Development of an Enzyme-Linked Immunosorbent Assay for the Organophosphorus Insecticide Bromophos

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A competitive enzyme-linked immunosorbent assay (ELISA) was developed for the quantitative detection of the organophosphorus insecticide bromophos. Three bromophos analogues (haptens) were synthesized and were coupled to carrier proteins to use as immunogens or coating antigens. Rabbits were immunized with either one of two haptens coupled to bovine serum albumin (BSA) for production of polyclonal antibodies, and the sera were screened against one of the haptens coupled to ovalbumin (OVA). Using the serum with highest specificity and an enzyme tracer, an antibody-coated ELISA was developed, which showed an IC_{50} of 40 ng/mL with a detection limit of 7 ng/mL. The antibodies in this assay showed negligible cross-reactivity with other organophosphorus pesticides except with the insecticides chlorpyrifos and fenitrothion.

Key Words : Bromophos, Insecticide, Immunoassay, 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 fast, sensitive, and cost-effective tools for environmental analysis.[2-4]

Bromophos [*O,O*-dimethyl *O*-(4-bromo-2,5-dichlorophenyl) phosphorothioate] is a non-systemic organophosphorus insecticide and acaricide, which is effective against a wide range of insects.[5] The most sensitive and toxicologically relevant effect after administration of bromophos is the inhibition of acetylcholinesterase activity in erythrocytes and brain.[6] An ELISA for this pesticide has not yet been reported.

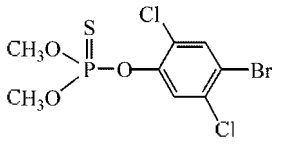
The development of an immunoassay requires the production of antibodies to the analyte. Since pesticides are small molecules, pesticide derivatives, namely haptens, must be synthesized and coupled to carrier proteins to induce antibody production. One type of hapten for organophosphorus pesticides is one with an amino carboxylic acid bridge at thiophosphate group, which has been used successfully in the development of ELISA for several organophosphorus pesticides.[7-12] We have developed a novel method for the synthesis of such haptens, which is much easier than the previous one.[12] This paper describes the application of this method to the synthesis of haptens for bromophos from which specific polyclonal antibodies to bromophos were obtained. Using the antibodies, a sensitive and selective ELISA for bromophos was developed.

Experimental Section

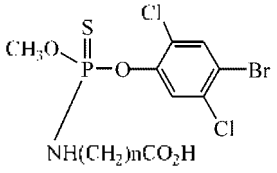
Reagents and Instruments. Organophosphorus pesticides including bromophos were purchased from Dr. Ehrenstorfer (Augsburg, Germany). BSA, OVA, peroxidase labeled goat anti-rabbit IgG, Freund's complete and incomplete adjuvants, and Sephadex G-25 were purchased from Sigma (St. Louis, USA). Tetramethylbenzidine was obtained from Boehringer Mannheim (Mannheim, Germany). Other chemicals were from Aldrich (Milwaukee, USA). Analytical (silica gel F254) and preparative TLC plates (silica gel, 1 mm) were purchased from Merck (Darmstadt, Germany). The dialysis membrane (MW cutoff 12000-14000) was obtained 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 Vmax microplate reader from Molecular Devices (Menlo park, USA). 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, qn, m, and ar represent singlet, doublet, triplet, quintet, multiplet, and aromatic, respectively.

Hapten Synthesis. The haptens used for immunization and enzyme tracer are presented in Figure 1. The synthetic routes for the haptens are illustrated in Figure 2. The procedure for the synthesis of Hapten A was as follows.

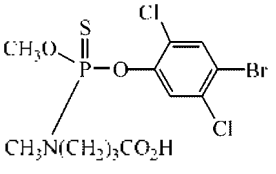
***O*-Methyl *O*-(4-Bromo-2,5-dichlorophenyl) Phosphorochloridothioate (3).** The starting material 4-bromo-2,5-dichlorophenol (**2**) was synthesized by a published proce-



Bromophos



Hapten A : n = 3
Hapten B : n = 5



Hapten C

Figure 1. Structures of the haptens for bromophos used for immunization and enzyme-tracer.

