in the present study. **Table 1** shows the structures of parathion and its four haptens. Hapten **5** was *p*-nitrobenzoic acid. Haptens **1** and **2** had been prepared in a previous study (*17*). Haptens **3** and **4** were synthesized using the same procedure as that for hapten **2** (*17*) but with *o*-cresol and *m*-cresol, respectively. The residue was subjected to preparative TLC [silica gel, petroleum ether:ethyl acetate:formic acid (70:30:1), R_f 0.45]. Hapten **3**: ^{1}H NMR ($CDCl_3$): δ 7.32 (1H, d, J = 8.0, Ar*H*), 7.15 (2H, m, Ar*H*), 7.06 (1H, m, Ar*H*), 4.20 (2H, m, CH_2OP), 3.36 (1H, s, N*H*), 3.17 (2H, m, CH_2NH), 2.49 (2H, m, CH_2COOH), 1.87 (2H, m, $CH_2CH_2CH_2$), 1.33 (3H, m, CH_3). Hapten **4**: ^{1}H NMR ($CDCl_3$): δ 7.19 (1H, m, Ar*H*), 7.04 (3H, m, Ar*H*), 4.18 (2H, m, CH_2OP), 3.28 (1H, s, N*H*), 3.18 (2H, m, CH_2NH), 2.46 (2H, m, CH_2COOH), 1.88 (2H, m, $CH_2CH_2CH_2$), 1.37 (3H, m, CH_3).

Preparation of Immunogens and Coating Antigens. The procedure for the preparation of the immunizing antigen (hapten **1**−BSA) and the coating antigens (haptens **1**− and **2**−OVA) had been described in a previous paper (*17*). Coating antigens (haptens **3**−**5**−OVA) were prepared by *N*-hydroxysuccinimide-active ester method, according to Manclus, et al. (*18*). The molar ratio of the initial hapten to protein was 20:1. Finally, conjugates were separated from uncoupled haptens by dialysis for 3 days using PBS. Conjugate formation was confirmed spectrophotometrically. UV−vis spectra showed qualitative differences between the carrier protein and the conjugates in the region of maximum absorbance of haptens. Molar ratios of the conjugates ranged from 2 to 15.

Mab Production and Characterization. *Immunization and Cell Fusion.* BALB/c female mice (8−10 weeks old) were immunized with hapten **1**−BSA conjugate. The immunizing strategy was the same as that reported previously (*19*). One week after the last injection, mice were tail-bled, and titers of antisera were determined by indirect ELISA. After a resting period of at least 3 weeks from the last injection in adjuvant, mice selected to be spleen donors for hybridoma production received a final intraperitoneal injection of 100 μg of conjugate dissolved in physiological saline (without adjuvant). Three days later, cell fusion procedures were carried out essentially as described by Nowinski, et al. (*20*), and the details were described previously (*19*).

Hybridoma Selection and Cloning. Twelve to fourteen days after cell fusion, culture supernatants were screened with the coating antigen hapten **5**−OVA for the presence of antibodies that could recognize parathion. Selected hybridomas were subcloned by limiting dilution. Stable antibody-producing clones were expanded and stored in liquid nitrogen.

Mab Characterization. Selected antibody-producing clones were cultured in 150 mL flasks, and the supernatants were collected. The selectivity of these Mabs was investigated by indirect competitive ELISA using hapten **5**−OVA as the coating antigen. The antibody and coating antigen concentrations were optimized by checkerboard titration. OP standards were prepared in 10% methanol−PBS (CK) including parathion (1 μg/mL), methyl-parathion (1 μg/mL), fenitrothion (4 μg/mL), fenthion (10 μg/mL), isocarbophos (2 μg/mL), triazophos (10 μg/mL), chlorpyrifos (10 μg/mL), phoxim (10 μg/mL), malathion (10 μg/mL), and trichlorfon (5 μg/mL). The concentration of these OP pesticides was determined by the original standards in methanol. For each Mab, the reactivity to OP pesticides was expressed as the percentage of inhibition calculated by the following equation: inhibition = 100% × $[(A_{CK} - A_{OP\,concentration})/A_{CK}]$. Hybridomas that could produce Mabs with broad selectivity and high sensitivity were injected intraperitoneally into pristane-primed mice to produce ascites. Then, Mabs were separated and purified by salt precipitation (with caprylic acid−ammonium sulfate) as described by Svendsen et al. (*21*).

