Development of an ELISA for the Organophosphorus Insecticide Isofenphos

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Received August 27, 2001

A selective enzyme-linked immunosorbent assay (ELISA) for the insecticide isofenphos was developed. Three different analogues (haptens) of isofenphos were synthesized and were coupled to carrier proteins through the pesticide thiophosphate group to use as immunogens or coating antigens. Rabbits were immunized with one of the haptens coupled to BSA for production of polyclonal antibodies and the sera were screened against each of the other two haptens coupled to ovalbumin (OVA). Using the sera of highest specificity, an antigen-coated ELISA was developed, which showed an I_{50} of 96 ng/mL with the detection limit of 2 ng/mL. The antibodies showed negligible cross-reactivity with other organophosphorus pesticides and the phenol metabolite of isofenphos, which makes the developed assay suitable for the selective detection of isofenphos. An antibody-coated ELISA was also developed, which showed an I_{50} of 580 ng/mL with a detection limit of 70 ng/mL.

Keywords : Isofenphos, Insecticide, Enzyme-linked immunosorbent assay, ELISA.

Introduction

Due to the widespread use of pesticides, there is a growing concern over the environmental contamination caused by their residues. The current methods such as gas chromatography and high-performance liquid chromatography have been used successfully for analysis of many pesticides.[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 are being demonstrated as a suitable alternative to the traditional methods that can meet such demands. They began recently to gain acceptance as a fast, sensitive, and cost-effective tool for environmental analysis.[2]

Isofenphos (1-methylethyl 2-[[ethoxy[(1-methylethyl)amino]-phosphinothioyl]oxy]benzoate) is an organophosphorus insecticide used for the control of various crop pests.[3] The toxicological effect after administration of isofenphos is inhibition of acetylcholinesterase activity.[3] The objective of this study is the development of an ELISA for isofenphos. ELISA for this pesticde has not yet been developed.

The development of an immunoassay requires the production of antibodies to the analyte. Since pesticides are small molecules, pesticide derivatives, namely haptens, must be synthesized and coupled to carrier proteins to induce antibody production. One type of hapten for organophosphorus pesticides is the one with an aminocarboxylic acid bridge at thiophosphate group, which has been used successfully for the development of ELISA for several organophosphorus pesticides.[4-6] We have developed a novel method for the synthesis of such haptens, which is easier than the previous one. In this study, this new method was applied to the synthesis of haptens for isofenphos, from which specific antibodies to isofenphos were obtained. Using the antibodies, sensitive and selective ELISAs for isofenphos were developed.

Experimental Section

Reagents and instruments. Organophosphorus pesticides were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Isopropyl salicylate, ethyl dichlorothiophosphate, 4-aminobutyric acid, 3-(methylamino)butyric acid hydrochloride, DL-3-aminobutyric acid, *N*-hydroxysuccinimide, 4-dimethylaminopyridine, 1,3-dicyclohexylcarbodiimide, $CHCl_3$-*d*, silica gel for column chromatography (60-230 mesh) and Tween 20 were obtained from Aldrich (Milwaukee, USA). BSA (A-3059), OVA (A-2512), peroxidase labeled goat anti-rabbit IgG (A-6154), Freund's complete (F-5881) and incomplete (F-5506) adjuvants, and Sephadex G-25 were purchased from Sigma (St. Louis, USA). KLH (374805) was from Calbiochem (La Jolla, USA) and tetramethylbenzidine was from Boehringer Mannheim (Mannheim, Germany). Analytical (silica gel F254) and preparative TLC plates (silica gel, 1 mm) were obtained from Merck (Darmstadt, Germany). The dialysis membrane (MW cutoff 12000-14000) was a Spectra/Por product from Spectrum Laboratories (Rancho Dominguez, USA). Microtiter plates (Maxisorp, 439454) were purchased from Nunc (Roskilde, Denmark). ELISA plates were washed with a Model 1575 ImmunoWash from Bio-Rad (Hercules, USA) and well absorbances were read with a V_{max} microplate reader from Molecular Devices (Menlo Park, USA). UV-Vis spectra were recorded on a Varian Cary 3 spectrophotometer. NMR spectra were obtained with a Bruker (Rheinstetten, Germany) ARX spectrometer (300 MHz). Chemical shift values are given relative to internal tetramethylsilane. Coupling constants are expressed in Hz and the abbreviations s, d, t, q, qn, sp, m, and ar represent singlet, doublet, triplet, quartet, quintet, septet, multiplet, and aromatic, respectively.

