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Monoclonal Antibody-Based Enzyme-Linked Immunosorbent Assays for the Organophosphorus Insecticide *O*-Ethyl *O*-4-Nitrophenyl Phenylphosphonothioate (EPN)

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This study aimed at developing competitive direct and indirect enzyme-linked immunosorbent assays (ELISAs) for the organophosphorus insecticide *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN) using a monoclonal antibody (mAb). Of the five EPN derivatives (haptens) prepared for use as an immunogen or as a competitor, two of them were used as the immunogen for the production of the mAbs. By using the antibody with the highest specificity and a coating antigen (hapten−OVA conjugate), a competitive indirect ELISA was developed, which showed an IC_{50} of 2.9 ng/mL with a detection limit of 0.3 ng/mL. A competitive direct ELISA using a different antibody and an enzyme tracer was also developed, which showed an IC_{50} of 0.6 ng/mL with a detection limit of 0.09 ng/mL. The mAbs in both assays showed negligible cross-reactivity with other organophosphorus pesticides. The recoveries of EPN from spiked samples determined by the developed ELISA ranged from 59 to 143%. Dilution of the samples improved the recovery. The assay performance of the present ELISAs based on the mAb was compared with that of the EPN ELISAs based on polyclonal antibodies (pAbs) that had been developed previously and was found to be better in dynamic response.

KEYWORDS: EPN; insecticide; immunoassay; monoclonal antibody; ELISA

INTRODUCTION

Analytical methods involving gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been used successfully for the analysis of many pesticides (*1*). However, these classical methods require a high cost, skilled analysts, and time-consuming sample preparation steps. Therefore, there is a growing demand for more rapid and economical methods for determining pesticide residues. Immunoassays began recently to gain acceptance as an alternative to the traditional methods that can meet such demands as they are fast, sensitive, and cost-effective tools for detecting trace amounts of chemicals such as pesticides (*2*).

O-Ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN) is an organophosphorus insecticide, which is effective against a wide range of insects (*3*). The most sensitive and toxicologically relevant effect after the administration of EPN is the inhibition of acetylcholinesterase activity (*4*). EPN is also reported to be an endocrine-disrupting chemical with estrogenic and antiandrogenic activity (*5*). Analysis of EPN is carried out by multiresidue methods using GC (*6*).

This paper describes the development of mAb-based ELISAs for EPN. An ELISA for this pesticide based on pAbs has been developed previously by the current authors (*7*), but an ELISA based on a mAb has not yet been reported. Although mAbs are difficult to prepare due to a complicated and time-consuming procedure, they are increasingly used in immunoassays because they have the merit that their use makes available virtually unlimited amounts of specific antibodies. However, mAbs tend to be of lower affinity than their corresponding counterparts by about an order of magnitude (*8*). Recently, we have developed several pesticide ELISAs based on both pAbs and mAbs (*9*−*12*) and have observed certain differences in assay performance between them. This paper describes such differences and discusses the possibility of using mAbs for merits other than those mentioned above.

Whereas a pAb is a heterogeneous mixture of antibody molecules, a mAb is homogeneous. Therefore, mAbs are presumed to be more specific than pAbs. Because this assumption appears not to have been verified, we attempted to test it in this study on an experimental basis. Another postulation we considered to be plausible on the basis of our comparison of the standard curves of pAb- and mAb-based ELISAs for the same pesticides was that mAbs would give steeper standard curves than pAbs. The slope of the standard curve indicates the dynamic response of an analytical method. Therefore, the additional merits of using mAbs that we attempt to identify are assay performance with regard to assay specificity and dynamic response. We tested this hypothesis by comparing the assay performance of mAb-based and pAb-based ELISAs for the three pesticides that we developed in this study and in previous studies (*9*−*12*). Comparison of the assay specificity

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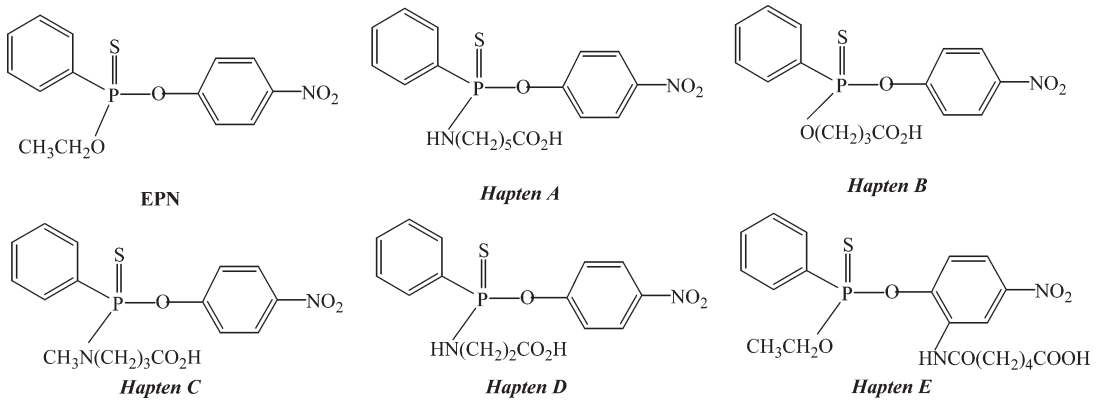


Figure 1. Structures of haptens for EPN.

was evaluated on the basis of the cross-reactivity of antibodies. Dynamic response, also called "calibration sensitivity", was then evaluated by investigating the standard curves with regard to the slope of the curve and the clarity of plateau formation.

