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

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A competitive enzyme-linked immunosorbent assay (ELISA) was developed for the quantitative detection of organophosphorus insecticide cyanophos. An analogue (hapten) of cyanophos was synthesized and was coupled to BSA to produce polyclonal antibodies from rabbits. The antisera were screened against another hapten coupled to ovalbumin (OVA). Using the sera of highest specificity, an antigen-coated ELISA was developed, which showed an I_{50} of 310 ng/mL with the detection limit of 20 ng/mL. The antibodies showed negligible cross-reactivities with other organophosphorus pesticides except for parathion-methyl, which makes the assay suitable for the selective detection of cyanophos.

Keywords : Cyanophos, Insecticide, Immunoassay, Enzyme-linked immunosorbent assay, ELISA.

Introduction

As a consequence of the widespread use of pesticides, detection of their residues in food and in the environment has become an important issue in analytical science. 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, skilled analysts, and time-consuming sample preparation. Therefore, there is a growing demand for more rapid and economical methods for determining pesticide residues. Immunochemical techniques that have been used extensively in clinical laboratories, began recently to gain acceptance as a fast, sensitive, and cost-effective alternative to the traditional techniques for detecting trace amounts of chemicals such as pesticides.[2]

Cyanophos [*O*-(4-cyanophenyl) *O,O*-dimethyl phosphorothioate] is an organophosphorus insecticide, which is effective against Aphididae, Coccidae, Diaspididae, Lepidoptera, etc. in various fruits and vegetables.[3] It is also used to control locusts and various sanitary pests. The toxicological effect after administration of cyanophos is inhibition of acetylcholinesterase activity.[3] The objective of this study is the development of an ELISA for cyanophos. 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 in the development of ELISA for several organophosphorus pesticides.[4-6] We have developed a novel method for the synthesis of such haptens, which requires fewer steps than those of the previous ones. In this study, this new method was applied to the synthesis of haptens for cyanophos, from which specific antibodies to cyanophos were obtained. Using the antibodies, a selective ELISA for cyanophos was developed.

Experimental section

Chemicals and instruments. Cyanophos was purchased from Chem Service (West Chester, USA). Other pesticides were obtained from Dr. Ehrenstorfer (Augsburg, Germany). 3-(methylamino)butyric acid hydrochloride, 4-aminobutyric acid, *N*-hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide, chlorform-*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), Freunds complete (F-5881) and incomplete (F-5506) adjuvants, and Sephadex G-25 were purchased from Sigma (St. Louis, USA). 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 Immuno-Wash 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 (Palo Alto, USA) 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 d, t, q, qn and ar represent doublet, triplet, quartet, quintet and aromatic, respectively.

Synthesis of haptens. The haptens used for immunization and antigen coating are presented in Figure 1. The synthetic

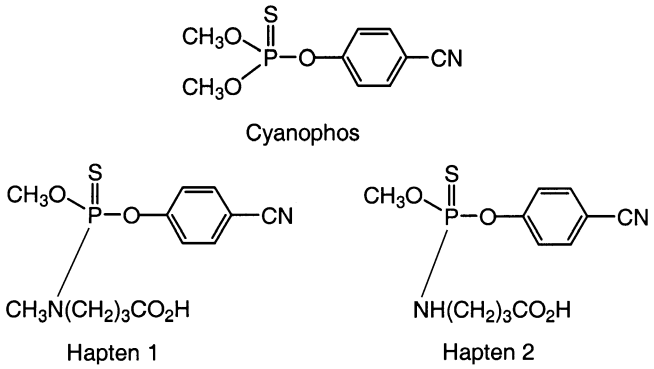


Figure 1. Structures of the haptens for cyanophos used for immunization (Hapten 1) and antigen coating (Hapten 2).

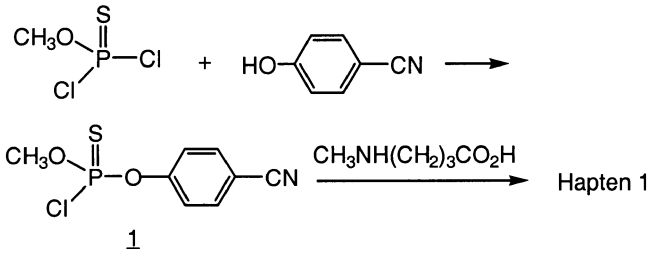


Figure 2. Synthetic route for Hapten 1.

routes for Hapten 1 are illustrated in Figure 2. The procedures for each step are as follows.

