Development of an ELISA for the Organophosphorus Insecticide Chlorpyrifos

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A selective enzyme-linked immunosorbent assay (ELISA) for the insecticide chlorpyrifos was developed. Four haptens for chlorpyrifos were synthesized and two of them were used as immunogens after coupling to keyhole limpet hemocyanin by two different approaches. Rabbits were immunized with either of them and the sera were screened against 4 haptens coupled to ovalbumin (OVA). Using the sera of highest specificity, an antigen-coated ELISA was developed, which shows an I_{50} of 160 ppb with a detection limit of 10 ppb. An antibody-coated ELISA was also developed, which shows an I_{50} of 20 ppb with a detection limit of 0.1 ppb. The antibodies showed negligible cross-reactivity with other organophosphorus pesticides except for insecticides chlorpyrifos-methyl and bromophos-ethyl, which makes these assays suitable for the selective detection of chlorpyrifos.

Keywords : Chlorpyrifos, Insecticide, Organophosphorus, Immunoassay, ELISA.

Introduction

Due to the widespread use of pesticides, there is an increasing concern over food and environmental contamination caused by their use. The current methods such as gas chromatography and high-performance liquid chromatography have been used successfully, with great sensitivity and reliability, for analysis of many pesticides.[1] However, these classical methods require a high capital expenditure 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 been emerging as an alternative to traditional methods to meet such demands. Immunochemical techniques that have been used extensively in clinical laboratories, began recently to gain acceptance as a fast, sensitive, and cost-effective tool for environmental analysis.[2]

Chlorpyrifos [*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate] is a broad spectrum organophosphorus insecticide, and is used extensively in both agricultural and domestic settings.[3] It is moderately toxic to mammalian species, but extremely toxic to bees and a wide range of aquatic species.[4] Therefore, there is an increasing demand for its more comprehensive monitoring.

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 proteins to induce antibody production. Manclús *et al.*[5,6,7] developed an enzyme-linked immunosorbent assay (ELISA) for chlorpyrifos using several different haptens. One type of haptens used was the one with an aminocarboxylic acid bridge at thiophosphate group, which has been used successfully in the development of ELISA for several other organophosphorus pesticides.[8,9,10] Using different strategies to develop ELISAs for chlorpyrifos, we found that the method used by Manclús *et al*. for coupling these haptens to proteins caused a serious problem, i.e., hydrolysis of the haptens. Thus, we adopted different approaches for safe conjugation of the haptens to proteins. Borate buffer, pH 8.7, is the most frequently used medium for the conjugation of haptens to proteins,[6] since the functional groups (lysine and histidine) of proteins reacting with haptens exist as nucleophilic free amine at this pH. We used instead distilled water as the medium which would allow an amine to exist in an unprotonated state more than pH 7 buffer would. The conjugates prepared by this method were found to be suitable as the immunoreagents. Using the sera against these conjugates, a sensitive and selective ELISA for chlorpyrifos was developed.

Experimental Section

Reagents and instruments. Organophosphorus pesticides, including chlorpyrifos, were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Diethyl chlorothiophosphate, 4-aminobutyric acid, 6-aminocaproic acid, 2,4,5-trichlorophenol, 3-(methylamino)butyric acid hydrochloride, *N*-hydroxysuccinimide, 1,3-dicylohexylcarbodiimide, 4-dimethylaminopyridine, $CHCl_3$-*d*, silica gel for column chromatography (60-230 mesh) and Tween 20 were obtained from Aldrich (Milwaukee, USA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, BSA (A-3059), OVA (A-2512), alkaline phosphatase labeled goat anti-rabbit IgG (A-3687), peroxidase labeled goat anti-rabbit IgG (A-6154), *p*-nitrophenyl phosphate (N-9389), Freund's complete (F-5881) and incomplete (F-5506)

adjuvants, and Sephadex G25 were purchased from Sigma (St. Louis, USA). *N*-hydroxysulfosuccinimide was from Pierce (Rockford, 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, 442404) were purchased from Nunc (Roskilde, Denmark). ELISA plates were washed with a Model 1575 ImmunoWash, and well absorbances were read with a Model 550 plate reader, both from Bio-Rad (Hercules, USA). UV-Vis spectra were recorded on a Varian Cary 3 spectrophotometer. NMR spectra were obtained with a Bruker ARX spectrometer (300 MHz). Chemical shift values are given relative to internal tetramethylsilane. Coupling constants are expressed in Hz and the abbreviations d, t, q, qn, m, and ar represent doublet, triplet, quartet, quintet, multiplet, and aromatic, respectively.