Hapten synthesis. The haptens used for immunization and antigen coating are presented in Figure 1. The synthetic route for hapten 1 is illustrated in Figure 2. Other haptens

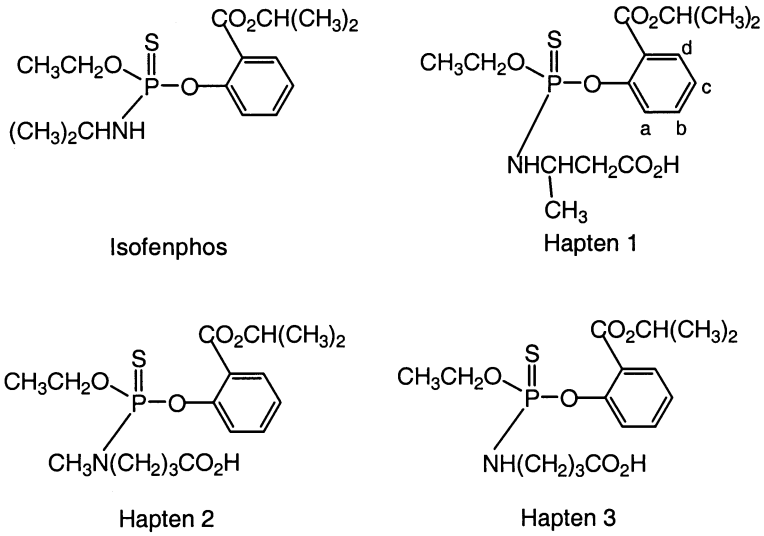

Figure 1. Structures of the haptens for isofenphos used for immunization (Hapten 1), antigen coating (Hapten 2) and enzyme tracer (Hapten 2 and Hapten 3).

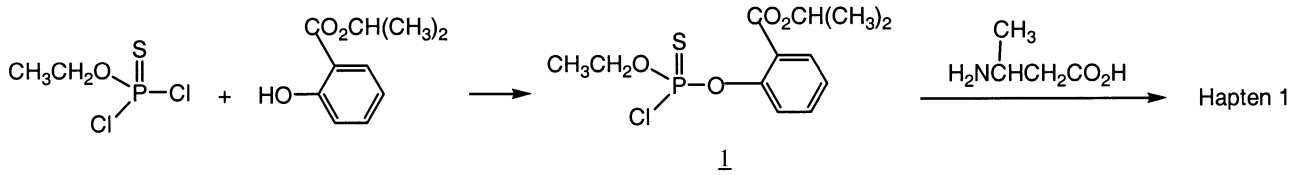

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Figure 2. Synthetic route for Hapten 1.

were synthesized by the same route using the necessary aminocarboxylic acids.

1. To a stirred mixture of 2.96 g (17 mmol) of ethyl dichlorothiophosphate, 5 g of finely ground K_2CO_3 and 10 mL acetonitrile was added dropwise 1.96 g (11 mmol) of isopropyl salicylate dissolved in 20 mL of acetonitrile. After stirring for 40 min at room temperature, the mixture was filtered through celite and the solvent was removed under reduced pressure. The residue was subjected to column chromatography (silica gel, 10 : 1 hexane-ethyl acetate) to give 2.15 g (61%) of the product as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.91 (H_d, d x d, J = 7.7 & 1.2, ar), 7.55 (H_a, d x d, J = 8.5 & 1.5, ar), 7.48 (H_c, t x t, J = 8.4 & 1.6, ar), 7.32 (H_b, t x t, J = 7.5 & 1.5, ar), 5.25 (1H, sp, J = 6.3, $(CH_3)_2C\underline{H}$), 4.48 (2H, q, J = 7.1, $C\underline{H}_2CH_3$), 1.47 (3H, t, J = 7.1, $CH_2C\underline{H}_3$), 1.38 (6H, d, J = 6.3, $CH(C\underline{H}_3)_2$).