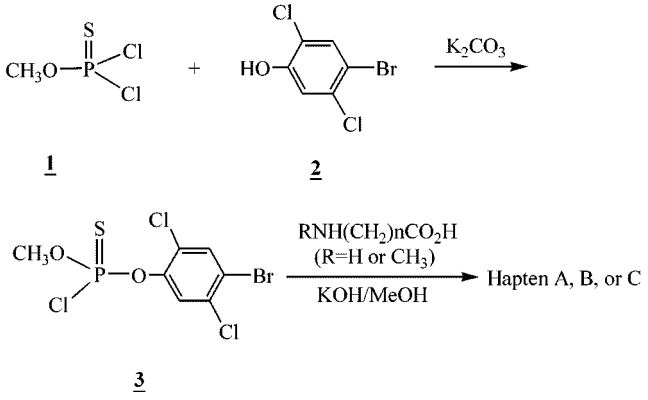


Figure 2. Synthetic route for haptens.

dure.[13] A solution of **2** (4 g, 17 mmol) in 5 mL of acetonitrile was added dropwise to a stirred mixture of 1.6 g (9.6 mmol) of **1**.[14] 15 g of finely ground K_2CO_3 and 20 mL of acetonitrile. After stirring for 1 h 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, 6 : 1 hexane/benzene, R_f: 0.60) to give 1.6 g (44%) of the product (**3**) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.73 (1H, s, ar), 7.59 (1H, s, ar), 4.06 (3H, d, J = 14.3, CH_3OP).

Hapten A. To a stirred solution of 570 mg (1.5 mmol) of **3** in 0.5 mL of methanol cooled in an ice-water bath was added dropwise a solution of 322 mg (4.0 mmol) of KOH and 170 mg (1.65 mmol) of 4-aminobutyric acid in 1.7 mL of methanol. After stirring for 5 min, the reaction mixture was filtered and extracted with 1 N HCl-chloroform. The extract was dried over $MgSO_4$, and the solvent was evaporated. Column chromatography (silica gel, 19 : 9 : 1 chloroform/ethyl acetate/acetic acid, R_f: 0.41) of the residue gave 258 mg (37%) of a white solid. ^{1}H NMR ($CDCl_3$): δ 7.66 (1H, d, J = 1.0, ar), 7.63 (1H, d, J = 1.6, ar), 3.82 (3H, d, J = 14.1, CH_3OP), 3.49 (1H, m, NH), 3.19 (2H, t×d, J = 12.5 & 6.7, $NHC\underline{H}_2$), 2.45 (2H, t, J = 7.1, $CH_2C\underline{O}_2$), 1.88 (2H, qn, J = 7.0, $CH_2C\underline{H}_2CH_2$).

Hapten B and C were synthesized by the same procedure as that for Hapten A, using 6-aminocaproic acid and 4-(*N*-methylamino)butyric acid, respectively.

Hapten B. The yield was 65%. TLC R_f: 0.42 (silica gel, 19 : 9 : 1 chloroform/ethyl acetate/acetic acid). ^{1}H NMR ($CDCl_3$): δ 7.66 (1H, d, J = 1.0, ar), 7.64 (1H, d, J = 1.6, ar), 3.82 (3H, d, J = 14.2, CH_3OP), 3.30 (1H, m, NH), 3.18 (2H, t×d, J = 10.5 & 6.6, $NHC\underline{H}_2$), 2.36 (2H, t, J = 7.0, CH_2CO_2), 1.57 (6H, m, $CH_2(C\underline{H}_2)_3CH_2$).

Hapten C. The yield was 33%. TLC R_f: 0.48 (silica gel, 19 : 9 : 1 chloroform/ethyl acetate/acetic acid). ^{1}H NMR ($CDCl_3$): δ 7.66 (1H, d, J = 1.0, ar), 7.53 (1H, d, J = 1.6, ar), 3.77 (3H, d, J = 14.0, CH_3OP), 3.35 (2H, m, $NC\underline{H}_2$), 2.87 (3H, d, J = 11.3, CH_3N), 2.43 (2H, t, J = 7.4, $C\underline{H}_2CO_2$), 1.92 (2H, qn, J = 7.3, $CH_2C\underline{H}_2CH_2$).