MATERIALS AND METHODS

Chemicals and Instruments. Organophosphorus pesticides including EPN were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG and sorbitan monolaurate (Tween 20) were obtained from Sigma (St. Louis, MO). Tetramethylbenzidine (TMB) was obtained from Boehringer Mannheim (Mannheim, Germany). Microtiter plates (Maxisorp) were purchased from Nunc (Roskilde, Denmark). ELISA plates were washed with a 1575 Immuno-wash from Bio-Rad (Hercules, CA), and well absorbances were read with a Vmax microplate reader obtained from Molecular Devices (Menlo Park, CA). GC measurements were made using a Clarus 600 gas chromatograph with an electron capture detector (ECD) from Perkin-Elmer (Waltham, MA).

Synthesis of Haptens and Hapten−Protein Conjugates. The haptens used for immunization, antigen coating, and enzyme labeling are described in **Figure 1**. The synthetic procedures for these haptens have been previously reported (*7*). Hapten A was attached to keyhole limpet hemocyanin (KLH) to be used as the immunogen. Haptens A−E were attached to ovalbumin (OVA) for use as coating antigens. Haptens A−E were conjugated with HRP for use as enzyme tracers. The conjugation method used was the active ester method (*7*, *9*). Two hapten/HRP molar ratios (10 and 50) were employed in the synthesis of enzyme tracers.

Production of MAbs. Procedures for the production of mAbs against the hapten−KLH conjugates, that is, immunization, cell fusion, hybridoma selection, and cloning, were similar to those previously described in our papers (*10*). The immunogens used were hapten A−KLH and hapten B−KLH conjugates. Procedures performed differently were as follows. Homologous as well as several heterologous coating antigens were used in noncompetitive indirect ELISAs for hybridoma screening. Ascites rather than the culture supernatants were used as the antibody reagent. To obtain ascites, female BALB/c mice were given a "priming" intraperitoneal injection of 0.5 mL of tetramethylpentadecane (pristine oil). Ten days later, the mice were given an intraperitoneal injection of approximately 10^7 hybridoma cells. Ascites fluids were harvested by peritoneal tap with an 18-gauge needle on the seventh day after the cells were introduced, followed by purification by ammonium sulfate precipitation.

Competitive Indirect ELISA. Checkerboard assays, in which antibodies were titrated against various amounts of the coating antigen, were used to measure the reactivity of antibodies and to select an appropriate antigen coating and antibody dilutions for competitive indirect assays. The procedure for the checkerboard assays was the same as that for competitive assays (see below) except that addition of the pesticide standard or sample is omitted at the competition step.

From the results of the checkerboard assays, three antibodies were selected as the most suitable ones (6A, 4A, and 8B). Then, to select the most suitable coating antigen, competitive assays were performed under various combinations of immunoreagents at several concentration levels. The concentrations of the antibodies and the coating antigen chosen were further optimized. Additionally, the tolerance of ELISA to various water-miscible organic solvents used to dissolve pesticides was tested for assay optimization. For this test, standard pesticide solutions were prepared in various concentrations of acetone, acetonitrile, or methanol (10, 20, 40, and 80% in PBS, which became 5, 10, 20 and 40%, respectively, after combination with equal volumes of diluted antibody). The effect of buffering capacity of the assay solution on ELISA performance was also studied using different concentrations of phosphate ion in 20% methanol−PBS to dissolve the pesticide (10, 90, 190, and 390 mM phosphate, which became 10, 50, 100, and 200 mM, respectively, after combination with equal volumes of diluted antibody). The influence of pH of the assay solution was also studied.

The procedure of the competitive assay was as follows. All incubations except that for antigen coating were carried out at room temperature (25 °C) and, after each incubation, the plates were washed four times with PBST (10 mM PBS containing 0.05% Tween 20, pH 7.4). Microtiter plates were coated with hapten−OVA (200−1000 ng/mL, 100 μL/well) in carbonate−bicarbonate buffer (50 mM, pH 9.6) by overnight incubation at 4 °C. The plates were blocked by incubation with 1% gelatin in PBS (200 μL/well) for 1 h. Serial dilutions of the analyte in methanol−PBS (50 μL/well) were added, followed by 50 μL/well of antibody diluted (1/1000−1/10000) with PBS. After incubation for 1 h, 100 μL/well of a diluted (1/6000) goat anti-mouse IgG−HRP was added. The mixture was incubated for 1 h, and 100 μL/well of a TMB solution (400 μL of 0.6% TMB−DMSO and 100 μL of 1% H_2O_2 diluted with 25 mL of citrate−acetate buffer, pH 5.5) was added. The reaction was stopped after an appropriate time (typically 10 min) by adding 50 μL of 2 M H_2SO_4, and the absorbance was read at 450 nm.