Competitive Indirect ELISA. All incubations were carried out at 37 °C, and the plates were washed four times with PBST (10 mM PBS containing 0.05% Tween 20, pH 7.4) after each incubation, unless specified otherwise. For competition assays, the antibody and coating antigen concentrations were optimized by checkerboard titration. Standards were prepared with 10% methanol−PBS by serial dilutions from a stock solution in methanol. The ELISA was run as described in a preceding paper (*22*). Briefly, microtiter plates were coated with the optimized concentrations of antigens in CBS (100 μL/well) by incubation for 2 h and then blocked with 2% skimmed milk in PBS (300 μL/well) for 30 min of incubation. Afterward, serial dilutions (50 μL/well) of the analyte in 10% methanol−PBS were added, followed by adding 50 μL/well of Mab at a previously determined concentration. The mixture solution was allowed to incubate for 1 h, and then, 100 μL per well of diluted (1/10000) $G\alpha M^{HRP}$ was added. After another 1 h incubation, 100 μL/well of OPD substrate solution (10 mg of OPD and 30 μL of 30% H_2O_2 diluted with 25 mL of phosphate−citrate buffer at pH 5.4) was added. After it was incubated for 15 min, the reaction was stopped by adding 50 μL of 2 M H_2SO_4, and the absorbance at 490 nm was measured.

Competitive curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoid curves and linear equations were simulated by means of Microsoft Excel 2003. From the equations, IC_{50} and IC_{10} values (i.e., analyte concentrations at which the binding of the antibody to the coating conjugate were inhibited by 50 and 10%) were determined to be taken as the assay sensitivity and the limit of detection (LOD), respectively.

Cross-Reactivity (CR) Determinations. To evaluate the selectivity of the Mab, homologous and heterologous ELISAs were established to detect parathion and several related compounds. CR was calculated by the following equation:

$$CR = [IC_{50}\ (\text{parathion})/IC_{50}\ (\text{compound})] \times 100\%$$

Sample Analysis. Determination of spiked samples was performed by interpolating their mean absorbance values (three well replicates) in the standard curve run on the same plate. Purple-clayed paddy soil was

Table 2. Titers of Antiserum Using Homologous and Heterologous Coating Antigens

	titer[a]		
mouse number	coated with OVA	coated with hapten **1**−OVA	coated with hapten **5**−OVA
1	40	2.56×10^5	400
2	20−40	$>2.56 \times 10^5$	400−800

[a] Antiserum dilution was considered suitable, while the OD 490 nm value was approximately 1.0. Coating antigen concentration = 10 μg/mL.

collected (in Hangzhou, China), dried, and passed through a 60 mesh sieve. Twenty grams of soil sample, free from OP pesticide residues (checked by gas chromatography, the detection limits, calculated by using a signal-to-noise ratio of 3, were in the range of 0.002−0.006 mg/kg) was put into glass jars and spiked with 400 μL of parathion standard. The soil was mixed thoroughly with a stainless steel spatula for 5 min and then allowed to equilibrate for 12 h before being aliquoted into 10 replicates of 2 g of samples. Subsequently, acetonitrile (4 mL) was added and vortexed for 1 min, and then, the fortified sample was centrifuged for 5 min at 4000 rpm. Two milliliters of the supernatant was transferred and evaporated by gentle blowing with nitrogen. The residue was dissolved with 10% methanol−PBS. After the mixture was vigorously shaken for 5 min by a rotation shaker, a 50 μL portion was used for the selected ELISA. In addition, the spike recoveries of other individual OP pesticides and the mixture of OP pesticides were also tested.

