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Development of a Microtiter Plate ELISA and a Dipstick ELISA for the Determination of the Organophosphorus Insecticide Fenthion

YOUNG AE CHO,† YOO JUNG KIM,‡ BRUCE D. HAMMOCK,§ YONG TAE LEE,‡ AND HYE-SUNG LEE*,†

Department of Food Science and Nutrition, Kyungpook National University, Daegu 702-701, Republic of Korea; Department of Biochemistry, Yeungnam University, Geungsan 712-749, Republic of Korea; and Department of Entomology and Cancer Research Center, University of California, Davis, California 95616

In previous studies, polyclonal antibodies against the organophosphorus insecticide fenthion were obtained and an indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed for this pesticide. In this study, using these antibodies and an enzyme tracer, direct competitive ELISAs for fenthion in microtiter plate and dipstick formats were developed. The microtiter plate ELISA showed an IC_{50} value of 1.2 μg/L with a detection limit of 0.1 μg/L. The antibodies showed negligible cross-reactivity with other organophosphorus pesticides. The use of the dipstick format using Immunodyne as a support membrane allowed the quick visual detection of fenthion in concentrations >10 μg/L. The IC_{50} value of the dipstick format using reflectance detection was 15 μg/L with a detection limit of 0.5 μg/L. The recoveries of fenthion from spiked vegetable samples using the two formats without any prior enrichment or cleanup steps were 87−116%.

KEYWORDS: Fenthion; insecticide; immunoassay; enzyme-linked immunosorbent assay; ELISA; dipstick

INTRODUCTION

Current analytical methods involving gas and liquid chromatography for the detection of pesticide residues are sensitive and reliable (*1*). However, they require a high cost and skilled analysts and involve time-consuming sample preparation steps. Therefore, there is a growing demand for more rapid and economical methods for determining pesticide residues. Immunoassays have recently emerged as an alternative to the traditional methods that can meet such demands (*2*, *3*). ELISAs are usually performed in laboratories using microtiter plates and, thus, are not suitable for field tests. In contrast, dipstick immunoassays allow a qualitative on-site determination of analytes. Dipstick immunoassays usually follow the standard ELISA procedure but use a membrane as antibody-coating support and rely on color development as seen with the naked eye. Their use as a diagnostic tool for detecting pesticides is becoming more common (*4*−*11*).

In our previous studies, we obtained polyclonal antibodies against the organophosphorus insecticide fenthion and developed a competitive antigen-coated (indirect) ELISA for fenthion (*12*). In this study, using the antibodies and an enzyme tracer, we developed a competitive antibody-coated (direct) ELISA for fenthion in microtiter plate and dipstick formats.

Fenthion [*O*,*O*-dimethyl *O*-[3-methyl-4-(methylthio)phenyl] phosphorothioate] is an organophosphorus insecticide with contact, stomach, and respiratory action and is used against many sucking and biting pests, especially fruit flies, leafhoppers, leaf miners, stem borers, and cereal bugs (*13*). The most sensitive and toxicologically relevant effect after administration of fenthion is inhibition of acetylcholinesterase activity (*13*).

MATERIALS AND METHODS

Chemicals and Instruments. Organophosphorus pesticides including fenthion were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Horseradish peroxidase (HRP), anti-HRP antibody, bovine serume albumin (BSA), gelatin (porcine skin), chicken egg albumin (OVA), casein, protein A, goat anti-rabbit IgG, poly(oxyethylenesorbitan) monolaurate (Tween 20), and dioctylsulfosuccinate (DSS) were purchased from Sigma (St. Louis, MO). Tetramethylbenzidine (TMB) was obtained from Boehringer Mannheim (Mannheim, Germany). The dialysis membrane (MW cutoff 12000−14000) was obtained from Spectrum Laboratories (Rancho Dominguez, CA). Biodyne B and Immunodyne ABC membranes were acquired from Pall (Pall filtrationstechnik GmbH, Dreieich, Germany). Ultrabind membrane was purchased from Pall (Germany Gelman Sciences GmbH, Rossdorf, Germany). Positively charged nylon membrane (Hybond N^+) was obtained from Amersham Int. Plc (Buckinghamshire, U.K.). Nitrocellulose and Immobilon membranes were purchased from Millipore (Billerica, MA). Microtiter plates (Maxisorp, 442404 and 439454) were

* Author to whom correspondence should be addressed (telephone +82-53-950-6231; fax +82-53-950-6229; e-mail hslee@knu.ac.kr).
† Kyungpook National University.
‡ Yeungnam University.
§ University of California.