Hapten 1. To a stirred solution of 67 mg (0.21 mmol) of **1** in 0.2 mL of methanol cooled in an ice-water bath was added dropwise a solution of 31 m g (0.55 mmol) of KOH and 26 mg (0.25 mmol) of DL-3-aminobutyric acid in 260 μL methanol. After stirring for 5 min, the reaction mixture was filtered and the solvent was evaporated. Column chromatography (silica gel, 29 : 9 : 1 $CHCl_3$-ethyl acetate-acetic acid) of the residue gave 54 mg (66%) of a white solid. ^{1}H NMR ($CDCl_3$): δ 7.81 (H_d, d x d, J = 8.9 & 1.2, ar), 7.60 (H_a, d x qn, J = 8.2 & 1.5, ar), 7.48 (H_c, t x t, J = 7.9 & 1.8, ar), 7.21 (H_b, t x t, J = 7.5 & 1.1, ar), 5.25 (1H, sp, J = 6.2, $(CH_3)_2C\underline{H}$), 4.29 (1H, q, J = 9.5, $N\underline{H}CH$), 4.20 (2H, q, J = 7.1, $C\underline{H}_2CH_3$), 3.97 (1H, sp, J = 6.0, $NHC\underline{H}$), 2.45 (2H, t, J = 6.3, $C\underline{H}_2CO_2$), 1.38 (3H, t, J = 7.0, $CH_2C\underline{H}_3$), 1.37 (6H, d, J = 6.2, $CH(C\underline{H}_3)_2$), 1.28-1.34 (3H, $NHCHC\underline{H}_3$).

Hapten 2. This hapten was synthesized by the same procedure as that for Hapten 1 using **1** and 3-(methylamino)-butyric acid hydrochloride. Yield 42%. ^{1}H NMR ($CDCl_3$): δ 7.82 (H_d, d x d, J = 8.8 & 1.1, ar), 7.60 (H_a, d x qn, J = 8.3 & 1.6, ar), 7.48 (H_c, t x t, J = 7.8 & 1.8, ar), 7.22 (H_b, t x t, J = 7.6 & 1.2, ar), 5.24 (1H, sp, J = 6.2, $(CH_3)_2$ $C\underline{H}$), 4.19 (2H, q, J = 7.0, $C\underline{H}_2CH_3$), 3.14 & 3.54 (2H, d x qn, J = 12.4 & 7.6, NCH_2), 2.91 (3H, d, J = 11.5, CH_3N), 2.42 (2H, d x t, J = 7.6 & 2.6, CH_2COO), 1.91 (2H, qn, J = 7.2, $NCH_2C\underline{H}_2$), 1.39 (3H, t, J = 7.1, $CH_2C\underline{H}_3$), 1.37 (6H, d, J = 6.2, $CH(C\underline{H}_3)_2$).

Hapten 3. This hapten was synthesized by the same procedure as that for Hapten 1 using **1** and 4-aminobutyric acid. Yield 83%. ^{1}H NMR ($CDCl_3$): δ 7.80 (H_d, d, J = 7.9, ar), 7.56 (H_a, d, J = 8.3, ar), 7.48 (H_c, t x d, J = 7.9 & 1.7, ar), 7.21 (H_b, t x d, J = 7.6 & 1.1, ar), 5.24 (1H, sp, J = 6.3, $C\underline{H}(CH_3)_2$), 4.2 & 4.2 (2H, q, J = 7.1, $OC\underline{H}_2CH_3$), 4.03 (1H, m, J = 9.8, NH), 3.16 (2H, d x q, J = 6.7, NCH_2), 2.36 (2H, t, J = 7.5, $C\underline{H}_2COO$), 1.78 (2H, qn, J = 7.1, $CH_2C\underline{H}_2CH_2$), 1.38 (6H, d, J = 6.3, $CH(C\underline{H}_3)_2$), 1.35 (3H , t, J = 7.0, $OCH_2C\underline{H}_3$).

Preparation of hapten-protein conjugates. Hapten 1 was covalently attached to BSA to be used as the immunogen. Hapten 2 was conjugated to OVA or KLH to be used as coating antigens. Hapten 2 and Hapten 3 were conjugated to HRP to be used as enzyme tracers. The method of conjugation used was the active ester method.[4] The structure of

the active ester in case of Hapten 1 is shown in Figure 4. The procedure for the synthesis of the active ester of Hapten 1 is described below. The procedures for the synthesis of other active esters were similar.