Preparation of Hapten-protein Conjugates. Hapten A and B were covalently attached to BSA to be used as immunogens. Hapten B was also attached to OVA to be used as the coating antigen for serum screening. Hapten A, B, and C were conjugated to HRP to be used as enzyme tracers. The method of conjugation used was the active ester method.[7,15] The structure of the active ester in the case of Hapten A is shown in Figure 3. The procedure for the synthesis of this ester is described below. Other active esters were synthesized by the same procedure. To a solution of *N*-hydroxysuccinimide (28 mg, 0.24 mmol) dissolved in dichloromethane (5 mL) were added Hapten A (97 mg, 0.22 mmol), 4-dimethylaminopyridine (2.7 mg, 0.022 mmol), and *N,N*-dicyclohexylcarbodiimide (63 mg, 0.24 mmol). The mixture was stirred for 2 h and then filtered to remove the dicyclohexylurea, and the solvent was evaporated. Chromatography of the resultant oil on silica gel using chlorform/ethyl acetate/acetic acid (19 : 9 : 1 R_f: 0.52) followed by preparative TLC using the same eluent gave the active ester as a syrup (51 mg, 40%). ^{1}H NMR ($CDCl_3$): δ 7.67 (1H, s, ar), 7.62 (1H, d, J = 1.6, ar), 3.82 (3H, d, J = 14.8, CH_3OP), 3.57 (1H, m, N$\underline{H}$), 3.25 (2H, t×d, J = 13.3 & 6.6, $NHC\underline{H}_2$), 2.85 (4H, s, succinyl), 2.73 (2H, t, J = 6.9, $C\underline{H}_2CO_2$), 1.98 (2H, m, $CH_2C\underline{H}_2CH_2$).

Active Ester of Hapten B. Yield 34%. TLC R_f: 0.52 (silica gel, 19 : 9 : 1 chloroform/ethyl acetate/acetic acid). ^{1}H NMR ($CDCl_3$): δ 7.67 (1H, d, J = 0.8, ar), 7.64 (1H, d, J = 1.5, ar), 3.89 (3H, d, J = 14.3, CH_3OP), 3.40 (1H, m, NH), 3.16 (2H, t×d, J = 11.4 & 6.6, $NHC\underline{H}_2$), 2.85 (4H, s, succinyl), 2.64 (2H, t, J = 7.1, CH_2CO_2), 1.58 (6H, m, $CH_2(C\underline{H}_2)_3CH_2$).

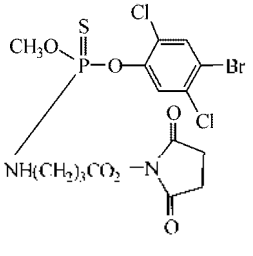


Figure 3. Structure of active ester of Hapten A.

Active Ester of Hapten C. Yield 33%. TLC R_f: 0.62 (silica gel. 19 : 9 : 1 chloroform/ethyl acetate/acetic acid). ^{1}H NMR ($CDCl_3$): δ 7.66 (1H, d, J = 0.9, ar), 7.53 (1H, d, J = 1.6, ar), 3.78 (3H, d, J = 14.1, CH_3OP), 3.39 (2H, m, $NC\underline{H}_2$), 2.89 (3H, d, J = 11.2, $C\underline{H}_3N$), 2.85 (4H, s, succinyl), 2.69 (2H, t, J = 7.5, $C\underline{H}_2CO_2$), 1.93 (2H, m, $CH_2C\underline{H}_2CH_2$).

The procedures for coupling haptens to the carrier proteins were as follows. To prepare hapten-BSA conjugates (immunogens), BSA (20 mg) 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 (16 mg, 0.03 mmol) dissolved in 0.1 mL of DMF was then added to the stirred protein solution, and stirring was continued overnight at 4 °C. Hapten-OVA conjugate (coating antigen) was prepared by the same procedure. Hapten-HRP conjugates (enzyme tracers) were prepared by the same procedure except that four hapten/protein molar ratios (5, 10, 20 and 50) were employed. The conjugates for immunogen and coating antigen were separated from the uncoupled haptens by gel filtration (Sephadex G-25) using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Finally, the eluates were dialyzed in water overnight and then freeze-dried. The conjugates for enzyme tracer were separated from the uncoupled haptens by dialysis in PBS for two days and in water for one day and then stored at 4 °C.