Hapten synthesis. The haptens used for immunization and antigen coating are presented in Figure 1. The synthetic routes for Hapten 1 and 2 are illustrated in Figure 2 and those for Hapten 4 in Figure 3. The synthetic route for Hapten 3 was similar to those for Hapten 1 and 2.

1. To a stirred mixture of 3.52 g (20 mmol) of ethyl dichlorothiophosphate, 10 g of finely ground K_2CO_3 and 5 mL acetonitrile was added dropwise 3.00 g (15 mmol) of 3,5,6-trichloro-2-pyridinol dissolved in 30 mL of acetonitrile. After stirring for 1 h at ambient temperature, the mixture was filtered through Celite and the solvent was removed under reduced pressure. The residue was subjected to column chromatography (silica gel, 4 : 1 hexane-benzene) to give 3.36 g (65%) of the product as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.91 (1H, d, J = 1.3, ar), 4.57-4.46 (2H, qxd, J = 11 & 7.1, $C\underline{H}_2CH_3$), 1.53-1.48 (3H, txd, J = 7.1 & 1.1, $CH_2C\underline{H}_3$).

Hapten 1. To a stirred solution of 0.50 g (1.47 mmol) of **1** in 3 mL of methanol cooled in an ice-water bath was added dropwise a solution of 0.205 g (3.23 mmol) of KOH and 0.166 g (1.61 mmol) of 4-aminobutyric acid in 1.7 mL methanol. After stirring for 10 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, 3 : 1 : trace chloroform-ethyl acetate-acetic acid) of the residue gave 320 mg (54%)

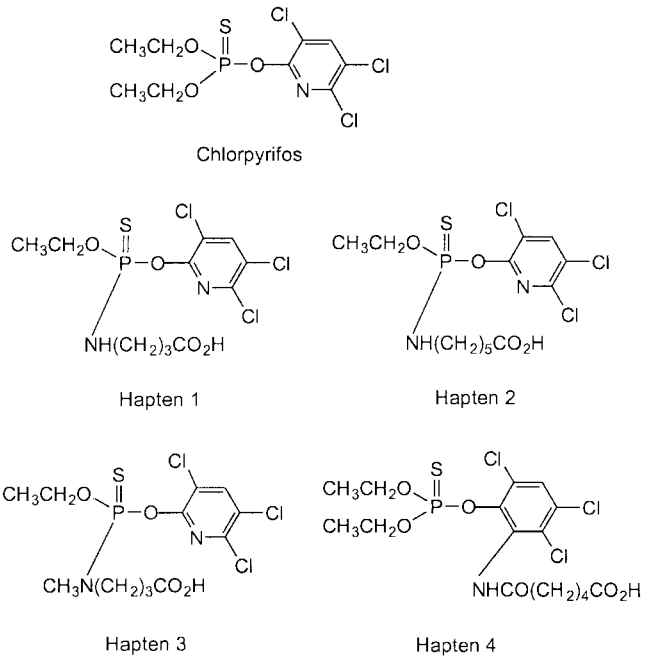


Figure 1. Structures of the haptens for chlorpyrifos ELISA used for immunization and antigen coating.

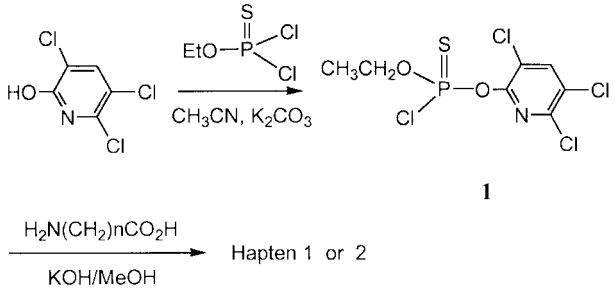


Figure 2. Synthetic route for Hapten 1 and Hapten 2.

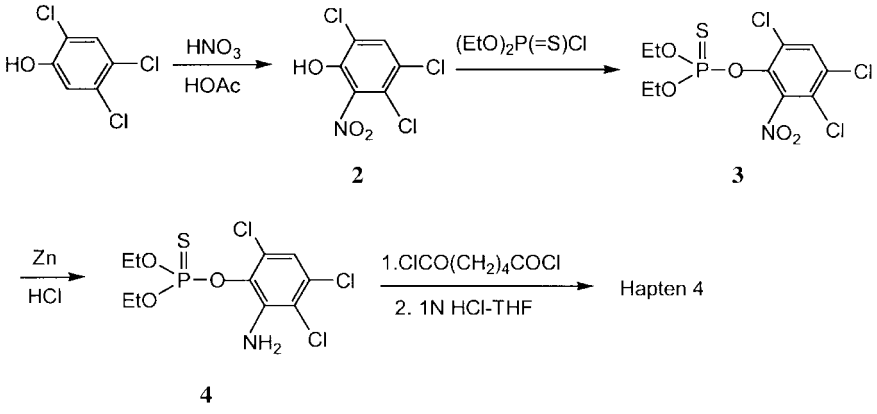


Figure 3. Synthetic route for Hapten 4.