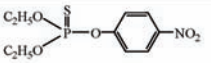
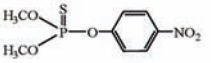
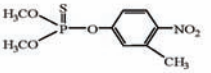
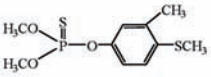
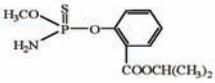
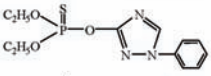
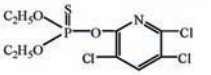
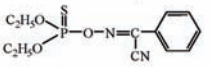
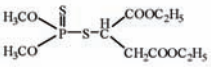
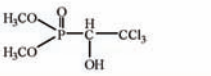
RESULTS AND DISCUSSION

Immune Response to Conjugates. In the immune system, a single B-lymphocyte produces a single type of antibody molecule. As a typical response to an immunizing conjugate, B-lymphocytes were found to produce several different antibody molecules, all directing to different parts of the immunizing conjugate (*23*). Antiserum (i.e., Pab) for a particular immunizing conjugate is always a mixture of antibodies produced by various clones of antibody-producing cells, while Mab is produced by one type of immune cells that are clones of a single parent cell. To investigate the suitability of the mouse's immune system to elicit appropriate antibodies, the antisera were collected and subsequently characterized for the presence of antibodies recognizing the conjugates (**Table 2**). The reactivity to OVA was set as a contrast. Because the molecular structures of parathion, methyl-parathion, and fenitrothion were very similar and they all contained a special nitro group in the aromatic ring, hapten **5**−OVA was used as a generic coating antigen to screen broad-selective Mabs. Results showed that mice sera displayed a high titer (2.56×10^5) level for homologous hapten conjugate (hapten **1**−BSA). Besides, the antisera containing Pabs recognized hapten 5-OVA with titers ranging from 400 to 800.

Mab Production and Characterization. In this study, haptens having a bridge at the thiophosphate group were used to prepare Mabs, and the aromatic group of parathion was selected as the determinant group. Because a single B-lymphocyte produces a single type of antibody molecule, screening for the hybridomas that could secrete broad-selective Mabs would be a pivotal process. In general, the standard screening method for the detection of hapten-specific antibodies was indirect homologous ELISA (*24*). That is, the same hapten is used for immunizing and coating, only conjugated with different proteins. Previously, we used the homologous ELISA to screen hybridomas (*19*), and the selected Mab exhibited high selectivity to parathion but little CR to methyl-parathion. Now, to choose broad-selective Mabs, indirect ELISA with a generic coating antigen (hapten **5**−OVA) was performed to screen hybridomas.

Two cell fusion experiments were conducted. After sceening, three selected Mabs (named 2F3, 5C10, and 2H6) showed

Table 3. Characterization of the Selected Mabs

Compound (concentration)	Structure	Inhibition[a] 2F3	5C10	2H6
Parathion (1 μg/mL)		High	High	High
Methyl parathion (1 μg/mL)		High	High	High
Fenitrothion (4 μg/mL)		Middle	Middle	High
Fenthion (10 μg/mL)		Low	Low	Low
Isocarbophos (2 μg/mL)		Middle	Middle	High
Triazophos (10 μg/mL)		Low	Middle	Middle
Chlorpyrifos (10 μg/mL)		Low	Low	Low
Phoxim (10 μg/mL)		Low	Low	Low
Malathion (10 μg/mL)		Low	Middle	Middle
Trichlorfon (5 μg/mL)		Low	Low	Low

[a] High, inhibition ≥70%; middle, inhibition from 50 to 70%; and low, inhibition ≤50%. OP standards were prepared in 10% methanol−PBS. The coating antigen used in this experiment was hapten **5**−OVA. Inhibition was determined from an average of three well replicates.

recognization to parathion, and their broad selectivity was investigated by testing the inhibitions from other OP pesticides under a fixed concentration. Inhibition results showed that Mab 2H6 had broader selectivity and higher sensitivity than the other two Mabs (**Table 3**). Then, the IC_{50} values of the seven OP pesticides, including parathion, methyl-parathion, fenitrothion, isocarbophos, triazophos, and malathion, were evaluated separately by indirect competitive ELISAs based on Mab 2H6 and hapten **5**−OVA. Sensitivity results of triazophos and malathion were low, with IC_{50} values larger than 2924.53 ng/mL. On the other hand, the IC_{50} values of the other four OP pesticides ranged from 57.24 to 308.20 ng/mL. These results indicated that Mab 2H6 was broad-selective to parathion, methyl-parathion, fenitrothion, and isocarbophos.