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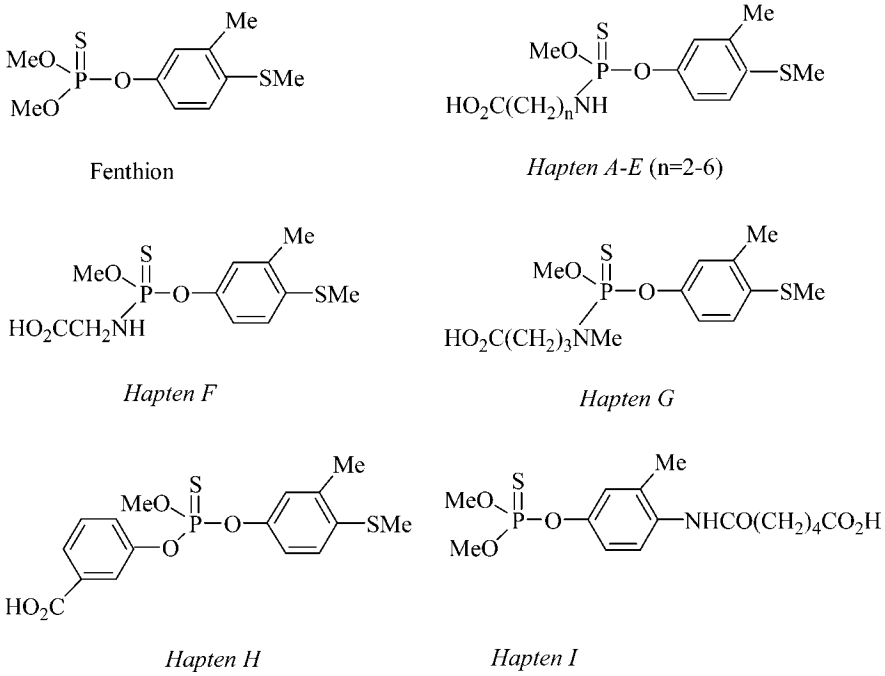


Figure 1. Structures of the fenthion haptens for preparing immunogens (haptens A–E) and enzyme tracers (haptens C and E–I).

purchased from Nunc (Roskilde, Denmark). ELISA plates were washed with a model 470175 ImmunoWash from Nunc (Roskilde, Denmark) and well absorbances were read with a model 550 plate reader from Bio-Rad (Hercules, CA). Reflectance was measured with an RQflex reflectometer from Merck (Darmstadt, Germany).

Synthesis of Haptens. The structures of the haptens that were used to prepare immunogens and enzyme tracers are presented in **Figure 1**. The procedures for the synthesis of haptens A−G are described in our previous paper (*12*). Hapten I was prepared as described by McAdam et al. (*14*).

Production of Antibodies. The production of the polyclonal antibodies against fenthion is described in our previous paper (*12*).

Synthesis of Fenthion—HRP Conjugates. Haptens C and E−I were conjugated to HRP to be used as enzyme tracers. The method of conjugation used was the active ester method (*14*), that is, synthesis of the succinimide ester of a hapten followed by coupling of the isolated ester to the protein. The procedure for the synthesis of the active esters is described in the previous paper (*12*). The reaction of the active esters with HRP was run at two hapten per HRP molar ratios: 12 and 60 for haptens C, E, and F; and 10 and 50 for haptens G, H, and I. The procedure was as follows. HRP (2 mg, 0.05 μmol) was dissolved in 1 mL of borate buffer (0.2 M, pH 8.7) to which 0.15 mL of DMF was added. A solution of an active ester dissolved in 50 μL of DMF was then added to the stirred protein solution, and stirring was continued for 24 h at 4 °C. The conjugates were separated from the uncoupled haptens by dialysis using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) followed by water. The final concentration of tracers was 0.67 g/L of buffer.

Direct ELISA on Microtiter Plates. *Checkerboard Titration.* Two-dimensional titrations, in which various dilutions of sera were titrated against various amounts of enzyme tracers, were used to have a rough estimate of their appropriate concentrations for competitive assays. The procedure for the checkerboard assays was the same as that for competitive assays (see below) except that only solvent (10% methanol/PBS) instead of pesticide solution was added at the competition step.