1. To a stirred mixture of 579 mg (3.50 mmol) of methyl dichlorothiophosphate,[7] 1 g of finely ground K_2CO_3 and 0.1 mL acetonitrile was added dropwise *p*-cyanophenol (278 mg, 2.34 mmol) dissolved in 0.1 mL of acetonitrile. After stirring for 30 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, 2 : 1 hexane-benzene) to give 341 mg (60%) of a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.73 (2H, d, J = 8.7, ar), 7.40 (2H, d, J = 8.7, ar), 4.03 (3H, d, J = 16.4, CH_3OP).

Hapten 1. To a stirred solution of 180 mg (0.72 mmol) of **1** in 0.2 mL of methanol cooled in an ice-water bath was added dropwise a solution of 155 mg (2.76 mmol) of KOH and 101 mg (0.86 mmol) of 3-(methylamino)butyric acid hydrochloride in 1.25 mL of methanol. After stirring for 7 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) of the residue gave 170 mg (71%) of a white syrup. ^{1}H NMR ($CDCl_3$): δ 7.64 (2H, d, J = 8.8, ar), 7.29 (2H, d, J = 8.3, ar), 3.74 (3H, d, J = 14.0, CH_3OP), 3.32 (2H, q x d, J = 13.7 & 6.8, NCH_2), 2.85 (3H, d, J = 11.1, CH_3N), 2.40 (2H, t, J = 7.4, CH_2CO), 1.89 (2H, qn, J = 7.3, $CH_2C\underline{H}_2CH_2$).

Hapten 2. This was synthesized by the same procedure as that for Hapten 1 using 4-aminobutyric acid. The yield was 59%. ^{1}H NMR ($CDCl_3$): δ 7.65 (2H, d, J = 8.5, ar), 7.34 (2H, d, J = 8.8, ar), 3.80 (3H, d, J = 14.1, CH_3OP), 3.46 (1H, qn, J = 7.2, NH), 3.16 (2H, q x d, J = 11.7 & 6.9, NCH_2), 2.44 (2H, t, J = 7.1, CH_2CO), 1.87 (2H, qn, J = 6.9, $CH_2C\underline{H}_2CH_2$).

Preparation of hapten-protein conjugates. Hapten 1 was covalently attached to BSA to use as an immunogen and Hapten 2 was coupled to OVA to use as a coating antigen. The procedure for the synthesis of Hapten 1-BSA conjugate using the active ester method[5] was as follows. To Hapten 1 (16 mg, 50 μmol) dissolved in DMF (0.2 mL) was added a solution of *N*-hydroxysuccinimide (8.6 mg, 75 μmol) and *N,N*-dicyclohexylcarbodiimide (15 mg, 75 μmol) in DMF (0.2 mL). The mixture was stirred for 2 h at room temperature, filtered, and then BSA (20 mg, 0.3 μmol) dissolved in 2 mL of borate buffer (0.2 M, pH 8.7) was added to the filtrate. After stirring at room temperature for 1.5 h, the conjugate was separated from the uncoupled hapten by gel filtration (Sephadex G-25), using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) as eluant. The eluates were freeze-dried. OVA and KLH conjugates (coating antigens) were prepared by the same procedure, however, they were purified by gel filtration followed by dialysis in water at 4 °C for 24 h.

Immunization of rabbits. Female New Zealand white rabbits were immunized with Hapten 1- or Hapten 2-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 7-10 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 aliquotted and stored at -70 °C.

Screening of antisera. Checkerboard assays (coated-antigen format), 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 competitive assays. The coated-antigen assays were performed as follows. All incubations were carried out at 37 °C unless stated otherwise. Microtiter plates were coated with Hapten 2-OVA (250 or 1000 μg/mL, 100 μL/well) in PBS (10 mM, pH 7.4) 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) for 1 h. After another washing step, 100 μL/well of antiserum previously diluted with PBST (1/500-1/2000) was added. After incubation for 1 h, the plates were washed and 100 μL/well of a diluted (1/2000 or 1/5000) goat antirabbit IgG-horseradish peroxidase was added. The mixture was incubated 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. The reaction was stopped after 6 min by adding 50 μL of 2 M H_2SO_4 and absorbance was read at 450 nm.