of a white solid. ^{1}H NMR ($CDCl_3$): δ 7.85 (1H, d, J = 0.9, ar), 4.39-4.29 (2H, qxd, J = 9.5 & 7.1, C$\underline{H}_2$CH$_3$), 3.58-3.50 (1H, m, NH), 3.30-3.19 (2H, m, NHC$\underline{H}_2$), 2.51 (2H, t, J = 7.2, C$\underline{H}_2$CO$_2$H), 1.93 (2H, qn, J = 6.9, CH$_2$C$\underline{H}_2$CH$_2$), 1.41 (3H, t, J = 7.1, CH$_2$C$\underline{H}_3$).

Hapten 2. This hapten was synthesized by the same method as Hapten 1 using **1** and 6-aminocaproic acid. Yield 53%. ^{1}H NMR ($CDCl_3$): δ 7.87 (1H, d, J = 1.0, ar), 4.42-4.30 (2H, qxd, J = 9.6 & 7.1, C$\underline{H}_2$CH$_3$), 3.52-3.41 (1H, m, NH), 3.25-3.12 (2H, m, NHC$\underline{H}_2$), 2.40 (2H, t, J = 7.3, C$\underline{H}_2$CO$_2$H), 1.75-1.25 [6H, m, J = 6.9, CH$_2$(C$\underline{H}_2$)$_3$CH$_2$], 1.43 (3H, t, J = 7.1, CH$_2$C$\underline{H}_3$).

Hapten 3. To a stirred solution of 0.50 g (1.47 mmol) of **1** in 3 mL of methanol cooled in an ice-water bath was added dropwise a solution of 0.205 g (3.23 mmol) of KOH and 0.166 g (1.61 mmol) of 4-(methylamino)butyric acid hydrochloride in 1.7 mL of methanol. After stirring for 10 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. The residue was subjected to column chromatography (silica gel, 3 : 1 : trace chloroform-ethyl acetate-acetic acid) to provide 320 mg (54%) of Hapten 3. ^{1}H NMR ($CDCl_3$): δ 7.82 (1H, s, ar), 4.42-4.30 (2H, qxd, J = 8.7 & 7.1, C$\underline{H}_2$CH$_3$), 3.38-3.28 (2H, m, NHC$\underline{H}_2$), 2.87 (3H, d, J = 12.4, C$\underline{H}_3$N), 2.46 (2H, t, C$\underline{H}_2$CO$_2$H), 1.93 (2H, qn, CH$_2$C$\underline{H}_2$CH$_2$), 1.43 (3H, t, J = 7.1, CH$_2$C$\underline{H}_3$).

2. To a stirred solution of 10 g (50 mmol) of 2,4,5-trichlorophenol in 50 mL of acetic acid cooled in an ice-water bath was added dropwise 3.46 g (55 mmol) of 70% nitric acid dissolved in 13 mL of acetic acid. After stirring for 10 min during which the colorless solution turned dark orange, the solvent was removed under reduced pressure. The product was purified by crystallization from benzene-hexane to provide 6.10 g of dark orange-colored solid. Evaporation of the filtrate and crystallization of the residue gave an additional 3.23 g of the product. Yield 76%. ^{1}H NMR ($CDCl_3$): δ 7.67 (1H, s, ar), 5.8-3.5 (1H, s, broad, OH).

3. A solution of **2** (100 mg, 0.41 mmol), diethyl chlorothiophosphate (108 mg, 0.57 mmol), and triethylamine (50 mg, 0.49 mmol) in 0.66 mL of anhydrous THF was stirred at 70 °C for 4 h. Diethyl chlorothiophosphate (52 mg, 0.28 mmol) and triethylamine (25 mg, 0.25 mmol) were added additionally to the reaction mixture and stirred for 1 h. The reaction mixture was filtered and the solvent was evaporated. The residue was subjected to preparative TLC (silica gel, 90 : 10 : 1 hexane-ethyl acetate-acetic acid) to provide 151 mg (93%) of the product as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 8.10 (1H, s, NHCO), 7.70 (1H, s, ar), 4.34-4.26 (4H, qxdxd, J = 9.6, 7.1 & 2.1, C$\underline{H}_2$CH$_3$), 1.40 (6H, t, J = 7.0, CH$_2$C$\underline{H}_3$).