Assay Protocol. All incubations except that for precoating the wells with a protein were carried out at room temperature. Microtiter plates were coated with 100 μL of a precoating agent in carbonate−bicarbonate buffer (0.05 M, pH 9.6) by overnight incubation at 4 °C. The wells were washed four times with PBST (10 mM PBS containing 0.05% Tween 20, pH 7.4) and incubated with 100 μL per well of the diluted antiserum for 1 h. After another washing step, 50 μL of the serial dilutions of the analyte or sample solution in PBS−organic solvent, together with 50 μL of the tracer, was added to the wells and incubated for an appropriate time. After a final washing step, 100 μL per well of 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 by adding 50 μL of 2 M H_2SO_4, and absorbance was read at 450 nm. Competition curves were obtained by plotting $\%B/B_0$ values against the logarithm of analyte concentration: $\%B/B_0 = (A - A_{xs}/A_0 - A_{xs}) \times 100$, where A is the absorbance at a given concentration of the analyte, A_0 is the absorbance at zero dose of the analyte, and A_{xs} is the absorbance at an excess of the analyte. Sigmoidal curves were fitted to a four-parameter logistic equation (*15*), from which IC_{50} values (concentration at which binding of the antibody to the enzyme tracer is inhibited by 50%) were determined.

Optimization of ELISA Procedure. The most suitable antiserum for the direct ELISA was selected from the competition experiments using each of the antisera diluted 1:10000 and the hapten C−HRP diluted 1:10000 as the tracer. The most suitable enzyme tracer for the direct ELISA was selected from the competition experiments using each of the tracers and the selected antiserum diluted 1:10000. Then, the concentrations of the antibodies and the tracer were optimized. Protein A and anti-rabbit IgG were tested for precoating the membrane. They were compared first by noncompetitive ELISA using them at the concentrations of 0, 0.2, 1, 2.5, 5, and 10 mg/L and then by competive ELISA using them at the concentrations of 0.2, 1, and 5 mg/L and pesticide standards in 10% methanol−PBS. The effect of incubation time for the competition step on ELISA was also investigated. 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 acetone, acetonitrile, or methanol of various concentration levels (5, 10, 30, and 50% in PBS, which became 2.5, 5, 15, and 25%, respectively, by combining with antisera diluted with PBS). The influence of ionic strength of the assay solution on ELISA performance was also studied using different concentrations (5, 10, 50, 100, 500, and 1000 mM) of phosphate in the assay solution of 10% methanol−PBS. The influence of pH of the assay solution, that is, 10% methanol−PBS buffer (10 mM), on ELISA performance was also studied.

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

Dipstick ELISA. *Preparation of Test Strips. Method A.* A section of membrane cut into square pieces (0.7 × 0.7 cm) was mounted onto a polystyrene plastic strip using double-sided adhesive tape. The diluted antibodies were spotted on the membrane: 2 μL for visual detection and 5 μL for the quantitative detection using a reflectometer. The antiserum used for the dipstick ELISA was the same as the one used for the microtiter plate ELISA but was purified by ammonium sulfate precipitation (*16*). Protein concentration (6.1 g/L) was determined according to the modified Lowry assay (*17*). After a 30 min drying period, the residual binding sites of the membrane were blocked by incubating the membrane in a protein solution for 30 min. After being washed two times with PBST, the membrane was dried for 30 min. These ready-to-use test strips could be stored at 4 °C for several weeks.

Method B. This method was used to make the visual detection of color development more accurate by integrating a negative control within each test (*18*). A section of membrane (0.6 × 1.5 cm) was mounted onto a polystyrene plastic strip, as described above (method A). The diluted antibodies were spotted on the lower part of the membrane (specific reaction zone) and anti-HRP antibodies on the upper part of the membrane (negative control zone). The dilution of anti-HRP antibodies was adjusted to make the color development at the specific reaction zone and that at the negative control zone similar. The blocking of the membrane was accomplished by the same procedure as that of method A.

Method C. This method was used only for the quantitative detection of color using a reflectometer to obtain a calibration curve. A section of membrane (10 × 10 cm) was cut and incubated with the diluted antibodies for 2 h at room temperature on a horizontal shaker with gentle agitation. After washing with PBST, the membrane was blocked by incubation with a protein solution for 1 h. After being dried for 30 min, the membrane was cut into 0.7 × 0.7 cm square pieces, which were then mounted onto a polystyrene strip. These test strips could be stored at 4 °C for several weeks.