Competitive Direct ELISA. A checkerboard assay, in which antibodies were titrated against various amounts of the enzyme tracers, was used to optimize the amount of antibody and enzyme tracer. The procedure for the checkerboard assays was the same as that for competitive assays (see below) except that addition of pesticide standard or sample is omitted at the competition step. After selection of the most suitable antibody and enzyme tracer from the checkerboard assays, their quantities for the competitive direct assays were optimized. The influence of kinds and concentrations of organic solvents, phosphate ion concentration, and pH of the assay buffer on ELISA performance was also studied using the same procedure as that for the indirect assay.

The assay procedure was as follows. All incubations except that for precoating the wells with IgG were carried out at room temperature (25 °C) and, after each incubation, plates were washed four times with PBST. Microtiter plates were precoated with anti-mouse IgG (5 μg/mL, 100 μL/well) in carbonate−bicarbonate buffer (50 mM, pH 9.6) by overnight incubation at 4 °C. The plates were loaded with 100 μL/well of the ascites dilutions (1/1000−1/10000) in PBS for 1 h. Serial dilutions of the analyte in methanol−PBS were added (50 μL/well) followed by 50 μL/well of a diluted (1/100−1/5000) enzyme tracer in 10 mM PBS. After incubation for 1 h, 100 μL/well of a TMB solution was added. The reaction was stopped after an appropriate time by adding 50 μL of 2 M H_2SO_4, and absorbance was read at 450 nm.

Determination of Cross-Reactivities. Several organophosphorus pesticides were tested for cross-reactivity using both the indirect and direct ELISA procedure described above. The cross-reactivity values were calculated as follows: (IC_{50} of EPN/IC_{50} of compound) × 100.

Analysis of Spiked Samples by ELISA. The pesticide-free vegetable samples were obtained from a local food supplier. Solutions of EPN in methanol for the fortification of vegetable and rice samples were prepared at 100, 200, 500, and 1000 ng/mL. To 1 g of the finely chopped leaves of the vegetables was added 1 mL of a spiking solution. After setting aside for 24 h, the vegetable leaves were incubated in 5 mL of methanol for 10 min with four vigorous shakes and then filtered through Whatman no. 1 filter paper. The container and the residues were rinsed with 5 mL of methanol and filtered, and the filtrate was combined with the previous filtrate. Methanol was evaporated to dryness under reduced pressure, and the residue was resuspended in 10 or 100 mL of 20% methanol−PBS (200 mM). The extract was analyzed by the indirect ELISA.

Analysis of Spiked Samples by GC. The spiked samples for GC analysis were the same as the spiked samples prepared for ELISA analysis. They were analyzed by GC at the Korea Food and Drug Administration at Daegu, Korea. The sample preparation procedure involved homogenization of spiked samples with acetonitrile, filtration, extraction of the filtrate with acetone/hexane (1:4), concentration of the organic phase, and elution on Florisil SPE with acetone/hexane (1:4). Quantification of EPN was carried out by GC using a DB-5 column (30 m × 0.25 mm i.d., 0.25 μm d_f) and ECD as the detector. The injection port temperature was 260 °C, and the detector temperature was 280 °C. Oven temperature was programmed from 80 °C (held for 2 min) to 280 °C at a rate of 10 °C/min. Nitrogen gas at 1.0 mL/min was used as the carrier gas.

RESULTS AND DISCUSSION

Production of MAbs. Among the six antisera from the mice previously injected with hapten A−KLH or hapten B−KLH, antisera M-1 (from hapten A−KLH) and M-5 (from hapten B−KLH) with the highest pAb titer (1.313 at 1/50000 dilution and 1.523 at 1/10000 after the second injection, respectively) were selected for cell fusion. Of the 96 wells of the fusion plates for each of the two antisera selected, a single well was selected, which shows the highest inhibition by EPN at 1 μg/mL in a homologous indirect ELISA. Cloning of the cells in the most inhibited well and screening of the hybridoma clones by indirect ELISA resulted in the selection of three hybridomas, 4A and 6A from hapten A and 8B from hapten B.

Competitive Indirect ELISA. The results of the experiments carried out to select the most suitable immunoreagents and their appropriate concentrations using various combinations of antibody dilutions and various amounts of the coating antigens are presented in **Table 1**. Only results with relatively high performance were presented. Antibody 8B diluted 1/1000 and the coating antigen hapten E−OVA at 500 ng/well were selected as the most suitable on the basis of the IC_{50} value that was the lowest. Hapten heterology is commonly used to eliminate problems associated with the strong affinity of the antibodies to the spacer arm that leads to no or poor inhibition by the target compound (*13*, *14*). Of the five coating haptens prepared, hapten B is homologous to the immunizing haptens in both position and structure of the bridge group, and haptens A, C, and D are homologous to the immunizing haptens in position of the bridge group, but heterologous in the structure of the bridge group. Hapten E is the most heterologous because it is heterologous in both position and structure of the bridge group. Therefore, the result is in agreement with the strategy of using the competitor with high hapten heterology to improve the assay sensitivity.