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

Methanol (%)	Abs_{max}	Slope	I_{50} (μg/mL)
10	0.814	0.578	2.817
20	0.752	1.084	1.535
30	0.873	1.056	4.576
40	0.953	2.364	6.754

[a]ELISA conditions: antiserum to Hapten 1-BSA, diluted 1/1000 with 100 mM PBST; coating antigen, Hapten 2-OVA, 100 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	7.5	1.526	1.488	2.523
50	15.0	1.208	0.865	2.802
100	16.5	1.034	0.650	0.524
200	16.5	0.887	0.664	1.550

[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 cyanophos and the antiserum diluted with different concentrations of PBST. [c]Time for color development.

Competitive indirect ELISA. Antigen coating and antibody concentration for the competitive assays were optimized. Also the tolerance of ELISA to methanol used to dissolve pesticides 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 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) used to dilute the antisera. The procedure of the competitive assay was as follows. To microtiter plates coated (100 ng/well) and blocked as described above, 50 μL/well of serial dilutions of the analyte in methanol-PBS (10 mM) was added, followed by 50 μL/well of the antiserum diluted 1/1000 with PBST. After incubation at 37 °C 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,[8] from which I_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) are determined.

Determination of cross-reactivities. The compounds listed in Table 1 were tested for cross-reactivity using the ELISA procedure described above. The cross-reactivity values were calculated as follows: (I_{20} of cyanophos/I_{20} of compound) × 100.

Results and Discussion

Hapten selection and synthesis. The phosphorothioate organophosphorus pesticides to which cyanophos belongs have a thiophosphate group in common and differ only in the structure of aromatic rings. Therefore, to achieve a high selectivity in cyanophos ELISA, it was desirable to synthesize immunogenic haptens having a bridge at the thiophosphate group preserving the aromatic ring unique to cyanophos. Hapten 1 was chosen as an immunogenic hapten on this ground. Several investigators used haptens having a bridge at the thiophosphate group to develop ELISAs for organophosphorus pesticides.[4-6] To synthesize such haptens, they used an aminocarboxylic acid protected at the carboxylic group to attach a bridge to phosphorus atom and then deprotected the bridge later. In our laboratory, a synthetic method for attaching an aminocarboxylic acid bridge to phosphorus without protection was developed. This method worked well for the synthesis of three haptens used in this study. The route which Skerrit[4-6] used to synthesize such haptens involves 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 displace 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.

ELISA. The checkerboard assays, in which the sera were titrated against varying amounts of the coating antigen (Hapten 2-OVA) was used to estimate the reactivity of the antisera and to optimize antigen coating and antibody concentration for the competitive assays. All of the four antisera obtained showed reasonably high recognition for the coating antigen. However, in competition experiments followed, the sera showed quite different degree of inhibition by the analyte for binding to the coating antigen. The one that exhibited the largest inhibition by the analyte was selected as the assay reagent. The optimum condition selected was the combination of this serum diluted 1/1000 and 100 ng/well of the coating antigen.

Since organic solvents are used 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 that 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.[9-12] Table 1 shows the effect of methanol concentration on the performance of the cyanophos ELISA. Increasing the concentration of methanol of the standard solutions affected not only the sensitivity of the assay but also the slope of the calibration curve. With increasing amounts of methanol, the sensitivity of the assay increased up to 20%