Active ester of Hapten 1. Hapten 1 (54 mg, 0.14 mmol) was dissolved in CH_2Cl_2 (0.2 mL) to which *N*-hydroxysuccinimide (17 mg, 0.15 mmol) dissolved in 3 mL of CH_2Cl_2 followed by *N,N*-dicyclohexylcarbodiimide (31 mg, 0.15 mmol) dissolved in 0.2 mL of CH_2Cl_2 and 4-dimethylaminopyridine (1.8 mg) were added. After stirring for 1 h, the mixture was filtered and the solvent was removed. Chromatography of the resultant oil on silica gel using 29 : 9 : 1 $CHCl_3$-ethyl acetate-acetic acid gave the active ester as a syrup (52 mg, 77%). ^{1}H NMR ($CDCl_3$): δ 7.81 (H_d, d x d, J = 7.7 & 0.8, ar), 7.59 (H_a, d, J = 8.3, ar), 7.49 (H_c, d x t, J=7.5 & 1.7, ar), 7.22 (H_b, t, J = 7.5, ar), 5.23 (H, sp, J = 6.2, $(CH_3)_2C\underline{H}$), 4.29 (1H, q, J = 9.5, N$\underline{H}$CH), 4.19 (2H, q, J=7.0 C$\underline{H}_2$CH$_3$), 4.10 (1H, sp, J = 5.1, NHC$\underline{H}$), 2.84 (2H, t, C$\underline{H}_2$CO$_2$), 2.85 (4H, s, succinyl), 1.39-1.19 ($CH_2C\underline{H}_3$, $CH(C\underline{H}_3)_2$, NHCHC$\underline{H}_3$).

Active ester of Hapten 2. Yield 32%. ^{1}H NMR ($CDCl_3$): δ 7.82 (H_d, d x d, J = 7.6 & 0.9, ar), 7.60 (H_a, d, J = 8.2, ar), 7.48 (H_c, d x t, J = 7.6 & 1.7, ar), 7.21 (H_b, t, J = 7.6, ar), 5.25 (1H, sp, J = 6.3, $(CH_3)_2C\underline{H}$), 4.18 (2H, q, J = 7.0 C$\underline{H}_2$CH$_3$), 3.17 & 3.54 (2H, d x qn, J = 11.8 & 7.5, NCH_2), 2.91 (3H, d, J = 11.2, CH_3N), 2.84 (4H, s, succinyl), 2.43 (2H, t x d, J = 7.3, CH_2COO), 1.91 (2H, qn, J = 7.3, $NCH_2C\underline{H}_2$), 1.38 (3H, t, J = 7.0, $CH_2C\underline{H}_3$), 1.37 (6H, d, J = 6.2, $CH(C\underline{H}_3)_2$).

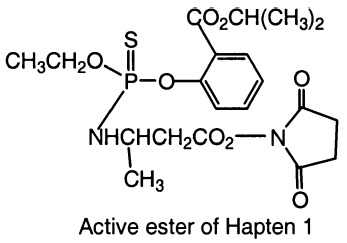
Active ester of Hapten 3. Yield 76%. ^{1}H NMR ($CDCl_3$): δ 7.81 (H_d, d, J = 7.8, ar), 7.57 (H_a, d x t, J = 8.3 & 1.4, ar), 7.49 (H_c, t x d, J = 7.8 & 1.7, ar), 7.22 (H_d, t x d , J = 7.5 & 1.2, ar), 5.23 (1H, sp, J = 6.2, $C\underline{H}(CH_3)_2$), 4.20 & 4.17 (2H, q, J = 7.1, O$\underline{CH}_2$CH$_3$), 3.22 (2H, d x q, J = 6.9, N$\underline{CH}_2$), 2.83 (4H, s, succinyl), 2.36 (2H, t, J = 7.5, CH_2COO), 1.88 (2H, qn, J = 7.1, $CH_2C\underline{H}_2CH_2$), 1.38 (6H, d, J = 6.2, $CH(C\underline{H}_3)_2$), 1.35 (3H, t, J = 6.7, $OCH_2C\underline{H}_3$).