Because organic solvents are commonly used for extraction in the analysis of pesticide residues in food and environmental samples and pesticides are hardly soluble in aqueous solvent, it is desirable to use organic solvent as a cosolvent of assay solution. Then, it is necessary to assess the effect of organic solvents on ELISA performance at the competition step. The effects of solvents (acetone, acetonitrile, methanol) on the ELISA system were evaluated by preparing standard curves in buffers containing various amounts of organic solvent. The results are presented in **Table 2**. These solvents significantly influenced assay performance. The speed of color development in the presence of acetone and acetonitrile was slower compared with that in the presence of methanol. It is interesting to note that although the maximum absorbance was decreased to a large extent by increasing the concentration of acetone and acetonitrile, the change was much less with methanol. IC_{50} values in the presence of acetone and acetonitrile were much higher than those in the presence of

Table 1. Standard Curve Characteristics[a] of the Indirect ELISA[b] with Different Combinations of Antibody and Coating Antigens

antibody	dilution	coating antigen (ng/well)	*A*	*B*	*C*	*D*
4A	1/5000	hapten B (100)	0.757	0.709	49	0.054
	1/1000	hapten C (200)	0.809	0.245	59	0.035
	1/1000	hapten E (1000)	0.616	0.615	12	0.018
6A	1/1000	hapten B (200)	0.811	0.781	18	0.195
	1/5000	hapten C (100)	0.820	0.680	12	0.097
	1/500	hapten E (1000)	0.861	0.664	10	0.090
8B	1/5000	hapten B (200)	0.838	0.685	16	0.012
	1/1000	hapten C (200)	0.864	0.737	18	0.048
	1/1000	hapten E (500)	1.003	0.909	4	0.014

[a] Maximal absorbance (*A*), slope (*B*), IC_{50} (*C*, ng/mL), and minimal absorbance (*D*) are values from the four-parameter curve fit program. The data are the means of duplicates. [b] Goat anti-mouse IgG−HRP diluted 1/6000 was used.

Table 2. Influence of Organic Cosolvent, Phosphate Buffer, and pH of the Assay Solution on Indirect Competitive ELISA[a]

variable		*A*	*B*	*C*	*D*	time[b] (min)
methanol (%)	5	0.931	0.783	4.5	0.001	7
	10	0.917	0.786	3.3	0.002	8
	20	0.928	0.893	3.2	0.002	8
	40	0.806	0.603	4.3	0.086	9
acetone (%)	5	0.745	0.954	8.4	0.008	10
	10	0.731	0.672	11.4	0.007	12
	20	0.446	0.703	13.1	0.012	15
	40[c]					
acetonitrile (%)	5	0.847	0.899	10.2	0.007	10
	10	0.869	0.885	14.2	0.012	11
	20	0.398	0.478	19.4	0.008	15
	40[c]					
PBS (mM)[d]	10	0.998	0.941	4.78	0.010	10
	50	0.979	0.990	4.85	0.006	10
	100	1.023	0.985	4.56	0.011	12
	200	1.008	0.992	4.42	0.011	13
	500	1.016	0.990	4.57	0.017	15
pH	6.0	0.844	0.985	5.71	0.007	8
	6.5	0.926	1.018	6.17	0.008	10
	7.0	0.961	0.989	6.44	0.012	10
	7.4	1.013	1.063	5.63	0.014	10
	8.0	0.815	1.042	5.37	0.042	18
	8.5	0.434	1.074	6.92	0.016	20

[a] Assay conditions: antibody to hapten B−KLH, diluted 1/1000 with 10 mM PBST; coating antigen, hapten E−OVA, 500 ng/well; goat anti-mouse IgG−HRP diluted 1/6000. Maximal absorbance (*A*), slope (*B*), IC_{50} (*C*, ng/mL), and minimal absorbance (*D*) are values from the four-parameter sigmoidal fitting. The data are the means of triplicates. [b] Time for color development. [c] Data fitting was impossible due to poor color development. [d] Final concentration of phosphate ions of the competition buffer containing 138 mM NaCl and 2.7 mM KCl.