Immunization of Rabbits. Female New Zealand white rabbits were immunized with Hapten A- or Hapten B-BSA. Routinely, 500 μg of the conjugate dissolved in 500 μL of PBS was emulsified with Freund's complete adjuvant (1 : 1 volume ratio) and 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 Freund's incomplete adjuvant and bled from seven to ten days later. After this, boosting and bleeding was 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 aliquoted and stored at -70 °C.

Screening of Antisera. Several dilutions of each serum were titrated against the coating antigen (Hapten B-OVA, 1000 ng/well) to measure the reactivity of antibodies. Flat-bottom polystyrene microtiter plates were coated with the coating antigen (10 μg/mL, 100 μL/well) in 50 mM carbonate-bicarbonate buffer (pH 9.6) by overnight incubation at 4 °C. The following day, the coated plates were washed five times with PBSTA (PBS containing 0.05% Tween 20 and 0.02% NaN_3, pH 7.4) and were blocked by incubation with 1% gelatin in PBS (200 μL/well) for 1 h at 37 °C. After another washing step 100 μL/well of antiserum diluted with PBSTA (1/10000-1/1250000) was added to the plate and incubated for 1 h at 37 °C. After another washing step 100 μL/well of goat anti-rabbit IgG conjugated with alkaline phosphatase diluted 1 : 2000 with PBSTA was added to the plate and incubated for 1 h at 37 °C. Then the plates were washed again, and 100 μL/well of *p*-nitrophenyl phosphate (1 mg/mL) dissolved in 10% diethanolamine buffer (pH 9.8) was added to the plate. After incubation at 37 °C for 30 min, the reaction was stopped by adding 50 μL of 3 N NaOH and absorbance was read at 405 nm.

Competitive Direct Assay. Checkerboard assays, in which various dilutions of the sera were titrated against varying amounts of enzyme tracers (Hapten A, B or C conjugated to HRP), were used to select the most suitable antiserum and enzyme tracer, and 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 instead of pesticide solution was added at the competition step. After selecting the most suitable antiserum and enzyme tracer from the checkerboard assays, their quantities for the competitive direct assays were 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 acetone, acetonitrile, or methanol of various concentration levels (2.5, 5, 10, 20 and 40% in PBS resulting in a final concentration of 1.25, 2.5, 5, 10, and 20%, respectively, by combining with the enzyme tracer diluted with 10 mM PBS). The influence of buffer concentration of assay solution on ELISA performance was also studied using different concentrations of phosphate in 5% methanol-PBS to dissolve the pesticide (10, 90, 190 and 390 mM phosphate resulting in the final concentration of 10, 50, 100 and 200 mM, respectively, by combining with the enzyme tracer diluted with 10 mM PBS). The influence of pH of the assay solution on assay parameters was also studied. The media of the assay solutions were 5% methanol-PBS buffer (50 mM) at various pH values.

The direct assays were performed as follows. All incubations except that for precoating the wells with protein A were carried out at 25 °C. Microtiter plates were coated 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 coated with 100 μL/well of the antiserum dilutions in PBS for 1 h. After another washing step, serial dilutions of the analyte in MeOH-PBS were added (50 μL/well) followed by 50 μL/well of an enzyme tracer previously diluted with PBS (500 ng/mL). After incubation for 1 h and 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) were 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 absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation,[16] from which IC_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) were determined.