The procedure for conjugation was as follows. To prepare BSA conjugates (immunogens), BSA (25 mg, 0.4 μmol) was dissolved in 2 mL of borate buffer (0.2 M, pH 8.7) to which 0.4 mL of DMF was added. A solution of an active ester (19 mg, 0.04 mmol) dissolved in 0.1 mL of DMF was then added to the stirred protein solution and stirring was continued for 1 h at room temperature and then at 4 °C overnight. OVA and KLH conjugates (coating antigens) were prepared by the same procedure. Conjugates were separated from unreacted haptens by gel filtration (Sephadex G-25) using PBS (10 mM, pH 7.4) as eluant, followed by dialysis in water at 4 °C overnight. Purified conjugate solutions were then freeze-dried. HRP conjugates (enzyme tracer) were prepared similarly using HRP and the hapten in 1/10 and 1/50 molar ratios. The conjugates were separated from uncoupled haptens by dialysis in water at 4 °C for two days.

Immunization of rabbits. Female New Zealand white rabbits were immunized with Hapten 1-BSA. Routinely, 500 μg of the conjugate dissolved in PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) emulsified with Freund's complete adjuvant (1 : 1 volume ratio) was injected intradermally at multiple sites on the back of each rabbit. After two weeks, each animal was boosted with an additional 500 μg of the conjugate emulsified with Freunds incomplete adjuvant and bled 7-10 days later. After this, boosting and bleeding were continued on a monthly basis. Serum was isolated by centrifugation, and sodium azide was added as a preservative at a final concentration of 0.02%. Serum was then aliquotted and stored at -70 °C.

Screening of antisera. Checkerboard assays, in which various dilutions of sera were titrated against varying amounts of the coating antigen were used to measure reactivity of antibodies and to select appropriate concentrations of coating antigens and antibodies for indirect competitive assays. The assay procedure was as follows. Microtiter plates were coated with Hapten 2-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 washed five times with PBST (10 mM PBS containing 0.05% Tween 20, pH 7.4) and were blocked by incubation with 1% gelatin in PBS (200 μL/well) at room temperature for 1 h. After another washing step, 100 μL/well of antiserum previously diluted with PBST (100 mM, 1/1000-1/2000) was added. After incubation at room temperature for 1 h, the plates were washed and 100 μL/well of a diluted (1/2000) goat antirabbit IgG-horseradish peroxidase was added. The mixture was incubated at room temperature for 1 h, and after another washing step, 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 and incubated at room temperature. The reaction was stopped after an appropriate time (10-20 min) by adding 50 μL of 2 M H_2SO_4 and absorbance was read at 450 nm.

Competitive indirect ELISA. To develop an antigen coated (indirect) ELISA, antigen coating and antibody concentration for competition assays were optimized again. Also the tolerance of ELISA to various concentrations of methanol used to dissolve the nonpolar pesticide was tested for assay optimization. For this test, standard solutions were prepared in a mixture of PBS (10 mM) and methanol in various proportions (10, 20, 30 and 40% methanol) by serial dilutions from a stock solution. The assay conditions were the same as that described above. The effect of ionic strength on ELISA performance was studied using different concentrations of PBST (10, 90, 190 and 390 mM phosphate resulting in the final concentration of 10, 50, 100 and 200 mM, respectively) to dilute the antisera and 10% methanol-PBS to dissolve the pesticide. The procedure for the competition assay was as follows. To microtiter plates coated and blocked as described above, 50 μL/well of serial dilutions of the analyte in methanol-PBS was added, followed by 50 μL/well of a previously determined antiserum dilution. After incubation at room temperature for 1 h, antibody binding was assessed as described above. Competition curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation,[7] from which I_{50} values (concentration at



Active ester of Hapten 1

Figure 3. Structure of the active ester of Hapten 1.

which binding of the antibody to the coating antigen is inhibited by 50%) are determined.