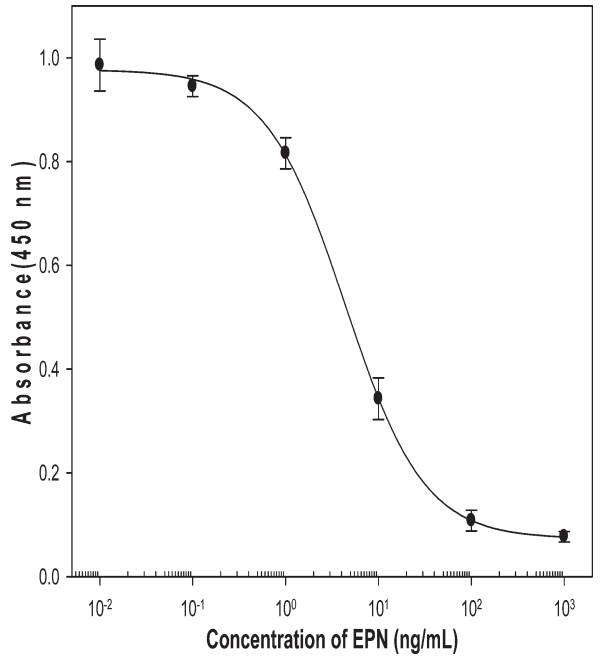


Figure 2. ELISA standard curve for EPN by indirect competitive assay. Assay conditions: antibody 8B raised against hapten B−KLH, diluted 1/1000; coating antigen, hapten E−OVA, 500 ng/well; goat anti-mouse IgG−HRP, 1/6000; organic cosolvent, 20% methanol; phosphate buffer, 200 mM; pH of assay solution, 7.4. Each point of the standard curve represents the mean of 16 determinations. Vertical bars indicate ± standard deviation about the mean.

methanol. This trend was observed repeatedly in our previous studies (*7*, *10*, *11*) and in other investigators' studies (*15*−*18*). Accordingly, we selected methanol as the most suitable cosolvent. **Table 2** shows the lowest IC_{50} value at 20% methanol.

Among the various assay parameters optimized, only the kind of organic solvent has profound influence on assay performance. Due to the nonpolar nature of EPN, it seems reasonable to assume that hydrophobic interactions are important in the antibody−analyte as well as antibody−coating antigen interactions. Because assay sensitivity is highest with methanol, it may be speculated that the ratio of antibody−analyte interaction to antibody−coating antigen interaction is highest in the medium containing methanol. Because increasing phosphate ion concentration does not enhance the assay sensitivity (see below), the higher interaction ratio appears to result from a certain factor other than the polarity of the medium.

Table 2 also presents the effect of the phosphate ion (buffer) concentration at the competition step on ELISA characteristics. Increasing the concentration of phosphate ions had little effect on assay sensitivity, in contrast to the large effect observed in pAb-based EPN ELISA (*7*). The optimum concentration selected was 200 mM phosphate, showing the lowest IC_{50} value. **Table 2** also presents the effect of pH of assay solution on ELISA. The physiological pH, pH 7.4, was selected as the optimum for the assay.

Figure 2 shows a typical inhibition curve obtained under these optimized conditions. The IC_{50} value of the assay was 2.9 ng/mL with a detection limit of 0.3 ng/mL (10% inhibition).

Competitive Direct ELISA. Antibody and enzyme tracer for direct ELISA were optimized by testing various combinations of antibodies and enzyme tracers at several concentration levels. The enzyme tracers with haptens B−E were excluded in the direct ELISA because they showed a very poor affinity to the antibody

Table 3. Influence of Organic Cosolvent, Phosphate Buffer, and pH of the Assay Solution on Direct Competitive ELISA[a]

variable		*A*	*B*	*C*	*D*	time[b] (min)
methanol (%)	5	0.804	0.920	0.78	0.007	6
	10	0.805	0.876	0.73	0.009	6
	20	0.820	0.781	0.59	0.013	8
	40	0.447	0.755	0.55	0.007	15
acetone (%)	5	0.921	0.761	2.92	0.015	8
	10	0.902	0.777	3.00	0.015	13
	20	0.371	0.750	9.20	0.001	20
	40[c]					
acetonitrile (%)	5	0.907	0.926	3.3	0.009	7
	10	0.922	0.897	3.8	0.010	9
	20	0.385	0.690	6.6	0.006	15
	40[c]					
PBS (mM)[d]	10	0.813	0.801	0.77	0.007	5
	50	0.904	0.935	0.71	0.006	7
	100	0.882	0.909	0.71	0.006	7
	200	0.889	0.863	0.70	0.008	7
	500	0.937	0.814	0.72	0.007	7
pH	6.0	0.834	0.913	0.82	0.006	6
	6.5	0.855	0.894	0.83	0.008	5
	7.0	0.927	0.876	0.78	0.007	5
	7.4	0.902	0.916	0.75	0.010	5
	8.0	0.923	0.792	0.69	0.013	6
	8.5	0.937	0.730	0.67	0.016	8

[a] Assay conditions: precoating with anti-mouse IgG (0.5 μg/well); antibody to hapten A−KLH, diluted 1/7500 with 10 mM PBS; enzyme tracer, hapten A−HRP (prepared at molar ratio 50:1, diluted 1:2500). Maximal absorbance (*A*), slope (*B*), IC_{50} (*C*, ng/mL), and minimal absorbance (*D*) are values from the four-parameter sigmoidal fitting. The data are the means of triplicates. [b] Time for color development. [c] Data fitting was impossible due to poor color development. [d] Final concentration of phosphate ions of the competition buffer containing 138 mM NaCl and 2.7 mM KCl.