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

Results and Discussion

Hapten Selection and Synthesis. A suitable hapten for immunization should preserve the structure of the target compound as much as possible. The phosphorothioate organophosphorus pesticides such as bromophos have a thiophosphate group in common and differ only in the structure of the aromatic rings. Therefore, to achieve a high selectivity in bromophos ELISA, it was desirable to synthesize immunizing haptens having a bridge at the thiophosphate group preserving the aromatic ring unique to bromophos. Haptens in this study have such structural features. Heldman *et al.* were the first to synthesize a hapten for OP pesticide with a spacer arm at the thiophosphate group.[17] However, a generic method was developed later by Skerritt *et al.*[18,19] The method was applied to the synthesis of haptens for the development of ELISAs of several organophosphorus pesticides.[7-11] This method requires a synthetic route involving seven steps including protection and deprotection of both amino and carboxyl groups. In an effort to simplify the synthetic process for this class of haptens we developed a simpler generic method which requires only two steps. It involves the reaction of *O*-methyl (ethyl) dichlorothiophosphate with a phenol and K_2CO_3 in acetonitrile and the reaction of the substitution product with an amino carboxylic acid (not protected) and KOH in methanol. This method was successfully applied to the synthesis of three haptens for bromophos (Figure 1). The reactions proceeded facily with relatively high yield; 44% and 33-65% in the first and second reaction, respectively. The reaction time was relatively short; 1 h and a few minutes for the first and second reaction, respectively. This method was successfully applied in this laboratory for the synthesis of haptens of several other OP pesticides such as chlorpyrifos,[12] diazinon, fenitrothion, parathion-methyl, cyanophos[20] and isofenphos.[21] and we applied for a patent. All the carboxylic acid haptens could be converted to the succinimide esters, active esters for coupling haptens to carrier proteins.

Direct Competitive Assay. The checkerboard assays, in which several dilutions of the sera were titrated against varying amounts of the enzyme tracers, were used to select the most suitable antiserum and enzyme tracer and to optimize enzyme tracer and antibody concentrations. The optimum combination selected was the combination of the serum from Hapten A-BSA (3rd boost) diluted 1/2000 and the tracer Hapten B-HRP prepared at 10 : 1 hapten/protein molar ratio and diluted to 500 ng/mL (25 ng/well).

Since the use of organic solvents for extraction and/or solid phase clean up is very common in the analysis of non-polar pesticide residues in food and environmental samples, it is desirable to assess the effect of organic solvents on ELISA performance. The effects of solvents (acetone, acetonitrile, and methanol) on the ELISA system were evaluated by preparing standard curves in buffers containing various amounts of solvent (1.25, 2.5, 5, 10, and 20% in PBS). The results are presented in Table 1. These solvents significantly influenced assay performance. The speed of color development (estimated from Abs_{max}) at the competition step decreased rapidly with increasing concentration of organic solvent resulting in more than 50% retardation of color development above 10% concentration. Based on the relatively high Abs_{max} value and the lowest IC_{50} value, methanol at 2.5% concentration was selected as the optimum composition for the assay. Several other workers reached the same conclusion as ours in that methanol caused the least negative effect on the performance of the assay.[22,23]

Table 1. Influence of organic cosolvent concentration of assay solution on assay parameters of direct ELISA[a]

	Concentration (%)	Abs_{max}	Slope	IC_{50} (ng/mL)
Acetone	1.25	0.892	1.775	75
	2.5	1.221	0.872	46
	5	0.478	1.137	191
	10	0.308	0.733	75
	20[b]			
Acetonitrile	2.5	1.860	0.958	137
	5	1.478	0.868	331
	10	0.922	1.078	196
	20	0.302	0.998	192
Methanol	1.25	0.894	0.711	92
	2.5	1.016	0.711	43
	5	0.667	0.983	207
	10	0.233	0.651	127
	20[b]			

[a]Assay conditions: precoating with protein A (0.5 μg well); antiserum to Hapten A-BSA, diluted 1 2000 with 10 mM PBS; enzyme tracer Hapten B-HRP, 500 ng/mL. Data are the means of duplicates. Parameters were obtained from the four-parameter sigmoidal fitting. [b]Data fitting was impossible due to poor color development.

Varying the concentration of the phosphate buffer in the competition solution had no significant effect on the assay sensitivity (data not shown). The optimum concentration selected was 50 mM phosphate which showed the lowest IC_{50} value. Table 2 presents the effect of pH of assay solution on ELISA. IC_{50} values were similar at the pH's from 6.0 to 7.4. Based on the relatively high A_{max} value and slope of the calibration curve, pH 7.0 was selected as the best one.

Figure 4 shows a typical inhibition curve obtained under the optimized condition. The IC_{50} value of the assay was 40

Table 2. Influence of pH of assay solution on assay parameters of direct ELISA[a]

pH	Abs_{max}	Slope	IC_{50} (ng/mL)
6.0	0.873	0.690	31
6.5	1.059	0.845	41
7.0	1.092	0.839	36
7.4	1.191	0.713	49
8.0	0.629	1.737	256
9.0	0.626	0.751	67
10.0	0.344	0.801	79

[a]Assay conditions were the same as those described in Table 1.