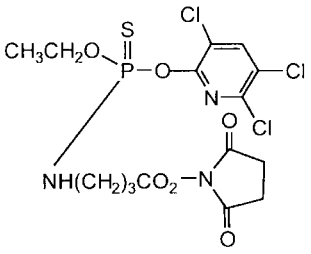
4. To a solution of **3** (1.74 g, 4.4 mmol) in 50 mL of ether was added 20 mL of 9 : 1 acetic acid-HCl. Zinc dust (4 g) was added to the mixture in portions maintaining a gentle reflux. After being refluxed for 45 min, the mixture was decanted from the reaction flask and zinc was washed with ether. The combined organic phase was then washed with water and dried over K_2CO_3. The solvent was evaporated under reduced pressure and the residue was subjected to TLC (silica gel, 4 : 1 hexane-ethyl acetate) to give 1.49 g (93%) of the product as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 6.91 (1H, s, ar), 4.76 (2H, s, NH_2), 4.36-4.26 (4H, qxd, J = 9.6 & 7.1, C$\underline{H}_2$CH$_3$), 1.39 (6H, t, J = 7.0, CH$_2$C$\underline{H}_3$).

Hapten 4. A solution of $\underline{4}$ (0.15 g, 0.41 mmol) and adipoyl chloride (0.45 g, 2.4 mmol) in 0.8 mL of toluene was stirred at 95 °C for 30 min under an anhydrous condition ($CaCl_2$ drying tube). The solvent was evaporated under reduced pressure and the residue was dissolved in 5 mL of 4 : 1 THF-1N HCl. After stirring at 60 °C for 50 min, the solution was cooled and 0.5 mL of ethyl acetate was added to the cooled solution. The organic layer was washed with water and dried over Na_2SO_4. The solvent was evaporated and the residue was subjected to preparative TLC (silica gel, 2 mm, 30 : 20 : 1 hexane-ethyl acetate-acetic acid) to provide 0.16 g (79%) of a white solid. ^{1}H NMR ($CDCl_3$): δ 8.06 (s, 1H, NH), 7.53 (1H, s, ar), 4.30-4.22 (4H, qxd, J = 9.6 & 7.0, C$\underline{H}_2$CH$_3$), 2.74 (2H, t, C$\underline{H}_2$CO$_2$), 2.51 (2H, t, C$\underline{H}_2$CONH), 1.93 (4H, m, CH$_2$C$\underline{H}_2$C$\underline{H}_2$CH$_2$), 1.40 (6H, t, J = 7.0, CH$_2$C$\underline{H}_3$).

Preparation of hapten-protein conjugates. The haptens were covalently attached to either KLH (immunogens), OVA (coating antigens) or HRP (enzyme tracer) using the active ester method.[8] The structure of the active ester in case of Hapten 1 is shown in Figure 4. As the haptens were found to hydrolyze rapidly at pH > 8, distilled water-DMF was used as the solvent for the conjugation. Hapten 4, which is quite stable near pH 8, was conjugated to the protein (OVA) in pH 8 borate buffer. Conjugation reactions in water-DMF were carried out by two different procedures: 1) the synthesis of active ester and then conjugation of isolated ester with protein and 2) *in situ* performing of active ester formation and conjugation. Hapten-KLH conjugates (immunogens) were prepared using both methods. Hapten-OVA and hapten-HRP conjugates were prepared only by method 1.

Conjugation *via* isolated active ester. 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 (210 mg, 0.52 mmol) was dissolved in dichloromethane (10 mL) to which *N*-hydroxysuccinimide (65 mg, 0.56 mmol) followed by *N*,*N*-dicyclohexylcarbodiimide (119 mg, 0.58 mmol) and 4-dimethylaminopyridine (7 mg, 0.06 mmol) were added. The



Active ester of Hapten 1

Figure 4. Structure of active ester of Hapten 1.

mixture was stirred for 1 h and filtered, and the solvent was removed. Chromatography of the resultant oil on silica gel using 65 : 35 : 1 benzene-ethyl acetate-acetic acid gave the active ester as a syrup (216 mg, 83%). ^{1}H NMR ($CDCl_3$): δ 7.84 (1H, d, J = 1.1, ar), 4.53-4.41 (2H, qxd, J = 10.2 & 7.1, $C\underline{H}_2CH_3$), 3.57-3.48 (1H, m, N$\underline{H}$), 3.28-3.20 (2H, m, $NHC\underline{H}_2$), 2.86 (4H, s, succinyl), 2.61 (2H, t, J = 7.2, $C\underline{H}_2CO_2H$), 1.89 (2H, qn, J = 7.0, $CH_2C\underline{H}_2CH_2$), 1.40 (3H, t, J = 7.0, $CH_2C\underline{H}_3$).