Competitive Indirect ELISA. In view of the haptens used for immunization and assay purposes, competitive ELISAs can be divided into homologous and heterologous formats (*25*). Some authors had discussed the effect of competitor heterology on the immunoassay performance and concluded that the degree of hapten heterology paralleled the degree of assay sensitivity to the target analyte (*25*, *26*). Thus, on an initial step, we explored the influence of the degree of hapten heterology on ELISA performance. When hapten **1** was used as an immunizing hapten, other designed haptens were set as competitors. In our previous study (*19*), five haptens (preservation or substituent of the special nitro group of parathion) were used to investigate the effect of the competitor structure on the selectivity and sensitivity of the monospecific Mab-based immunoassay. As a result, hapten **2** (the nitro group of parathion was substituted by an ethyl group) was selected as the optimal competitor (*19*) and, thus, was also used as a competitor in the current study. Besides, haptens **3** and **4**

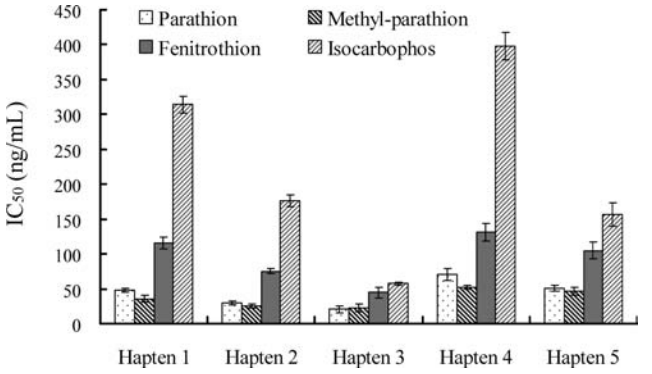


Figure 1. IC_{50} values of homologous and heterologous ELISAs against parathion, methyl-parathion, fenitrothion, and isocarbophos. Indirect ELISAs were developed using the Mab 2H6 and different coating antigens (haptens **1**−**5**−OVA). For each combination (antibody and coating antigen), competitive assays were performed at their appropriate concentrations. Data were obtained from three standard curves performed in different ELISA plates. Vertical bars indicated ±standard deviations of the mean.

Table 4. Sensitivity and LOD for OP Pesticides Using the Combination of Mab 2H6 and Hapten **3**−OVA

compound	IC_{50} value (ng/mL)[a]	CV (%)[b]	IC_{10} value (ng/mL)[a]	CV (%)[b]
parathion	20.32 ± 1.65	9.27	5.67 ± 0.87	17.46
methyl-parathion	21.44 ± 1.06	5.62	6.73 ± 0.94	15.88
fenitrothion	42.15 ± 3.10	8.39	8.42 ± 1.14	15.38
isocarbophos	58.85 ± 2.51	4.87	8.65 ± 1.50	19.78

[a] Results were the means and standard deviations of five determinations performed in different ELISA plates. [b] Coefficient of variation (CV).

(a methyl group in the aromatic ring at different position of the nitro group of parathion) were designed to further estimate the influence of the competitor structure on the performance of the broad-selective Mab-based ELISA.

With the broad-selective Mab 2H6, homologous and heterologous ELISAs were established to detect parathion, methyl-parathion, fenitrothion, and isocarbophos. Sensitivity results are shown in **Figure 1**. As for parathion, methyl-parathion, and fenitrothion, identified was the following order of IC_{50} values: hapten **4** > hapten **5** > hapten **1** > hapten **2** > hapten **3**. Also, the IC_{50} values for isocarbophos followed this order: hapten **4** > hapten **1** > hapten **2** > hapten **5** > hapten **3**. That is to say, as compared with hapten **1**-based homologous ELISA results, the heterologous systems with hapten **2** and hapten **3** both promoted the assay sensitivity to the four OP pesticides, while using hapten **4** could not improve the sensitivity to each analyte. These results revealed that employing an appropriate coating hapten could enhance the assay sensitivity of broad-selective ELISAs, which was in accord with the viewpoints mentioned by Zhang et al. (*27*) and Liu et al. (*15*).