Competitive direct assay. A checkerboard assay, in which various dilutions of sera were titrated against varying amounts of an enzyme tracer (Hapten 2 or Hapten 3 conjugated to HRP) was used to optimize the amount of enzyme tracer and antibody. The procedure was the same as that for competitive assays (see below) except that only solvent instead of pesticide solution was added at the competition step. The competitive coated-antibody assays under the optimized conditions were performed as follows. Microtiter plates were precoated with protein A (5 μg/mL, 100 μL/well) in carbonate-bicarbonate buffer (50 mM, pH 9.6) by overnight incubation at 4 °C. The plates were washed five times with PBST and were coated with 100 μL of the antiserum dilutions (1/1000 or 1/1500) in PBST for 1 h at room temperature. After another washing step, serial dilutions of the analyte in 10% MeOH-PBS were added (50 μL/well) followed by 50 μL/well of enzyme tracer previously diluted with PBS (10 or 13.3 μg/mL). After incubation at room temperature for 1 h and another washing step, 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. Competition curves were obtained by the same procedure as that for the indirect assays.

Determination of cross-reactivities. The compounds listed in Table 3 were tested for cross-reactivity by preparing standard curves using the indirect assays and determining their I_{20} values (concentration at which binding of the antibody to the enzyme tracer is inhibited by 20%). The cross-reactivity values were calculated as follows: (I_{20} of isofenphos/I_{20} of compound) × 100.

Results and Discussion

Hapten design and synthsis. A suitable hapten for immunization should preserve the structure of the target compound as much as possible. The majority of organophosphorus pesticides have the thiophosphate group in common and differ only in the structure of aromatic rings. Therefore, to achieve a high selectivity in isofenphos ELISA, it was desirable to synthesize immunogenic haptens having a bridge at the thiophosphate group preserving the aromatic ring unique to isofenphos. Hapten 1 was chosen as immunogenic haptens on this ground. Reactivity and selectivity of some of the antibodies were fairly high as described below.

The haptens for organophosphorus pesticides which have an aminocarboxylic acid bridge at thiophosphate group have been used successfully for the development of ELISAs for several organophosphorus pesticides.[4-6] We have developed a novel method for the synthesis of such haptens, which require fewer steps than the previous one. This method worked well for the synthesis of the haptens for isofenphos. The route which Skerrit[4-6] used to synthesize such haptens involve seven steps including protection and deprotection at both amino and carboxyl groups of the spacer arm. The route we use instead consists of only two steps with no protection and deprotection, *i.e.*, nucleophilic attack by phenol at the P atom of $ROP(=S)Cl_2$ to displce Cl, followed by nucleophilic substitution of the remaining Cl by an aminocarboxylic acid. The facile substitution of Cl by an aminocarboxylic acid could be attributed to the polar nature of methanol (reaction solvent), which would stabilize the polar transition state developed in the S_N2 reaction.

Screening of the Sera. All of the four antisera obtained showed reasonably high recognition for the coating antigen, however, they showed quite different degrees of inhibition by the analyte for binding to the coating antigen. The one that exhibited the most inhibition by the analyte was selected as the assay reagent.

Indirect ELISA. In addition to optimization of antigen coating (30 ng/well) and antiserum dilution (1/2000), the concentrations of methanol and buffer of the competition step were optimized. Since it is unavoidable to use an organic solvent in the extraction of nonpolar pesticides from food and environmental samples, it is desirable to assess the effect of organic solvents on ELISA performance. Several workers reached the same conclusion regarding this matter; methanol causes the least negative effect of the solvents they tested. However, their assays showed diverse direction and magnitude of response to increasing concentration of methanol.[8-11] Table 1 shows the effect of methanol concentration on ELISA performance. Increasing the concentration of methanol to the standards affected significantly both the sensitivity of the assay and the slope of the calibration curve, but caused little effect on the speed of the color development. A large decrease in the sensitivity was observed with increasing amounts of methanol and the lowest I_{50} value was found at 10% methanol. However, slope of the calibration

Table 1. Effects of methanol concentration on assay parameters of indirect ELISA[a]

Methanol (%)	Abs_{max}	Slope	I_{50} (μg/mL)
10	0.996	0.377	0.096
20	1.074	0.715	0.471
30	1.148	0.769	0.450
40	1.167	0.897	0.607

[a]ELISA conditions: antiserum to Hapten 1-BSA, diluted 1/2000 with 100 mM PBST; coating antigen, Hapten 2-OVA, 30 ng/well; goat antirabbit IgG-HRP diluted 1/2000. Data were obtained from the four-parameter sigmoidal fitting. Each set of data represents the average of three replicates.