Active ester of Hapten 2. Yield 31%. ^{1}H NMR ($CDCl_3$): δ 7.85 (1H, d, J = 1.1, ar), 4.40-4.27 (2H, qxd, J = 9.7 & 7.0, $C\underline{H}_2CH_3$), 3.53-3.45 (1H, m, N$\underline{H}$), 3.30-3.11 (2H, m, $NHC\underline{H}_2$), 2.84 (4H, s, succinyl), 2.63 (2H, t, J = 7.3, $C\underline{H}_2CO_2H$), 1.85-1.46 [6H, m, $CH_2(C\underline{H}_2)_3CH_2$], 1.41 (3H, txd, J = 7.1 & 0.76, $CH_2C\underline{H}_3$).

Active ester of Hapten 3. Yield 16%. ^{1}H NMR($CDCl_3$): δ 7.83 (1H, s, ar), 4.38-4.29 (2H, qxd, J = ca. 9 & 7.0, $C\underline{H}_2CH_3$), 3.48-3.31 (2H, m, $NC\underline{H}_2$), 2.88 (3H, d = 12.0, $C\underline{H}_3N$), 2.84 (4H, s, succinyl), 2.76-2.71 (2H, m, $C\underline{H}_2CO_2H$), 2.1-2.0 (2H, m, $CH_2C\underline{H}_2CH_2$). 1.42 (3H, t, J = 7.0, $CH_2C\underline{H}_3$).

Active ester of Hapten 4. Yield 27%. ^{1}H NMR($CDCl_3$): δ 8.08 (s, 1H, NH), 7.53 (1H, s, ar), 4.32-4.27 (4H, qxd, J = 9.0 & 7.0, $C\underline{H}_2CH_3$), 2.86 (4H, s, succinyl), 2.70 (2H, t, $C\underline{H}_2CO_2$), 2.50 (2H, t, $C\underline{H}_2CONH$), 1.92 (4H, m, $CH_2C\underline{H}_2C\underline{H}_2CH_2$), 1.40 (6H, t, J = 7.0, $CH_2C\underline{H}_3$).

The procedure for conjugation was as follows. To prepare KLH conjugates (immunogens), KLH (20 mg) was dissolved in 4 mL of distilled water to which 1.2 mL of DMF was added. A solution of hapten (0.01 mmol) and NaN_3 (final concentration of 0.02%) in 0.2 mL of DMF was then added to the stirred protein solution and stirring was continued for 1 or 2 days at ambient temperature. To prepare OVA conjugates (coating antigens), OVA (10 mg) was dissolved in 1 mL of distilled water or borate buffer (0.2 M, pH 8.0, for the conjugation of Hapten 4) to which 0.15 mL of DMF was added. A solution of hapten (4.4 or 11 μmol) and NaN_3 (final concentration of 0.02%) in 0.1 mL of DMF was then added to the stirred protein solution and stirring was continued for 1 or 2 days at ambient temperature. To prepare HRP conjugates (enzyme tracer), HRP (2 mg) was dissolved in 1 mL of distilled water or borate buffer (0.2 M, pH 8.0, for the conjugation of Hapten 4) to which 0.15 mL of DMF was added. A solution of hapten (0.5 or 2.5 μmol) in 0.1 mL of DMF was then added to the stirred protein solution and stirring was continued for 1 day at ambient temperature. Conjugates were separated from uncoupled haptens by gel filtration (Sephadex G-25), using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.0). Finally the eluates were dialyzed against water overnight.

***In situ* conjugation**. A published procedure[11] was used with a slight modification. To a stirred solution of KLH (20 mg) in 1 mL of distilled water was added a solution of hapten (0.009 mmol) in 0.1 mL of DMF, 1 mg (0.1 mmol) of sulfo-NHS and 20 mg (0.1 mmol) of EDC. Stirring was continued overnight at ambient temperature. Conjugates were purified by gel filtration on Sephadex G-25, using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.0) and dialysis against water.