According to the results above, the optimal competitor was hapten **3**, which gave the lowest IC_{50} values for all four OP pesticides. The detailed data of IC_{50} and IC_{10} values are listed in **Table 4**. IC_{50} values of parathion (20.32 ng/mL) and methyl-parathion (21.44 ng/mL) were similar, as well as those of fenitrothion (42.15 ng/mL) and isocarbophos (58.85 ng/mL), and their IC_{10} values were close to each other, ranging from 5.67 to 8.65 ng/mL. Concerning the reproducibility, the average CVs of the IC_{50} and IC_{10} values were 7.04 and 17.12%, respectively. Although the assays for parathion, methyl-parathion, and fenitrothion were less sensitive than those of other

Table 5. CR of OP Pesticides and Parathion-Related Compounds

	CR (%)				
compound	hapten **1**−OVA	hapten **2**−OVA	hapten **3**−OVA	hapten **4**−-OVA	hapten **5**−OVA
parathion	100	100	100	100	100
methyl parathion	**157.84**	**130.66**	**94.77**	**124.64**	**110.98**
fenitrothion	**46.04**	**42.38**	**48.21**	**49.50**	**47.16**
fenthion	<0.48[a]	<0.30[a]	<0.20[a]	<0.62[a]	<0.50[a]
isocarbophos	**15.12**	**17.33**	**34.53**	**15.94**	**32.15**
triazophos	<0.48[a]	<0.30[a]	**0.86**	<0.62[a]	<0.50[a]
chlorpyrifos	<0.48[a]	<0.30[a]	<0.20[a]	<0.62[a]	<0.50[a]
phoxim	<0.48[a]	<0.30[a]	<0.20[a]	<0.62[a]	<0.50[a]
malathion	<0.48[a]	<0.30[a]	**0.26**	<0.62[a]	<0.50[a]
trichlorfon	<0.48[a]	<0.30[a]	<0.20[a]	<0.62[a]	<0.50[a]
paraoxon	<0.48[a]	<0.30[a]	**1.13**	**0.77**	**0.63**
4-nitrophenol	<0.48[a]	<0.30[a]	**0.36**	<0.62[a]	<0.50[a]

[a] Although the IC_{50} value could not be determined accurately due to the limited solubility of the compounds at high concentrations, it was clear that inhibition was less than 50% at 10000 ng/mL. The antibody used in this experiment was the Mab 2H6.

reported, which were applied to detecting single pesticides (*19*, *28*, *29*), it was more sensitive than those for multipesticide determination (*13*−*15*). The fact that the immunizing hapten used in this study had more specificity than the generic haptens described in refs *13*−*15* probably contributed to this result. Furthermore, in spite of the widespread use of isocarbophos, few immunoassays for its detection had been reported before. The present heterologous ELISA with high sensitivity to isocarbophos would also be suitable for its residue determination.

CR Studies. Assay selectivity was evaluated by using a set of OP insecticides and metabolites with similar structures to parathion. Because some researchers reported that coating-antigen/Pab combinations could modify the immunoassay selectivity (*27*), five coating-antigen/Mab combinations were used to assess the immunoassay selectivity. Results showed that the different combinations of coating antigen/Mab could not modify the assay selectivity but affected the CR values (**Table 5**). The highest interference came from methyl-parathion (CR > 94.77%), followed by fenitrothion (CR > 42.38). However, a negligible CR was observed from the other OP pesticides whose molecules excluded or changed the nitro-aromatic group, except isocarbophos (CR > 15.12). This meant that the structure of the aromatic group was an important site for antibody recognition. The relatively high response to isocarbophos might attribute to the amino group on the phosphate moiety, which was similar to the "NH−" structure of the immunizing hapten. Moreover, the interferences by paraoxon and 4-nitrophenol were very low (< 1.13%), which implied that the presence of the phosphorothionate moiety was also essential to antibody recognition.

Sample Analysis. Recovery tests were performed using the combination of Mab 2H6 with hapten **3**−OVA to detect the OP residues spiked in soil samples. The determination of spiked samples with single pesticide was performed by interpolating their mean absorbance values in the corresponding OP standard curve run on the same plate. Control samples without OP pesticides were also systematically included in the analysis, and the absorbance was significantly lower than that of 10% methanol−PBS, which served as a control of the standard curve. It may be attributed to the matrix effect of soil samples. Dilution, which was considered as an effective means (*29*), significantly decreased matrix interference of samples, although it simultaneously caused reduction of assay sensitivity due to the shift of the dynamic range. Therefore, to compromise the sensitivity and the dynamic range of the immunoassay, soil samples were diluted to 1:2 (v/v) for the detection of parathion and methyl-parathion and to