Assay Protocol. The antibody-coated dipstick was incubated with a mixture of 200 μL of the standard or sample and 200 μL of the enzyme tracer in a test tube for an appropriate time at room temperature. The dipsticks were washed three times with PBST and then incubated with 400 μL of TMB−DSS (a mixture of 400 μL of 0.6% TMB−DMSO solution, 50 μL of 1% H_2O_2, and 1 mL of 0.8% DSS−methanol diluted with 3.6 mL of citrate−acetate buffer, pH 5.5) for an appropriate time. A positive sample results in reduced or completely inhibited color development. The color intensity of the spots was visually compared with the negative control, which exhibits the most intense color development. The minimal detection limit was defined as the lowest concentration level of fenthion that was scored "positive" in five tests by six test persons. The color intensity was also estimated by measuring the absorption of the colored product with a reflectometer. Reflectance was measured at 657 nm and converted to $\%B/B_0$ values. Average values were calculated from duplicate runs for each sample using two reflectometric measurements for each determination.

Optimization of Dipstick ELISA System. The dipstick ELISA was optimized in regard to the type of membrane, the proteins for precoating and blocking the membrane, the quantities of antibodies and enzyme tracers, the times for competition and color development, and the concentration of methanol used to dissolve the pesticide in assay solution. For the experiments carried out to select the best membrane and blocking agent, color development of only the negative control was examined. For the other experiments, color development of both the negative control and a pesticide-containing solution were examined.

Several types of membranes that were commercially available were tested for the suitability of immobilizing antibodies. The membranes tested were Biodyne B, Immunodyne ABC, Ultrabind, Hybond N^+, nitrocellulose, and Immobilon membrane. After the selection experiment, the effect of the pore size of the selected membrane (Immunodyne) on ELISA performance was examined using pore sizes of 0.45, 1.2, 3, and 8 μm.

The proteins tested for precoating the membrane were protein A and goat anti-rabbit IgG. Color development with and without membrane precoating was compared.

The proteins tested for blocking the membrane were OVA, BSA, gelatin, and casein (1%). The concentration of the selected protein (casein) was optimized by comparing the color developments at several concentrations of it (0.1−5%).

After these optimization experiments, a final result of the dipstick assay under optimized conditions and at several concentration levels of fenthion was obtained. The membranes prepared by using methods B and C were used.

Analysis of Fenthion in Food Samples. Rice and lettuce grown pesticide-free were finely ground and chopped, respectively. To 1 g of the ground or chopped sample was added a fortification solution (5 mL for microtiter plate ELISA, 3 mL for dipstick ELISA). After several vigorous shakes during a 10 min period, the suspension was filtered and 1 mL of the supernatant was diluted with PBS (1:10 for microtiter plate ELISA; 1:3 for dipstick ELISA). The diluted samples were analyzed by both the microtiter plate ELISA and the dipstick ELISA using the membrane prepared according to method C.

Table 1. Standard Curve Characteristics of the Microtiter Plate ELISA Using Different Enzyme Tracers[a]

enzyme tracer	ng/well	hapten/HRP molar ratio[b]	A_{max}	slope	IC_{50} (μg/L)	A_{min}
hapten C–HRP	1.7	6	1.086	0.585	1.558	0.014
	1.7	12	0.978	0.426	5.084	0.149
hapten E–HRP	1.7	6	1.368	0.670	7.111	0.008
	1.7	12	0.563	0.595	8.441	0.005
hapten I–HRP	17	10	1.452	0.578	2.193	0.033
	68	50	0.439	0.522	172.5	0.140

[a] Assay conditions: precoating with protein A (0.5 μg per well); antiserum raised against hapten C–KLH, diluted 1:10000 with 10 mM PBS. The parameters of the assays were obtained from the four-parameter sigmoidal fitting. Data are the means of triplicates. [b] The hapten to HRP molar ratio employed in coupling reaction between the two.

RESULTS AND DISCUSSION

Microtiter Plate ELISA. A prerequisite for a potent ELISA is availability of not only antibodies with a high affinity toward the analyte but also an enzyme tracer that exhibits a high activity and can be easily replaced by the analyte. The antibody used in this study was the one against hapten C−KLH, which was characterized in our earlier study as having a high affinity and specificity for fenthion (*12*). To select the most suitable enzyme tracer for the direct ELISA, optimum concentrations of the antiserum and enzyme tracers for ELISA were determined by the checkerboard assays in which several dilutions of the sera were titrated against various amounts of the enzyme tracers. Then, competition curves were obtained under the optimum condition using each of the six enzyme tracers (haptens C and E−I conjugated to HRP). The tracers containing haptens F, G, and H were found to be unsuitable for ELISA due to the poor color development they gave. Because their enzyme activities were found to be similar to those of the other tracers, poor color development must come from their weak affinities to the antibodies, rather than their lowered enzyme activity. The poor affinity of the tracers with haptens G and H is understandable because these haptens are heterologous to the immunizing hapten (hapten C) in the structure and length of the spacer arm. However, because the most heterologous hapten I performs relatively well on the assay, it appears that the enzyme acitivity of the tracers containing haptens F and G decreases after binding to the antibodies. The poor affinity of the tracer with hapten F, which is homologous in the site and structure of spacer arm, but heterologous in only spacer length, can be attributed to masking of the hapten determinant groups by the enzyme due to the very short spacer arm. **Table 1** shows the result of the ELISA using the tracers with relatively high affinity to the antibodies. The tracers prepared at lower hapten per enzyme