Immunization of rabbits. Female New Zealand white rabbits were immunized with Hapten 1- or Hapten 2-KLH. Routinely, 200 μg of the conjugate dissolved in 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 200 μg of the conjugate emulsified with Freund's 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. The titer of the serum from each animal was determined by measuring the binding of serial dilutions to microtiter plates coated with several different concentrations of the homologous coating antigens. Microtiter plates were coated with Hapten 1-OVA (1 μg/mL, 100 μL/well) in PBS (10 mM, pH 6.9) and incubated overnight at 4 °C while covered with adhesive plate sealers. The following day, the plates were washed five times with PBSTA (10 mM PBS containing 0.05% Tween 20 and 0.02% NaN_3, pH 7.4) and 100 μL/well of serum diluted with PBST (10 mM PBS containing 0.05% Tween 20, pH 6.9) was incubated for 1 h at 37 °C. The dilution ratios were 10000, 50000, 250000. After another washing step, 100 μL/well of goat anti-rabbit IgG, conjugated with alkaline phosphatase, diluted 1 : 2000 with PBST was added and incubated for 1 h. Then the plates were washed again, and *p*-nitrophenyl phosphate (1 mg/mL) dissolved in 10% diethanolamine buffer, pH 9.8 was added (100 μL/well). After incubation at 37 °C for 30 min, the reaction was stopped by adding 3 N NaOH (50 μL/well) and the absorbance was read at 405 nm.

Indirect competitive assay. A checkerboard assay, in which sera were titrated against varying amounts of a coating antigen (either one of the four haptens conjugated to OVA) was used to optimize antigen coating and antibody concentrations. The coated-antigen assays under the optimized conditions were performed as follows. Microtiter plates were coated with Hapten 1-OVA (2.5 μg/mL, 100 μL/well) in PBS (10 mM, pH 6.9) and incubated overnight at 4 °C. The plates were washed five times with PBST and were blocked by incubation with 1% gelatin in PBS (200 μL/well) at ambient temperature for 1 h. After another washing step, serial dilutions of the analyte standard in 10% MeOH-PBS were added (50 μL/well) followed by 50 μL/well of antiserum previously diluted with PBST (1/5000). After incubation at ambient temperature for 1 h, the plates were washed and 100 μL/well of a diluted goat anti-rabbit IgG-horseradish peroxidase (1/3000) was added. The mixture was incubated at ambient 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 ambient temperature. The reaction was stopped after 10 min 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.[12]

Direct competitive assay. A checkerboard assay, in which sera were titrated against varying amounts of an enzyme tracer (either one of the four haptens conjugated to HRP) was used to optimize the amount of enzyme tracer and antibody. The 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 PBS (10 mM, pH 6.9) by incubation at 4 ^{o}C overnight. The plates were washed five times with PBST (10 mM PBS containing 0.05% Tween 20, pH 7.4) and were coated with 200 μL of the antiserum dilutions (1/1000) in PBS for 1 h at ambient temperature. Instead of using PBST at pH 6.9 that was used for indirect assay, PBST at pH 7.4 was used since the conjugates were found later to be stable at this pH. 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 (1/1000). After incubation at ambient temperature for 1 h and another washing step, 100 μL/well of a TMB solution was added. The reaction was stopped after 10 min by adding 50 μL of 2 M H_2SO_4 and absorbance was read at 450 nm. Standards were run in quadruplicate. Competition curves were obtained by the same procedure as for the indirect assays.

Determination of cross-reactivities. The compounds listed in Table 2 were tested for cross-reactivity by preparing standard curves using the direct assays and determining their I_{50} values (concentration at which binding of the antibody to the enzyme tracer is inhibited by 50%). The cross-reactivity values were calculated as follows: (I_{50} of chlorpyrifos/I_{50} of compound) × 100.

Results and Discussion

Hapten selection. 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 chlorpyrifos ELISA, it was desirable to synthesize immunogenic haptens having a bridge at the thiophosphate group preserving the aromatic ring unique to chlorpyrifos. Hapten 1 and 2 were chosen as immunogenic haptens on this ground. Reactivity and selectivity of some of the antibodies were fairly high as described below.

Screening of the sera. The titers of the antisera raised against Hapten 1 and Hapten 2 are shown in Table 1. In general, the antibodies raised against Hapten 1 showed higher titers than Hapten 2. The antisera against the immunogens prepared using the isolated active esters (976, 445, 834 and 581) showed reasonably high titers, but the antisera against the immunogens prepared by *in situ* conjugation (593 and 228) showed virtually no titer values.