Table 6. Recoveries of OP Pesticides from Spiked Soil Samples

analyte	spiked (ng/mL)	measured (ng/mL)[a]	recovery (%)	CV (%)
parathion	20	14.10 ± 1.94	70.52	12.13
	50	42.99 ± 2.84	85.98	5.84
methyl-parathion	20	18.42 ± 2.48	92.09	11.89
	50	39.38 ± 3.16	78.76	7.10
fenitrothion	50	36.70 ± 2.10	73.40	5.06
	100	80.92 ± 6.06	80.92	6.62
isocarbophos	50	51.64 ± 6.79	103.27	11.63
	100	99.31 ± 11.35	99.31	10.10
mixture of parathion + methyl-parathion	10 + 10	18.71 ± 1.49	93.55	7.06
	10 + 20	20.82 ± 1.05	69.40	4.46
	20 + 10	22.34 ± 0.68	74.48	2.68
	20 + 20	34.49 ± 1.98	86.22	5.07
mixture of the four OP pesticides	10 + 10 + 10 + 10	55.20 ± 9.35	137.99	14.98

[a] Data were derived from determinations of three samples at each spike level.

1:5 (v/v) for the detection of fenitrothion and isocarbophos. Recovery results are summarized in **Table 6**. The mean recoveries at two spiked levels were 78.25, 85.42, 77.16, and 101.29% for parathion, methyl-parathion, fenitrothion, and isocarbophos, respectively. The mean assay CV was also satisfactory, which was 8.80% on average and ranged from 5.06 to 12.13%.

Additionally, soil samples were fortified with the mixtures of the four OP pesticides at a final concentration of 10 + 10 + 10 + 10 ng/mL. Determined by the established immunoassay using the parathion standard curve, the mean recovery value was 137.99%, beyond the normal range of 70−120% [the criteria for assessment of analytical methods (*30*)]. This might be ascribed to the differences in assay sensitivities to these four OP pesticides, especially the disparity between parathion/methyl-parathion and fenitrothion/isocarbophos. Because the developed ELISA exhibited very close sensitivity to parathion and methyl-parathion in comparison with fenitrothion and isocarbophos, simultaneous determination of the former two pesticides spiked in soil samples was performed using the parathion standard curve. Soil samples were spiked with parathion + methyl-parathion standard mixtures at corresponding final concentrations of 10 + 10, 10 + 20, 20 + 10, and 20 + 20 ng/mL. The recovery and intra-assay CVs were 80.91 and 4.82% on average, ranging from 69.40 to 93.55% and from 2.68 to 7.06%, respectively. These results confirmed the potential of the broad-selective Mab-based immunoassay for semiquantitative screening of an unknown mixture of parathion and methyl-parathion.

ABBREVIATIONS USED

OP, organophosphorus; Mab, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; Pab, polyclonal antibody; OVA, ovalbumin; BSA, bovine serum albumin; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; CBS, carbonate-buffered saline; IC_{50} and IC_{10}, analyte concentration at which the binding of the antibody to the coating conjugate was inhibited by 50 and 10%; CR, cross-reactivity; MRL, maximum residue limit; CV, coefficient of variation; LOD, limit of detection.

LITERATURE CITED

(1) Roex, E. W. M.; Keijzers, R.; van Gestel, C. A. M. Acetylcholinesterase inhibition and increased food consumption rate in the zebrafish, danio rerio, after chronic exposure to parathion. *Aquat. Toxicol.* **2003**, *64*, 451–460.

(2) Yaqoob, M. A.; Mumtaz, M. W.; Mukhtar, H.; Anwar, F.; Shahzad, A. Determination of organophosphorous pesticide residue in water samples using gas chromatography-electron capture detector. *Asian J. Chem.* **2008**, *20*, 2064–2068.

(3) Pyo, D.; Lee, J.; Choi, E. Trace analysis of microcystins in water using enzyme-linked immunosorbent assay. *Microchem. J.* **2005**, *80*, 165–169.

(4) Watanabe, E.; Miyake, S.; Ito, S.; Baba, K.; Eun, H.; Ishizaka, M.; Endo, S. Reliable enzyme immunoassay detection for chlorothalonil: Fundamental evaluation for residue analysis and validation with gas chromatography. *J. Chromatogr. A* **2006**, *1129*, 273–282.