molar ratios provided higher assay sensitivity (lower IC_{50} values) and faster color development (higher A_{max}). The lowest IC_{50} value was observed with the completely homologous tracer (hapten C−HRP) prepared at a hapten per enzyme molar ratio of 6. Further experiments using hapten C−HRP prepared at different hapten per protein molar ratios (1.2, 3.6, 6, 12, 60, and 120) confirmed that the ratio of 6 is the best one. This molar ratio is considerably lower than usually employed in the conjugation of other proteins, which may be attributed to the availability of only two lysine residues for the conjugation on the peroxidase molecule.

It is generally accepted that heterology between immunizing and competitor hapten is an important factor for improving the sensitivity of an immunoassay. Heterology results in weaker recognition of antibodies to the competitor, thus allowing analyte at lower concentrations to compete with the competitor hapten. However, the affinity of the competitor to the antibody must be high enough for rapid color development. Therefore, in our case, the affinity of the competitor for the antibody appeared to be more important than the hapten heterology for improving ELISA sensitivity. We have repeatedly observed this trend in our studies on the development of direct ELISA for several organophosphorus pesticides (*19*, *20*).

Protein A and anti-animal IgG are the most frequently used precoating agents for antibody-coated ELISA, because they bind to the Fc region of the antibody molecule, leaving the Fab region more accessible to the antigen molecule. They were tested at several concentrations. In both cases, increasing the concentration of the protein caused an initial increase followed by a plateau in the rate of color development. Maximum absorbance was reached at 2.5 and 5 mg/L with protein A and anti-rabbit IgG, respectively. Protein A was better than anti-rabbit IgG in trapping antibodies because absorbance with the former protein was ~3-fold higher than that with the latter protein. From the results of the competition experiments using protein A at various concentration levels, 5 mg/L was selected as the most suitable concentration on the basis of the IC_{50} value that was the lowest.

Because the use of organic solvents for extraction and/or solid phase cleanup is very common in the analysis of nonpolar pesticide residues in food samples, it is desirable to assess the effect of organic solvents on ELISA performance. The effects of organic solvents (acetone, acetonitrile, and methanol) in assay solution on the ELISA system are presented in **Figure 2**. These solvents significantly influenced assay performance. Color development at the competition step decreased with increasing concentration of acetone and acetonitrile, resulting in >50% retardation of color development at 15 and 25% concentrations. Such an effect was not observed with methanol. IC_{50} values in the presence of acetone and acetonitrile were much higher than those in the presence of methanol. Therefore, methanol was the most suitable cosolvent for the direct assay, in agreement with the results of several other studies (*19*−*22*). The optimum concentration of methanol selected was 5%, where A_{max} per IC_{50} value was the highest. The effect of the concentration of the phosphate ion (5−1000 mM) on ELISA performance was not remarkable. The optimum concentration selected was 10 mM, at which A_{max} per IC_{50} value was the highest. The effect of the pH (5−10) of the assay solution on ELISA was remarkable only at the extreme pH values (5 and 10), where A_{max} per IC_{50} value is much smaller. The physiological pH, pH 7.4, was selected as the best one on the basis of the highest A_{max} per IC_{50} value.

The optimum conditions selected consisted of the antiserum raised against hapten C−KLH, diluted 1:10000, precoating the plate with 0.5 μg per well of protein A, the enzyme tracer hapten C−HRP at 1.7 ng per well, and an assay solution of 5% methanol−PBS (10 mM, pH 7.4). A typical standard curve for fenthion obtained under the optimum condition is presented in **Figure 3**. The IC_{50} value was 1.2 μg/L.