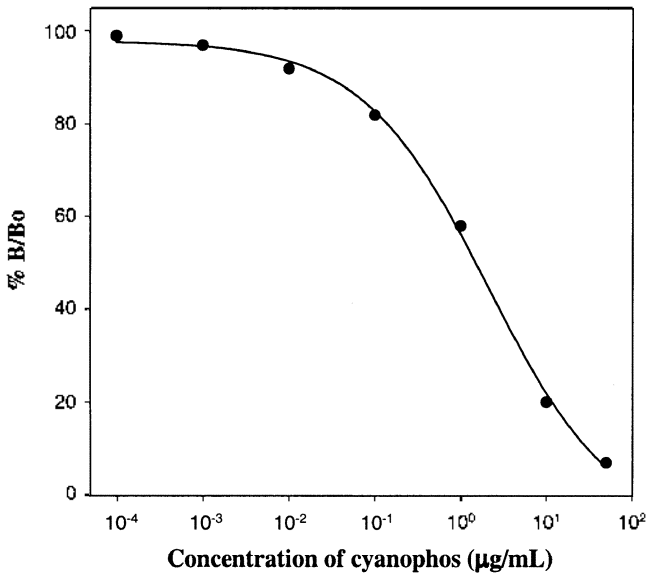


Figure 3. Calibration graph for cyanophos by indirect competitive ELISA using the antisera against Hapten 1-BSA, the coating antigen Hapten 2-OVA (100 ng/well) and gelatin blocking agent (1%). Standards were prepared in 10% methanol-PBS (10 mM) and the sera were diluted 1/1000 with 390 mM PBST. $\%B/B_0 = (A - A_{ss}/A_0 - A_{ss}) \times 100$, 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.

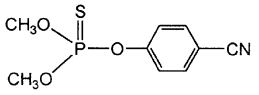
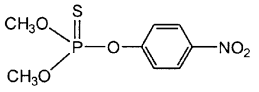
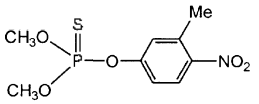
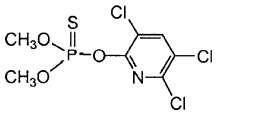
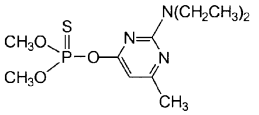
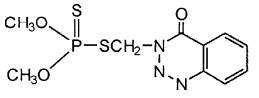
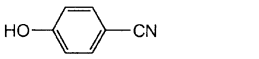
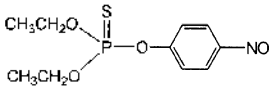
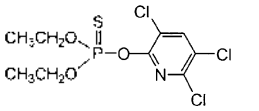
methanol and then decreased, and the slope of the curve continued to decrease. The lowest I_{50} value was found at 20% methanol.

Table 2 presents the effect of the buffer concentration of the competition medium on ELISA characteristics. Increasing the concentration of the phosphate caused large increase of sensitivity up to 100 mM phosphate and then decrease of sensitivity, which differ from the results of several previous studies,[13,14] in which sensitivity continued to increase with increasing amount of phosphate. Due to the nonpolar nature of cyanophos, 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. Since increased 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 3 shows a typical inhibition curve obtained after optimization. The lowest I_{50} value observed was 310 ng/mL with a detection limit of about 20 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 cyanophos in food samples at levels near maximum residue limits. Temporarily established MRLs of cyanophos in Japan for fruits and vegetables are 0.2 and 0.05 ppm, respectively.

Cross-reactivity studies. Several organophosphorus pesticides as well as the metabolite of cyanophos (phenol) were tested for cross-reactivities. Table 1 shows the cross-reactivities that were found by the indirect assay described above, expressed in percentage of the I_{20} of cyanophos. The interference to the assay was negligible except for parathion-methyl. The appreciable cross-reactivity for this pesticide is understandable as it has a small substituent only at the *para* position of the benzene ring as cyanophos does. It may be concluded that the competitive ELISA developed in this study is suitable for the selective detection of cyanophos, with the exception of parathion-methyl.

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

Pesticide	Structure	I_{20} (μg/mL)[b]	CR (%)[c]
Cyanophos		0.27	100
Parathion-methyl		0.63	43
Fenitrothion		4.1	6.6
Chlorpyrifos-methyl		> 100	< 0.27
Pirimiphos-methyl		> 100	< 0.27
Azinphos-methyl		> 100	< 0.27
4-Cyanophenol		> 100	< 0.27
Parathion-ethyl		> 100	6.6
Chlorpyrifos-ethyl		> 100	< 0.27

[a]Determined by indirect ELISA using antiserum to Hapten 1-BSA and coating antigen Hapten 2-OVA. [b]I_{20} values of the pesticides below chlorpyrifos-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 100 ppm. [c]Cross-reactivity(%) = (I_{20} of cyanophos/I_{20} of other compound) × 100.

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