Indirect ELISA. The two dimensional titration of the antisera 581 and 976 against the four coating antigens revealed that Hapten 1-OVA is the one with the highest and widest range of recognition for the antibody. Figure 5 shows the results of the competition experiments using the antisera of high titers and Hapten 1-OVA (coating antigen). Antiserum 976 that exhibited the highest titer in serum screening showed virtually no recognition for the analyte (no competition) not only at 1/5000 serum dilution, but also at 1/10000 dilution. In addition, the antibodies showed a very high background due to strong binding of the antibody to the blocking protein BSA. The combination of antiserum 581 (2nd boost)/Hapten 1-OVA seemed to be the most promising for indirect immunoassays. Using this combination, antigen-coated ELISA was optimized with regard to the dilution of antiserum and coating antigen, incubation time and the type of blocking protein. Figure 6 shows the inhibition curve obtained after optimization. The assay showed an I_{50} value of 160 ppb with a detection limit of about 10 ppb, which is a rather low sensitivity. In addition to this, this assay has the problem of too high a background.

Table 1. Titers of antisera determined by indirect ELISA[a] using Hapten 1-OVA as the coating antigen

Antiserum[b] (1:10,000)	Boost[c]				
	1st	2nd	3rd	4th	5th
976 (1)	0.1040	2.0115	2.9065	2.0165	
445 (1)	–0.0415	0.9035	1.1400	1.8020	
834 (1)	0.7280	1.0855	1.7110	0.8425	
581 (2)	0.2940	1.2780	0.2835	0.7135	0.8195
593 (2)	0.0345	0.1175	0.1055	0.1365	0.2090
228 (2)	0.2570	0.1465	–0.0025	–0.0010	0.0125

[a]Used alkaline phosphatase-conjugated anti-rabbit IgG as the secondary antibody. Blocking of the wells was omitted. [b]The figures 1 and 2 in parenthesis indicate that the antisera were obtained from Hapten 1 and Hapten 2, respectively. [c]Absorbance of antiserum-absorbance of control serum.

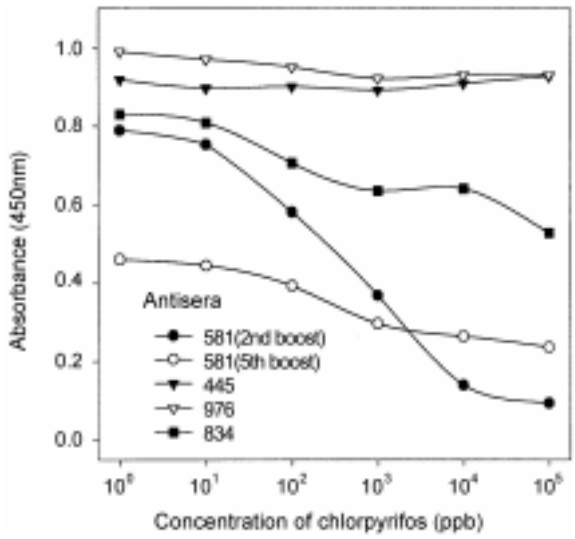


Figure 5. Standard curves for chlorpyrifos using the antisera of high titers. Coating antigen: hapten 1-OVA, 250 ng/well, Blocking: 1% gelatin.

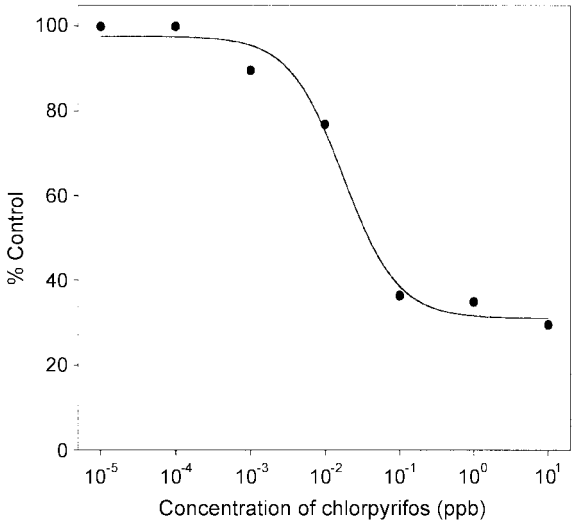


Figure 6. Standard curve for chlorpyrifos by indirect competitive ELISA using antiserum 581, the coating antigen Hapten 1-OVA (250 ng/well) and gelatin blocking agent (1%).