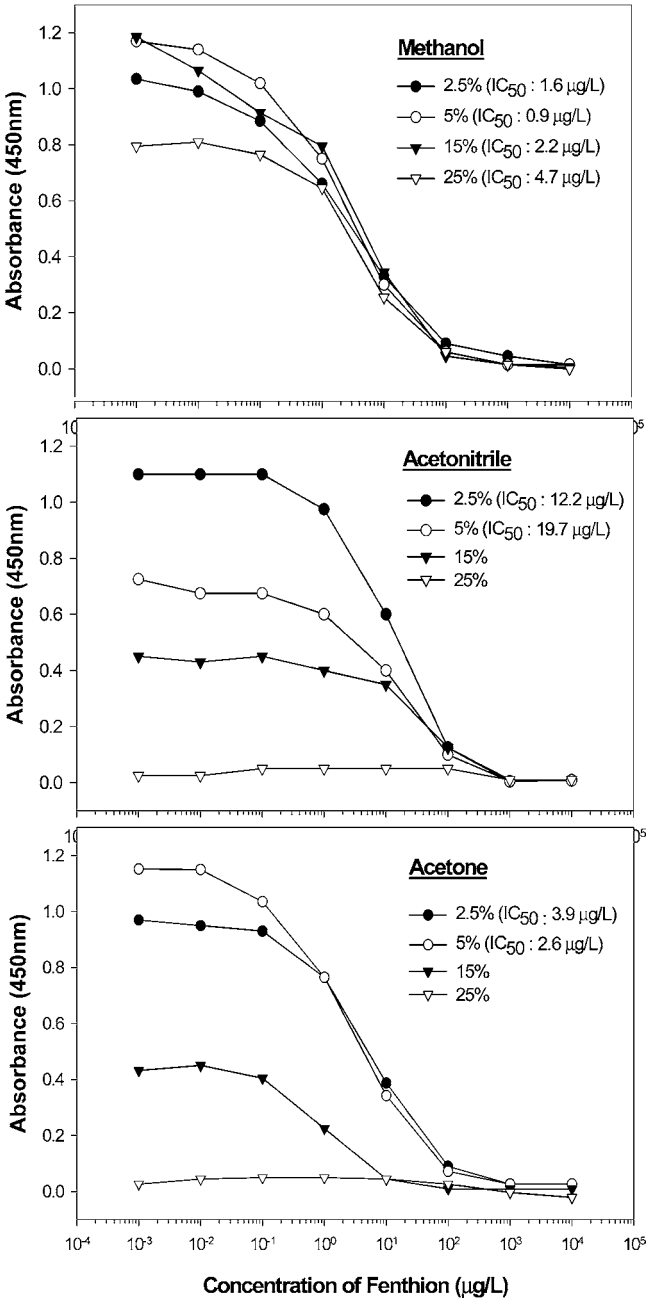


Figure 2. Influence of organic solvents on fenthion detection by the microtiter plate ELISA. Reagent concentrations: precoating agent protein A, 0.5 μg per well; antiserum raised against hapten C–KLH, diluted 1:10000, enzyme tracer (hapten C–HRP) at 3.4 ng per well.

Cross-Reactivity Studies. Several organophosphorus pesticides as well as the metabolite of fenthion were tested for cross-reactivities. **Table 2** shows the cross-reactivity that was found by the direct ELISA. The ELISA showed negligible cross-reactivity with all of the substances tested. It may be concluded that the competitive ELISA that was developed is suitable for the sensitive and selective detection of fenthion.

Dipstick ELISA Format. To develop an immunoassay in a dipstick format, the choice of a suitable antibody support material is of crucial importance. Of the membranes tested for the suitability of immobilizing the antibodies on their surface,

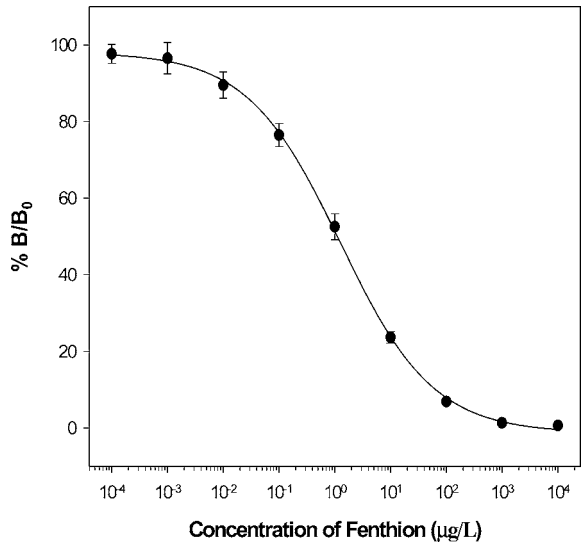
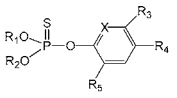
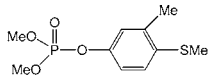
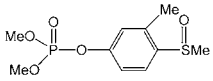
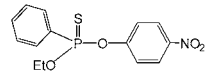
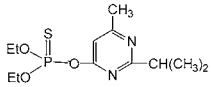
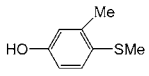


Figure 3. Competition curves for fenthion by the microtiter plate ELISA. Reagent concentrations: same as described in the caption of **Figure 2** except that the enzyme tracer was diluted to 1.7 ng per well. Each point represents the mean of 16 determinants. Vertical bars indicate ±SD about the mean.