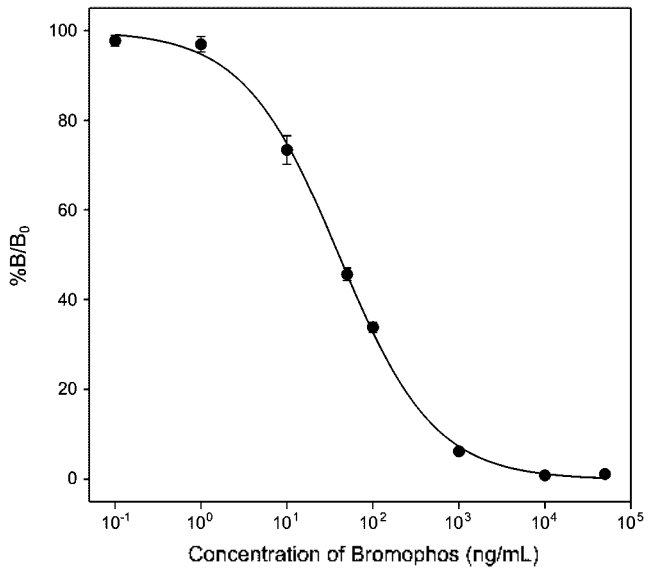
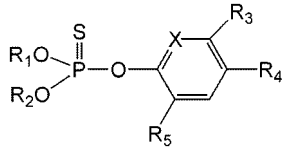
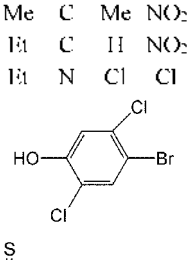
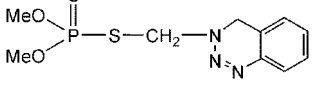
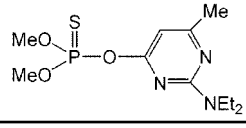


Figure 4. ELISA inhibition curves for bromophos by direct competitive assay. Assay conditions were the same as those described in Table 1. $\%B/B_0 = (A-A_{xs}/A_0-A_{xs}) \times 100$, where A is the absorbance at a given concentration of the analyte, A_0 is the absorbance at zero dose of the analyte, and A_{xs} is the absorbance at an excess of the analyte. Each point represents the mean of 24 determinations. Vertical bars indicate ± SD about the mean.

Table 3. Cross-reactivity of compounds structurally related to bromophos determined by direct competitive ELISA[a]



Compound	Structure						IC_{50}[b] (μg mL)	CR[c] (%)
	R_1	R_2	X	R_3	R_4	R_5		
Bromophos	Me	Me	C	Cl	Br	Cl	0.034	100
Parathion-methyl	Me	Me	C	H	NO_2	H	10	0.3
Fenitrothion	Me	Me	C	Me	NO_2	H	0.308	11
Parathion	Et	Et	C	H	NO_2	H	50	0.07
Chlorpyrifos	Et	Et	N	Cl	Cl	Cl	0.322	11
4-Bromo-2,5-dichlorophenol							4.66	0.68
Azinphos-methyl							50	0.07
Pirimiphos-methyl							50	0.07

[a]Assay conditions were the same as those described in Table 1. [b]IC_{50} values of parathion, azinphos-methyl and pirimiphos-methyl could not be determined accurately due to the limited solubility at high concentrations, however, it was clear that inhibition was less than 50% at 50 μg mL. [c]cross-reactivity (%) = (IC_{50} of bromophos/IC_{50} of other compound) 100.

ng/mL with a detection limit of 7 ng/mL (20% inhibition).

Cross-Reactivity Studies. Several organophosphorus pesticides as well as the metabolite of bromophos (phenol) were tested for cross-reactivities. Table 3 shows the cross-reactivity that was found by the direct assay described above, expressed in percentage of the IC_{50} of bromophos. The interference to the assays was negligible except with chlorpyrifos and fenitrothion. The appreciable cross-reactivities of antibodies for these pesticides are understandable, because they have similar aromatic structure as bromophos. It may be concluded that the competitive ELISA that was developed is suitable for the sensitive and selective detection of bromophos, with the exception of chlorpyrifos and fenitrothion.

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