(5) Piao, Y. Z.; Kim, Y. J.; Kim, Y. A.; Lee, H. S.; Hammock, B. D.; Lee, Y. T. Development of ELISAs for the class-specific determination of organophosphorus pesticides. *J. Agric. Food. Chem.* **2009**, *57*, 10004–10013.

(6) Skerritt, J. H.; Hill, A. S.; Beasley, H. L.; Edward, S. L.; Mcadam, D. P. Enzyme-linked-immunosorbent-assay for quantitation of organophosphate pesticides - fenitrothion, chlorpyrifos-methyl, and pirimiphos-methyl in wheat-grain and flour-milling fractions. *J. AOAC Int.* **1992**, *75*, 519–528.

(7) Jin, R. Y.; Guo, Y. R.; Wang, C. M.; Wu, J. X.; Zhu, G. N. Development of a bispecific monoclonal antibody to pesticide carbofuran and triazophos using hybrid hybridomas. *J. Food Sci.* **2009**, *74*, T1–T6.

(8) Shu, T. W.; Wen, J. G.; Yi, R. G.; Guo, N. Z. Preparation of a multihapten antigen and broad specificity polyclonal antibodies for a multiple pesticide immunoassay. *Anal. Chim. Acta* **2007**, *587*, 287–292.

(9) Xu, Z. L.; Xie, G. M.; Li, Y. X.; Wang, B. F.; Beier, R. C.; Lei, H. T.; Wang, H.; Shen, Y. D.; Sun, Y. M. Production and characterization of a broad-specificity polyclonal antibody for o,o-diethyl organophosphorus pesticides and a quantitative structure-activity relationship study of antibody recognition. *Anal. Chim. Acta* **2009**, *647*, 90–96.

(10) tenHoeve, W.; Wynberg, H.; Jones, W. T.; Harvey, D.; Ryan, G. B.; Reynolds, P. H. S. Syntheses of haptens containing dioxaphosphorinan methoxyacetic acid linker arms for the production of antibodies to organophosphate pesticides. *Bioconjugate Chem.* **1997**, *8*, 257–266.

(11) Johnson, J. C.; Van Emon, J. M.; Pullman, D. R.; Keeper, K. R. Development and evaluation of antisera for detection of the o,o-diethyl phosphorothionate and phosphorothionothiolate organophosphorus pesticides by immunoassay. *J. Agric. Food. Chem.* **1998**, *46*, 3116–3123.

(12) Alcocer, M. J. C.; Dillon, P. P.; Manning, B. M.; Doyen, C.; Lee, H. A.; Daly, S. J.; O'Kennedy, R.; Morgan, M. R. A. Use of phosphonic acid as a generic hapten in the production of broad specificity anti-organophosphate pesticide antibody. *J. Agric. Food. Chem.* **2000**, *48*, 2228–2233.

(13) Liang, Y.; Liu, X. J.; Liu, Y.; Yu, X. Y.; Fan, M. T. Synthesis of three haptens for the class-specific immunoassay of o,o-dimethyl organophosphorus pesticides and effect of hapten heterology on immunoassay sensitivity. *Anal. Chim. Acta* **2008**, *615*, 174–183.

(14) Jang, M. S.; Lee, S. J.; Xue, X. P.; Kwon, H. M.; Ra, C. S.; Lee, Y. T.; Chung, T. Production and characterization of monoclonal antibodies to a generic hapten for class-specific determination of organophosphorus pesticides. *Bull. Korean Chem. Soc.* **2002**, *23*, 1116–1120.

(15) Liu, Y.; Lou, Y.; Xu, D.; Qian, G. L.; Zhang, Q.; Wu, R. R.; Hu, B. S.; Liu, F. Q. Production and characterization of monoclonal antibody for class-specific determination of o,o-dimethyl organophosphorus pesticides and effect of heterologous coating antigens on immunoassay sensitivity. *Microchem. J.* **2009**, *93*, 36–42.

(16) Mercader, J. V.; Montoya, A. Performance of two monoclonal immunoassays in mixtures of cross-reacting dithiophosphorus pesticides. *Talanta* **2007**, *74*, 52–58.