(< 0.5 in absorbance over 30 min in the checkerboard assay). The optimum combination selected was antibody 6A from hapten A diluted 1/7500 and the tracer hapten A−HRP prepared at a 50:1 hapten/protein molar ratio and diluted 1/2500. Hapten A is homologous to immunizing hapten in both the position and structure of the bridge group. We repeatedly observed that direct ELISAs with homologous combinations showed better performance compared to those with heterologous ones (*7*, *19*, *20*). Here, we propose a hypothesis that there is no relationship between the assay sensitivity and hapten heterology in direct ELISAs.

The effects of various parameters on the ELISA system are presented in **Table 3**. Methanol was the most suitable cosolvent, in agreement with the results of several other studies (*21*−*23*). It may be speculated that the ratio of antibody−analyte interaction to antibody−enzyme tracer interaction is highest in the medium containing methanol. **Table 3** shows that assay sensitivity continues to improve with increasing concentration of methanol. Twenty percent was selected as the optimum concentration, because color development was so slow with 40% methanol. The optimum concentration of the phosphate buffer selected was 50 mM, which showed the strongest dynamic response (slope). The physiological pH, pH 7.4, was selected as the best one on the basis of the same criterion as for phosphate buffer.

Figure 3 shows a typical inhibition curve obtained under optimized conditions. The IC_{50} value of the assay was 0.6 ng/mL with a detection limit of 0.09 ng/mL (10% inhibition).

Cross-Reactivity Studies. Several organophosphorus pesticides were tested for cross-reactivity. **Table 4** shows the cross-reactivities that were determined by both the indirect and direct assays,

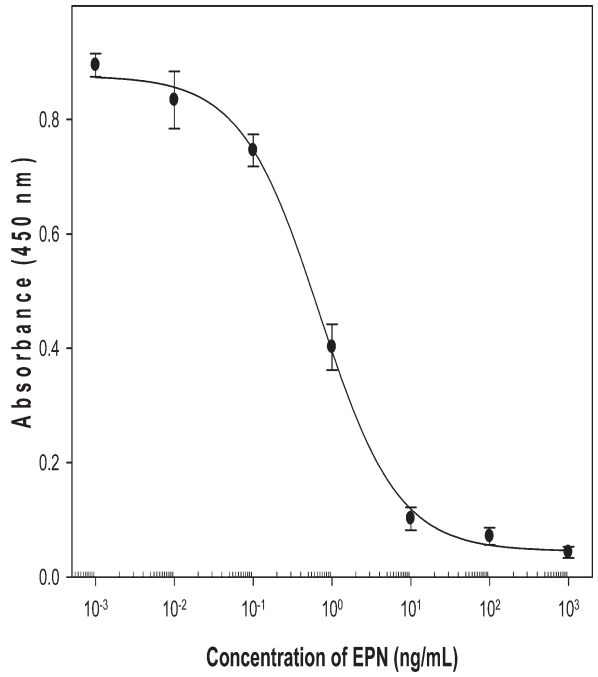


Figure 3. ELISA standard curve for EPN by direct competitive assay. Assay conditions: precoating agent, goat anti-mouse IgG, 0.5 μg/well; antibody 6A raised against hapten A−KLH, diluted 1/7500; enzyme tracer, hapten A−HRP prepared at molar ratio of 50:1 and diluted 1:2500; organic cosolvent, 20% methanol; phosphate buffer, 50 mM; pH of assay solution, 7.4. Each point of the standard curve represents the mean of 16 determinations. Vertical bars indicate ± standard deviation about the mean.

expressed in a percentage of the IC_{50} of EPN. The interference with all of the pesticides tested was negligible.

Comparison between MAb-Based and PAb-Based ELISA. It has been over three decades since immunoassays began to be developed for detecting pesticides, but studies in this field are still actively carried out (*24*−*27*). It is now certain that most widely used pesticides can be detected by immunoassay methods. However, contrary to the initial expectations, few pesticide immunoassays developed so far have been approved for official use. Therefore, we have to draw attention to the limitations of immunoassay methods and find ways to overcome the limitations. The difficulties in resolution of matrix interference and in standardization of reagents and procedures are among the various limitations of pesticide immunoassays. With regard to standardization of reagents, comparison of the capacity of pAb and mAb in immunoassay is desirable.