Direct ELISA. Two-dimensional titration of antiserum 581 against the four enzyme tracers showed that Hapten 4-HRP is the one with the highest and widest range of recognition for the antibody. All the enzyme tracers except Hapten 3-HRP showed a quite strong binding to the antibodies, however inhibition of the binding by the analyte was appreciable only in case of Hapten 4-HRP. Higher inhibition of the binding of Hapten 4-HRP by the analyte (greater competition) could be attributed to the weaker binding of this enzyme tracer to the antibodies due to the heterogeneity of hapten structure. Hapten 4 is unique among the four haptens in that it has no nitrogen in the ring. Antibody-coated ELISA using the combination of antiserum 581/Hapten 4-HRP was optimized with regard to the dilution of antiserum and enzyme tracer, and incubation time. Figure 7 shows the inhibition curve obtained after optimization. The assay showed an I_{50} value of about 20 ppb with a detection limit of about 0.1 ppb, which is much better than that of indirect ELISA in sensitivity. The I_{50} value and the detection limit of the ELISA previously reported were approximately 4 and 1 ppb, respectively.[6] Therefore, the present ELISA is somewhat less sensitive, but shows lower detection limit compared to the previous one. Several organophosphorus pesticides as well as chlorpyrifos metabolites were tested for cross-reactivities. Table 2 shows the cross-reactivity that was found by the direct assay, expressed in percentage of the I_{50} of chlorpyrifos. In all cases, the interference to the assay was negligible except for chlorpyrifos-methyl and bromophos-ethyl. The appreciable cross-reactivities for these pesticides are understandable as they have the same or similar aromatic structure as chlorpyrifos. It is well known that chlorpyrifos is metabolized in human body and in the environment into the pyridinol (3,5,6-trichloro-2-pyridinol).[13] Although the pyridinol has the same aromatic structure as that of chlorpyrifos, its I_{50} value indicates that it can not be an interference in the analysis of chlorpyrifos by ELISA. It may be concluded that the direct ELISA that was developed is

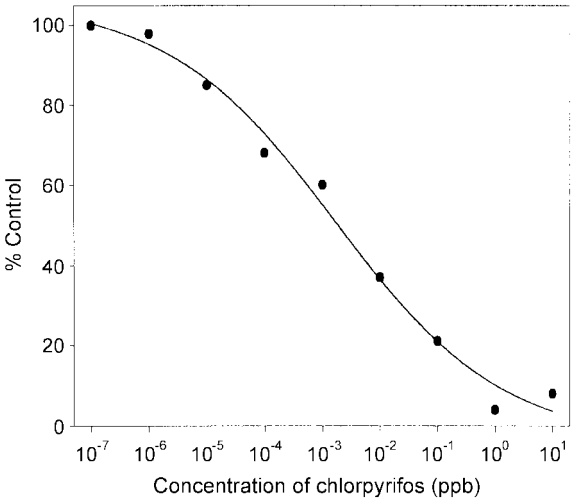
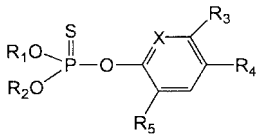
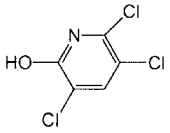
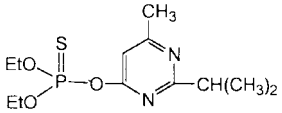
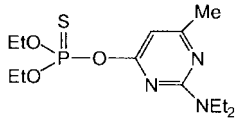
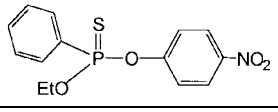


Figure 7. Standard curve for chlorpyrifos by direct competitive ELISA using antiserum 581, the coating antigen Hapten 4-OVA (250 ng/well) and gelatin blocking agent (1%).

Table 2. Cross-reactivity of compounds structurally related to chlorpyrifos, as determined by direct competitive ELISA[a]



Compound	Structure						I_{50} (ppm)	CR[b] (%)
	R_1	R_2	X	R_3	R_4	R_5		
Chlorpyrifos	Et	Et	N	Cl	Cl	Cl	0.026	100
Chlorpyrifos-methyl	Me	Me	N	Cl	Cl	Cl	0.039	66.6
Bromophos-ethyl	Et	Et	C	Cl	Br	Cl	0.167	15.6
Bromophos-methyl	Me	Me	C	Cl	Br	Cl	0.567	4.58
Dichlofenthion	Et	Et	C	H	Cl	Cl	0.850	3.05
Parathion	Et	Et	C	H	NO_2	H	14.3	0.18
Parathion-methyl	Me	Me	C	H	NO_2	H	21.3	0.12
Fenitrothion	Me	Me	C	Me	NO_2	H	5.99	0.43
3,5,6-Trichloro-2-pyridinol							1.14	2.27
Diazinon							22.8	0.11
Pirimiphos-ethyl							0.425	6.1
EPN							> 100	< 0.03

[a]Antiserum to Hapten 1-KLH, 581, diluted 1/1000; enzyme tracer: Hapten 4-HRP. [b]Cross-reactivity (%) = (I_{50} of chlorpyrifos/I_{50} of other compound) × 100.

suitable for the sensitive and selective detection of chlorpyrifos, with the exception of chlorpyrifos-methyl and bromophos-ethyl.

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