(17) Liu, Y. H.; Jin, M. J.; Gui, W. J.; Cheng, J. L.; Guo, Y. R.; Zhu, G. N. Hapten design and indirect competitive immunoassay for parathion determination: Correlation with molecular modeling and principal component analysis. *Anal. Chim. Acta* **2007**, *591*, 173–182.

(18) Manclus, J. J.; Primo, J.; Montoya, A. Development of enzyme-linked immunosorbent assays for the insecticide chlorpyrifos. 1. Monoclonal antibody production and immunoassay design. *J. Agric. Food. Chem.* **1996**, *44*, 4052–4062.

(19) Wang, C. M.; Liu, Y. H.; Guo, Y. R.; Liang, C. Z.; Li, X. B.; Zhu, G. N. Development of a mcab-based immunoassay for parathion and influence of the competitor structure. *Food Chem.* **2009**, *115*, 365–370.

(20) Nowinski, R. C.; Lostrom, M. E.; Tam, M. R.; Stone, M. R.; Burnette, W. N. Isolation of hybrid cell-lines producing monoclonal antibodies against the p15(e) protein of ecotropic murine leukemia viruses. *Virology* **1979**, *93*, 111–126.
(21) Svendsen, L.; Crowley, A.; Ostergaard, L. H.; Stodulski, G.; Hau, J. Development and comparison of purification strategies for chicken antibodies from egg-yolk. *Lab. Anim. Sci.* **1995**, *45*, 89–93.
(22) Gui, W. J.; Jin, R. Y.; Chen, Z. L.; Cheng, J. L.; Zhu, G. N. Hapten synthesis for enzyme-linked immunoassay of the insecticide triazophos. *Anal. Biochem.* **2006**, *357*, 9–14.
(23) Hennion, M. C.; Barcelo, D. Strengths and limitations of immunoassays for effective and efficient use for pesticide analysis in water samples: A review. *Anal. Chim. Acta* **1998**, *362*, 3–34.
(24) Cervino, C.; Weber, E.; Knopp, D.; Niessner, R. Comparison of hybridoma screening methods for the efficient detection of high-affinity hapten-specific monoclonal antibodies. *J. Immunol. Methods* **2008**, *329*, 184–193.
(25) Kim, Y. J.; Cho, Y. A.; Lee, H. S.; Lee, Y. T. Investigation of the effect of hapten heterology on immunoassay sensitivity and development of an enzyme-linked immunosorbent assay for the organophosphorus insecticide fenthion. *Anal. Chim. Acta* **2003**, *494*, 29–40.
(26) Holthues, H.; Pfeifer-Fukumura, U.; Sound, I.; Baumann, W. Evaluation of the concept of heterology in a monoclonal antibody-based ELISA utilizing direct hapten linkage to polystyrene microtiter plates. *J. Immunol. Methods* **2005**, *304*, 68–77.
(27) Zhang, Q.; Wu, Y. R.; Wang, L. M.; Hu, B. S.; Li, P. W.; Liu, F. Q. Effect of hapten structures on specific and sensitive enzyme-linked immunosorbent assays for n-methylcarbamate insecticide metolcarb. *Anal. Chim. Acta* **2008**, *625*, 87–94.
(28) Kolosova, A. Y.; Park, J. H.; Eremin, S. A.; Park, S. J.; Kang, S. J.; Shim, W. B.; Lee, H. S.; Lee, Y. T.; Chung, D. H. Comparative study of three immunoassays based on monoclonal antibodies for detection of the pesticide parathion-methyl in real samples. *Anal. Chim. Acta* **2004**, *511*, 323–331.
(29) Kim, Y. J.; Kim, Y. A.; Lee, Y. T.; Lee, H. S. Enzyme-linked immunosorbent assays for the insecticide fenitrothion - influence of hapten conformation and sample matrix on assay performance. *Anal. Chim. Acta* **2007**, *591*, 183–190.
(30) Sullivan, J. J.; Chen, Y. G.; Goh, K. S. Performance assessment and validation of a paramagnetic particle-based enzyme-linked immunosorbent assay for chlorpyrifos in agricultural runoff waters. *J. Agric. Food. Chem.* **2007**, *55*, 6407–6416.

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