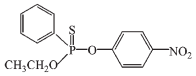
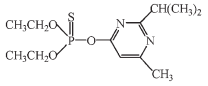
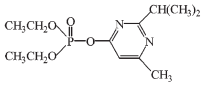
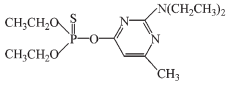
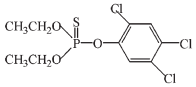
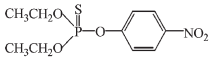
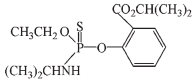
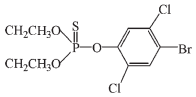
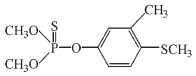
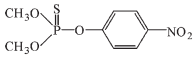
MAbs are increasingly used in immunoassays because they can be produced in unlimited quantities. We have developed several pesticide ELISAs based on both pAbs and mAbs (*9*−*12*), and comparison of their standard curves caused us to note that they look different in some aspects. Thus, we began to presume that the use of mAb might have merits other than the unlimited supply of antibodies. We assumed that the additional merits of using mAb may be better assay performance with regard to assay specificity (*28*) and dynamic response. We attempted to test this hypothesis in this study by comparing the assay performance of mAb-based EPN ELISAs developed in this study with those of pAb-based EPN ELISAs previously developed. We also extended the comparison to the ELISAs of organophosphorus pesticides isofenphos and fenitrothion that we had developed previously using both pAb and mAb (*9*−*12*).

Specificity in assay performances can be evaluated by examining the cross-reactivity of antibodies. The cross-reactivity values of the two most cross-reactive pesticides in each of the pAb- and mAb-based ELISAs for the three pesticides are presented in **Table 5**. In the case of EPN, mAb-based ELISA in both indirect and direct formats showed much better assay specificity than the pAb-based ELISAs. In the case of isofenphos, the difference was slight. The exception was the indirect ELISA for fenitrothion. The immunizing haptens for the mAb- and pAb-based ELISA for fenitrothion were slightly different in structure. Therefore, comparisons in the case of fenitrothion ELISAs were considered to be somewhat inappropriate for testing the hypothesis. More reliable data would be those in McAdam et al.'s paper on the pAb- and mAb-based ELISAs for fenitrothion (*29*). The corresponding data in their paper with pesticides excluding pesticide metabolites were (1, 1) versus (1.5, 0.5) and (8, 2) versus (3, 0.6) in indirect and direct ELISA, respectively. Their result's agreement with the hypothesis is only with the direct ELISA. Their result's disagreement is higher if pesticide metabolites are included. Overall, despite the clear correlation in the case of EPN ELISAs, we should note that the data included in this study are not sufficient to test the hypothesis that a mAb-based ELISA performs better than a pAb-based ELISA with respect to assay specificity. Theoretically, a mAb selected by clone isolation would not necessarily show an exclusive affinity to the antigen. A clone may be selected which secretes a mAb with a high affinity to an epitope that is not unique to the antigen as well as to an epitope that is unique to the antigen. Such a mAb would be less specific than a mAb with an exclusive affinity only to an epitope that is unique to the antigen. In the case of EPN, a mAb with a high affinity only to the aromatic rings that is unique to EPN would show better specificity than a mAb with a strong affinity to both the aromatic rings and the thiophosphate group [P(=S)—O−], which is common among phosphorothioate organophosphorus pesticides. Therefore, a mAb would show high specificity only when an appropriate clone is selected.

For an analytical method to be sensitive, the detectable concentrations must be low and dynamic response must be strong. The sensitivity of an ELISA is usually expressed as the IC_{50} value, which is the median value of the detectable concentration range. The dynamic response, also called "calibration sensitivity", is the change in the response signal per unit change in analyte concentration and, thus, is the slope of the calibration curve (*30*). Higher dynamic response means narrower detectable concentrations; however, it cannot be a problem because detectable concentrations can be easily adjusted by changing the dilution factor of assay mixtures. As indicated by McAdam et al. (*29*), the steepness of a standard curve is important for sensitive detection by ELISAs; however, it is rarely presented in the papers on ELISA development. We repeatedly observed that the standard curves of mAb-based ELISAs are steeper than those of pAb-based ELISAs (*9*−*12*), which indicates higher dynamic response with mAbs. **Table 5** presents the slope of the standard curves of mAb- and pAb-based ELISAs of the three pesticides. The slope of the standard curves of a mAb-based ELISA is higher than the corresponding polyclonal counterparts with no exception. Therefore, it may be concluded that a mAb-based ELISA is better than a pAb-based ELISA in dynamic response. The same trend was observed in McAdam et al.'s paper (*29*).

For a wide range of concentrations to be detected, plateaus must be clearly formed in the standard curves. Therefore, we believe that the concept of dynamic response must include clarity in the plateau formation, and an index indicating the dynamic response in this aspect must be devised. We have observed that the plateau is more clearly formed in the standard curves of mAb-based ELISAs compared to pAb-based ELISAs. There is no exception among the 12 ELISAs listed in **Table 5** (*9*−*12*).