Table 2. Effects of buffer concentration on assay parameters of indirect ELISA[a]

Buffer (mM)[b]	Incubation Time (min)[c]	Abs_{max}	Slope	I_{50} (μg/mL)
10	8	1.466	0.660	2.216
50	13	1.531	0.601	0.954
100	17	1.291	0.679	0.337
200	21	0.975	0.761	0.191

[a]ELISA conditions were the same as those described in Table 1. Data were obtained from the four-parameter sigmoidal fitting. Each set of data represents the average of three replicates. [b]Final concentration of phosphate buffer of the competition media after mixing 10% methanol-10 mM PBS containing isofenphos and the antiserum diluted with different concentrations of PBST. [c]Time for color development.

curve became slower at lower concentration of methanol.

Table 2 presents the effect of the concentration of the phosphate of the competition step on ELISA characteristics. Increasing the concentration of the phosphate caused a large improvement in the sensitivity of the assay, which is in agreement with the results of several previous studies.[12,13] Due to the nonpolar nature of isofenphos, it seems reasonable to assume that hydrophobic interactions are important in the antibody-analyte binding. Therefore, increased ionic strength of the medium would enhance the antibody-analyte binding, which could explain the behavior observed.[14] Since increasing buffer concentration causes a remarkable retardation of the color development, selecting optimum buffer concentration would depend on the sensitivity and speed of color development of the assay developed.

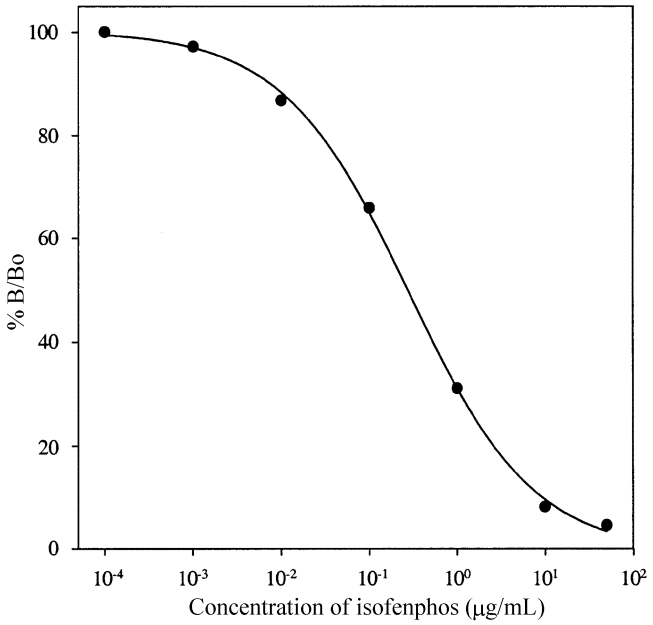


Figure 4. Inhibition curve for isofenphos by indirect competitive ELISA using antiserum to Hapten 1-BSA, diluted 1/2000, the coating antigen Hapten 1-OVA (30 ng/well) and gelatin blocking agent (1%). $\%B/B_0 = (A-A_{ss}/A_0-A_{ss}) \times 100$, where A is the absorbance, A_0 is the absorbance at zero dose of the analyte, and A_{ss} is the absorbance at an excess of the analyte.