Table 2. Cross-Reactivity of Compounds Structurally Related to Fenthion Determined by Direct Competitive ELISA[a]



Compound	Structure						I_{50} (μg/L)	CR(%)[b]
	R_1	R_2	X	R_3	R_4	R_5		
Fenthion	Me	Me	C	Me	SMe	H	1.20	100
Fenitrothion	Me	Me	C	Me	NO_2	H	261	0.46
Parathion	Et	Et	C	H	NO_2	H	3000	0.04
Parathion-methyl	Me	Me	C	H	NO_2	H	300	0.40
Bromophos-methyl	Me	Me	C	Cl	Br	Cl	2400	0.05
Dichlofenthion	Et	Et	C	H	Cl	Cl	2400	0.05
Chlorpyrifos-methyl	Me	Me	N	Cl	Cl	Cl	30000	0.004
Oxidized fenthion							923	0.13
Oxidized fenthion							203	0.59
EPN							857	0.14
Diazinon							30000	0.004
Dimethylchloro-Thiophosphate							$>10^5$	0
4-(Methylthio)-*m*-cresol							2400	0.05

[a] Assay conditions were the same as those described in **Figure 3**. [b] Cross-reactivity (%) = (IC_{50} of fenthion/IC_{50} of other compound) × 100.

nitrocellulose and Immobilon membranes, which are known to bind proteins via hydrophobic interactions, exhibited initially a strong color precipitate, but the color leached into the solution and disappeared almost completely in a few minutes. The intensity of the dot color did not change within a few minutes with other membranes. Immunodyne membrane, which has active functional groups on its surface that covalently bind proteins, was selected as the most suitable for this study on the basis of the color intensity, which was strongest. After the selection of a membrane, the effect of the pore size (0.45−8 μm) of the Immunodyne membrane was examined. With increasing pore size, the size of the dot increased, the color intensity decreased, and the background color increased. Therefore, the pore size of 0.45 μm was selected as the most suitable.

Precoating the membrane with protein A or anti-mouse IgG prior to antibody immobilization resulted in a decrease of color intensity and an increase of background color development. Immunodyne membrane seemed to have enough surface area to compensate for the random attachment of antibodies. Therefore, the precoating step was omitted.

Because the adsorptive surface area of membranes is 100−1000 times greater than plastic (*23*), nonspecific binding of enzyme tracer is a more serious problem in dipstick immunoassay than in polystyrene plate assay. Therefore, it is essential to block the antibody-immobilized membrane. The order of signal per noise ratio for several blocking agents tested (1%) was casein > BSA > OVA > gelatin > control. In an experiment carried out to select the most suitable concentration of the selected blocking agent (casein), nonspecific binding of enzyme tracer was lowest at the concentration of 2%.

Because the color developed on the membrane is relatively unstable, the timing of color development should be considered critical. For the times tested for color development (1−10 min), signal per noise ratio showed an initial increase up to 4 min followed by a slow decrease. A color development time of 5 min was chosen as the optimum.

Among the times tested for competition (5−60 min), 20 min was chosen as the optimum. A prolonged time resulted in higher background noise with no enhancement of color intensity, whereas a shorter time resulted in a weaker signal.

The effect of methanol in the competition assay solution on the dipstick assay was evaluated by obtaining standard curves at several concentration levels of methanol (5, 10, 15, 25, 35, and 50%). The color intensity was nearly the same at all concentrations up to 15% and then continued to decrease. A concentration of 15% was chosen as the optimum.

Optimization of the concentrations of tracer and antibody was achieved by checkerboard assay. The optimum dilutions of the antibody and the tracer were 1:16 and 1:8000, respectively, for the semiquantitative assays (visual detection using the membranes prepared by using methods A and B) and 1:100 and 1:40000, respectively, for the quantitative assays (reflectance detection using the membranes prepared by using method C).