Table 4. Cross-Reactivity of Organophosphorus Pesticides Determined Indirect and Direct Competitive ELISA[a]

Compound	Structure	Indirect ELISA		Direct ELISA	
		IC_{50}[b] (ng/mL)	CR (%)[c]	IC_{50}[b] (ng/mL)	CR (%)[c]
EPN		2.5	100	0.6	100
Diazinon		862	0.3	ni	0
Diazoxon		ni	0	ni	0
Pirimiphos-ethyl		ni	0	ni	0
Chlorpyrifos		ni	0	ni	0
Parathion		1430	0.1	1.0×10^5	0
Isofenphos		ni	0	ni	0
Bromophos-ethyl		ni	0	ni	0
Fenthion		ni	0	ni	0
Parathion-methyl		2150	0.1	2.0×10^6	0

[a] Assay conditions were the same as those described in the legend of **Figure 2** (indirect ELISA) or **3** (direct ELISA). [b] ni, no inhibition. [c] CR (cross-reactivity) (%) = (IC_{50} of EPN/IC_{50} of other compound) × 100.

Table 5. Comparison of Assay Performances with Regard to Specificity and Dynamic Response between PAb-Based and MAb-Based ELISAs for Three Organophosphorus Pesticides

	slope[a]				cross-reactivity[b]			
	indirect		direct		indirect		direct	
pesticide	pAb	mAb	pAb	mAb	pAb	mAb	pAb	mAb
isofenphos[c]	0.76	1.29	0.91	1.13	0.8, 0.6	0.37, 0.36		
fenitrothion[d]	0.89	1.44	0.61	1.07	0.92, 0.73	7.4, 5.6	8.8, 1.2	6.3, 3.3
EPN	0.59	1.02	0.61	0.87	1.5, 1.2	0.3, 0.1	5.9, 0.5	0.0, 0.0

[a] Slope of the standard curve at the concentration of IC_{50} value. [b] Cross-reactivity values of the two most interfering pesticides among the pesticides tested in common. [c] Data from refs *9* and *10*. PAb-based direct ELISA for isofenphos was not developed. [d] Data from refs *11* and *12*. The immunizing hapten for the mAb-based ELISA of fenitrothion was slightly different from that for the pAb-based ELISA.

Table 6. Recovery of EPN Spiked into Vegetables[a]

		recovery (%) by indirect ELISA		
sample	spiked concentration (ng/g)	10 mL[b]	100 mL[b]	GC
lettuce	100	63 ± 1.9	99 ± 4.5	90 ± 1.1
	200	72 ± 4.2	83 ± 10.7	94 ± 0.8
	500	78 ± 6.6	85 ± 12.4	99 ± 0.8
	1000	93 ± 3.2	81 ± 16.1	101 ± 0.5
kale	100	63 ± 3.0	99 ± 8.6	92 ± 1.2
	200	59 ± 2.2	99 ± 9.0	89 ± 1.1
	500	99 ± 0.5	81 ± 11.0	95 ± 0.8
	1000	143 ± 4.3	100 ± 7.8	99 ± 0.5

[a] Data are the means of triplicates. [b] Volume of the final extract.

Therefore, a mAb-based ELISA is better in dynamic response with regard to clarity of plateau formation.

This study shows better assay performance results of mAb-based ELISAs in dynamic response in regard to both the slope and clarity of plateau formation. However, the pAbs have merits in that they can be prepared easily and at low cost and they are usually better in affinity to the antigen, which is the main determinant of assay sensitivity in competitive assays. Therefore, the choice between the two types of antibody would be based on the time and cost for production and the purpose of the users. For example, a mAb would be more suitable for the long-term manufacture of assay kits and other applications (*28*). If the purpose is screening sera for the presence of a heterogeneous protein such as gonadotropins, a pAb would be preferable.

Recovery Studies. Two kinds of vegetables were spiked with EPN and analyzed by the indirect ELISA. Samples were prepared by extraction of 1 g of spiked crops with methanol, followed by evaporation of the solvent and dissolution of the residue in 10 or 100 mL of 20% methanol−PBS. Recoveries are presented in **Table 6**. Vegetable samples extracted into 10 mL of methanol−PBS produced recoveries that were generally underestimated, especially at lower concentrations. Recoveries from the samples extracted into 100 mL of methanol−PBS were more satisfactory. Thus, it appears that the dilution of the sample improved the recovery by reducing the matrix interference. Recovery values

determined by the ELISA were less satisfactory than those by GC in accuracy and precision. Because ELISA is a much more rapid method compared to GC, the choice between ELISA and GC would depend on the choice between rapidity and accuracy.

In conclusion, the indirect ELISA developed in this study can determine EPN residues in vegetable samples rapidly and easily by following both the simple and rapid extraction procedure and the ELISA procedure developed in this study. The comparative study regarding the capacity of the two types of antibodies shows that mAb-based ELISAs perform better than pAb-based ELISAs with regard to dynamic response in terms of both the slope and clarity of plateau formation.

ABBREVIATIONS USED

CR, cross-reactivity; ECD, electron capture detector; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IC_{50}, concentration of analyte giving 50% inhibition of the maximum absorbance; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; OVA, ovalbumin; pAb, polyclonoal antibody; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline−0.05% Tween 20; TMB, 3,3′,5,5′-tetramethylbenzidine.

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