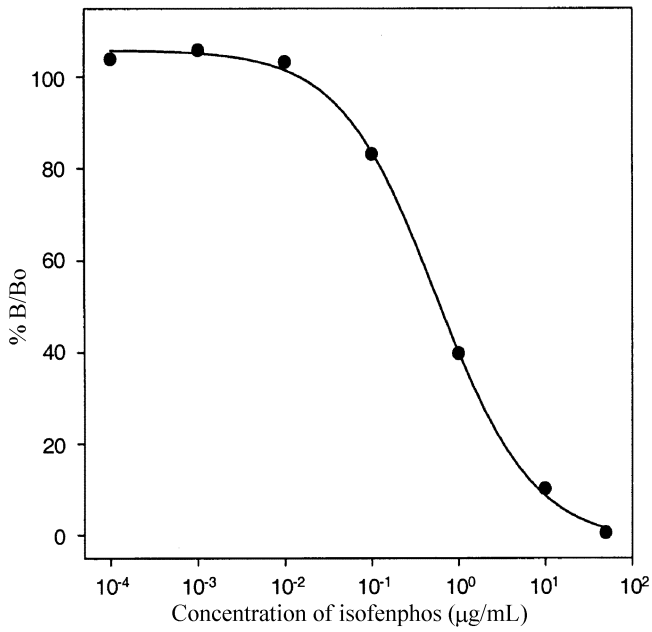


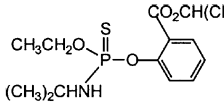
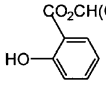
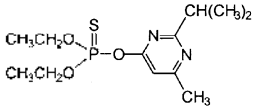
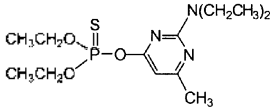
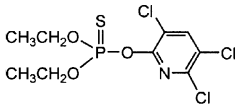
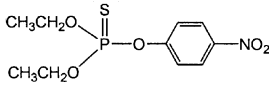
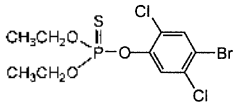
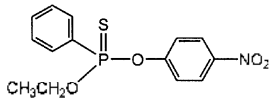
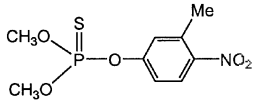
Figure 5. Inhibition curve for isofenphos by direct competitive ELISA using antiserum to Hapten 1-BSA, diluted 1/1500 and the enzyme tracer Hapten 2-HRP (1 μg/well). $\%B/B_0 = (A-A_{ss}/A_0-A_{ss}) \times 100$, where A is the absorbance, A_0 is the absorbance at zero dose of the analyte, and A_{ss} is the absorbance at an excess of the analyte.

Figure 4 shows a typical inhibition curve obtained after optimization. The lowest I_{50} value of the assay was 96 ng/mL with a detection limit of 2 ng/mL. This level of sensitivity is lower than those observed in the previously developed pesticide ELISAs with a relatively high sensitivity (I_{50} values below 10 ng/mL)[15]. However it is high enough for the quantitation of isofenphos in food samples at levels near maximum residue limits (20-200 ng/mL in Korea).

Direct ELISA. Enzyme tracers for direct ELISA were prepared by conjugation reaction of either Hapten 2 or Hapten 3 with HRP at 1 : 10 or 1 : 50 molar ratio. Hapten 2-HRP prepared at 1 : 10 molar ratio gave the best result. Antibody-coated ELISA using this enzyme tracer was optimized with regard to the dilution of antiserum and enzyme tracer. Figure 5 shows a typical inhibition curve obtained after optimization. The lowest I_{50} value of the assay was 285 ng/mL with a detection limit of 10 ng/mL. Since this assay showed a sensitivity considerably lower than that of the indirect ELISA, no further optimization was attempted.

Cross-reactivity of the assays. Several organophosphorus pesticides as well as isofenphos metabolites were tested for cross-reactivities. Table 3 shows the cross-reactivities that were found by the indirect assay, expressed in percentage ratio of I_{50} of the pesticide and isofenphos. In all cases, the interference to the assay was negligible. The small cross-reactivities of the antibodies to pirimiphos-ethyl and diazinon are understandable as they have 2- or 3-carbon alkyl groups at the aromatic ring as isofenphos does. It may be concluded that the indirect ELISA that was developed is suitable for the sensitive and selective detection of iso-

Table 3. Cross-reactivity profiles of the antibodies[a]

Pesticide	Structure	I_{20} (μg/mL)[b]	CR (%)[c]
Isofenphos		0.083	100
Isopropyl salicylate		> 100	< 0.08
Diazinon		13	0.6
Pirimiphos-ethyl		11	0.8
Chlorpyrifos-ethyl		>100	< 0.08
Parathion-ethyl		>100	< 0.08
Bromophos-ethyl		>100	< 0.08
EPN		>100	< 0.08
Fenitrothion		>100	< 0.08

[a]Determined by indirect ELISA using antiserum to Hapten 1-BSA and coating antigen Hapten 2-OVA. [b]I_{20} values of many of the pesticides could not be determined accurately due to the limited solubility at high concentrations, however, it was clear that inhibition was less than 20% at 100 ppm. [c]cross-reactivity (%) = (I_{20} of isofenphos/I_{20} of other compound) × 100

fenphos.

Acknowledgment. This work was supported by Korea Research Foundation Grant (KRF-99-005-D-000-55).

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