Compared to the corresponding microwell plate ELISA, dipstick assays reached equilibrium of the specific immunoreaction much more quickly. The total assay time for dipstick ELISA using antibody-coated membrane was ∼30 min. Ordinary dipstick assays require a negative control to discriminate "reduced color development" of positive samples. The assays using an "integrative negative control" (*18*), which places a negative control and reaction zone on the same strip and produces color development at the same time, reduce the test protocol as well as the materials required. Goat anti-HRP antibodies used in this study as an integrated negative control were found to be a simple and reproducible way to provide a negative control within each test. We found that an anti-fenthion antibody diluted 1:16, anti-HRP antibody diluted 1:1500, and tracer diluted 1:4000 were most suitable. **Figure 4** presents the results of the assay using an integrative negative control. A fenthion concentration of 10 μg/L caused a slight but distin-

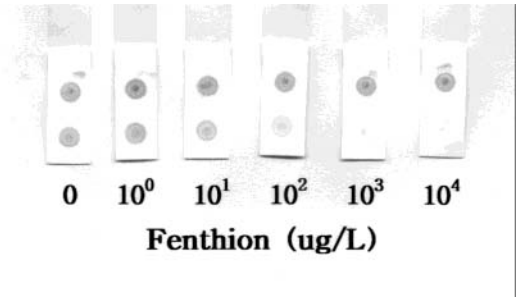


Figure 4. Result of the dipstick ELISA at different fenthion concentrations with the integrative negative control. Reagent concentrations: antibody prepared by ammonium sulfate precipitation of the antiserum raised against hapten C–KLH, diluted 1:16; enzyme tracer (hapten C–HRP), diluted 1:4000.

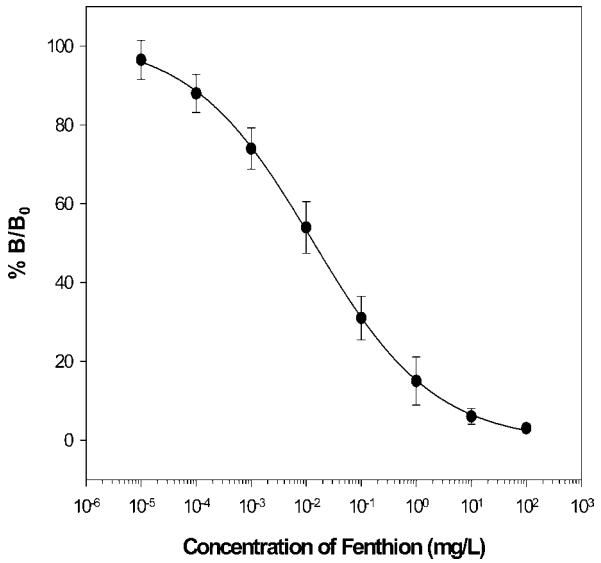


Figure 5. Competition curves for fenthion by the dipstick ELISA. Reagent concentrations: same as described in the caption of **Figure 4** except that antibody was diluted 1:100 and enzyme tracer was diluted 1:40000. Each point represents the mean of six determinants. Vertical bars indicate ±SD about the mean.

Table 3. Recovery of Fenthion Spiked into Food Samples[a]

fortified concn (μg/L)	microtiter plate ELISA		dipstick ELISA	
	rice	lettuce	rice	lettuce
0	88	94	96	90
0.01	90	95		
0.1	91	101		
1	95	101	87	93
10	94	116	95	105
100	92	102	110	98
1000			95	94

[a] Assay conditions were the same as those described in **Figure 3** (microtiter plate ELISA) or **5** (dipstick ELISA). Data are the means of triplicates.

guishable difference compared to the negative control. Therefore, the detection limit for fenthion using visual detection was considered to be 10 μg/L. Fenthion levels >1000 μg/ L resulted in a complete suppression of the color development.

Figure 5 presents the calibration curve for fenthion using reflectance detection. The IC_{50} value for fenthion was 15 μg/L with a detection limit of 0.5 μg/L.

Analysis of Fenthion in Food Samples. The recoveries of fenthion spiked into food samples determined by the ELISAs are presented in **Table 3**. The recoveries determined by the microtiter plate and dipstick ELISAs were 90−116 and 87−110%, respectively. The plot of the recovered pesticide against the spiked pesticide for all combinations gave regression lines with slope and intercepts very close to 1 (0.998−1.012) and 0 (−0.042−0.011), respectively. Therefore, there was no significant matrix effect in the determination of fenthion in food samples, and it can be stated that the samples can be analyzed directly by both of the ELISAs without the need of any cleanup steps.

In conclusion, the ELISAs developed in this study can be used as convenient tools for the rapid and pretreatment-free screening of fenthion residues in food samples.

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