Development of an Enzyme-Linked Immunosorbent Assay for the Organophosphorus Fungicide Tolclofos-methyl

Kyung Yi Park, Won Chul Park, Yoo Jung Kim, and Yong Tae Lee*

Department of Biochemistry and Institute of Natural Sciences, Yeungnam University, Gyongsan 712-749, Korea
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A simple synthetic method for haptens of organophosphorus (OP) pesticides with a spacer arm (aminocarboxylic acid) attached at the pesticide thiophosphate group was developed and was applied to the synthesis of haptens for the OP fungicide tolclofos-methyl. Using the haptens, a selective enzyme-linked immunosorbent assay (ELISA) for tolclofos-methyl was developed. One of the haptens was coupled to BSA to use as an immunogen. Rabbits were immunized with this conjugate to obtain polyclonal antibodies to tolclofos-methyl. The antisera were screened against another hapten coupled to ovalbumin (OVA). Using the serum with highest specificity, an antigen-coated ELISA was developed, which showed an IC_{50} of 160 ng/mL with the detection limit of 20 ng/mL. The antibodies showed negligible cross-reactivity with other OP pesticides. An antibody-coated ELISA was also developed, which showed an IC_{50} of 410 ng/mL with a detection limit of 130 ng/mL.

Key Words : Tolclofos-methyl, Fungicide, Enzyme-linked immunosorbent assay, ELISA

Introduction

Since the 1970s, OP pesticides have replaced the organochlorine insecticides previously used and are now widely used on agricultural crops to control a large variety of pests. However, the OP pesticides generally have higher acute toxicity than the chlorinated insecticides, which necessitates comprehensive monitoring programs for them. Tolclofos-methyl [*O*,*O*-dimethyl *O*-(2,6-dichloro-4-methylphenyl) phosphorothioate] is an OP fungicide used for the control of soil-borne diseases caused by *Rhizoctonia*, *Corticium*, and *Sclerotium* on potatoes, sugar beet, vegetables, cereals, bulb flowers, lawn turf, etc..[1] The toxicologically relevant effect after administration of tolclofos-methyl is inhibition of phospholipid biosynthesis, leading to inhibition of germination of spores and growth of fungal mycelium.[1]

Current analytical methods involving gas and liquid chromatography for the detection of pesticide residues are sensitive and reliable.[2] 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.[3]

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 OP pesticides is the one with an aminocarboxylic acid bridge at the thiophosphate group, which has been used for the development of ELISA for several OP pesticides.[4-12] We have developed a simple method for the synthesis of such haptens, which requires far fewer steps and can provide a wider range of hapten structures.[4-6] We previously applied this method to the synthesis of several OP pesticides including chlorpyrifos,[4] isofenphos,[5] and cyanophos.[6] In this study, we further confirmed the general applicability of this method by synthesizing haptens for the OP fungicide tolclofos-methyl by this method. Then, we raised antibodies against one of these haptens to develop an antigen-coated and an antibody-coated ELISA for tolclofos-methyl. An antibody-coated ELISA for this pesticide has already been developed by Kawada *et al.* and was patented,[13] however, an antigen-coated ELISA, a more popular format, has not yet been developed.

Experimental Section

Reagents and instruments. Tolclofos-methyl was purchased from Chem Service (West Chester, USA). Other OP pesticides were obtained from Dr. Ehrenstorfer (Augsburg, Germany). 2,6-dichloro-4-methylphenol, 4-aminobutyric acid, 3-(methylamino) butyric acid hydrochloride, *N*-hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide, and 4-dimethylaminopyridine were obtained from Aldrich (Milwaukee, USA). BSA, OVA, horseradish peroxidase (HRP) labeled goat anti-rabbit IgG, Freund's complete and incomplete adjuvants, Tween 20, and Sephadex G-25 were purchased from Sigma (St. Louis, USA). Tetramethylbenzidine (TMB) 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) were purchased from Nunc (Roskilde, Denmark). ELISA plates were washed with a Model 1575 ImmunoWash, and

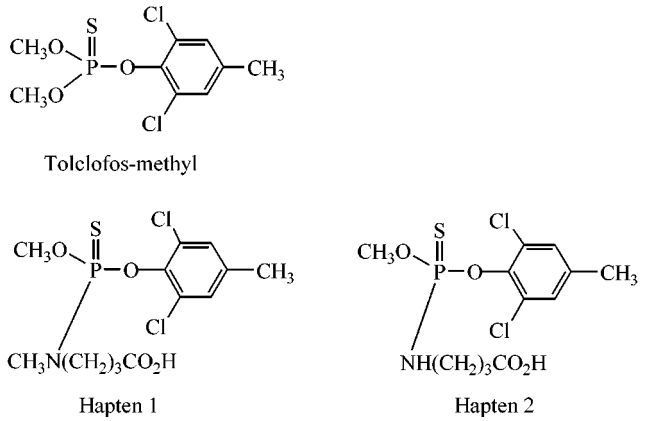


Figure 1. Structures of the haptens for tolclofos-methyl used for immunization (Hapten 1), antigen coating (Hapten 2) and enzyme tracer (Hapten 2).

well absorbances were read with a Model 550 plate reader, both from Bio-Rad (Hercules, 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, q, qn, sx, m, and ar represent singlet, doublet, triplet, quartet, quintet, sextet, 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. The procedure for the synthesis of Hapten 1 was as follows.

1. To a stirred mixture of 1.03 g (6.3 mmol) of methyl dichlorothiophosphate,[14] finely ground K_2CO_3 and 10 mL acetonitrile was added dropwise 0.93 g (5.2 mmol) of 2,6-dichloro-4-methylphenol dissolved in 3 mL of acetonitrile. After stirring for 1 h at room temperature, the mixture was filtered and the solvent was removed under reduced pressure. The residue was subjected to column chromatography (silica gel, 10 : 1 hexane-ethyl acetate) to give 1.23 g (67%) of the product as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.18 (2H, s, ar), 4.08 (3H, d, J = 16.5, CH_3OP), 2.33 (3H, d, J = 2.3, CH_3-ar).

Hapten 1. To a stirred solution of 0.23 g (0.76 mmol) of **1** in 1 mL of methanol cooled in an ice-water bath was added dropwise a solution of 0.16 g (2.9 mmol) of KOH and 0.14 g (0.91 mmol) of 3-(methylamino)butyric acid hydrochloride

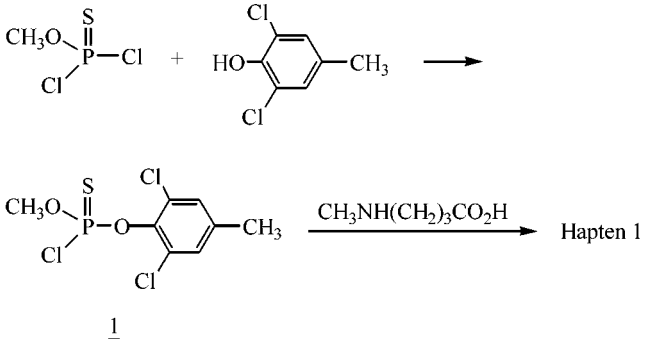


Figure 2. Synthetic route for Hapten 1.

in 0.16 mL 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 followed by preparative TLC using the same solvent gave 0.25 g (78%) of a white solid. ^{1}H NMR ($CDCl_3$): δ 7.13 (2H, s, ar), 3.85 (3H, d, J = 14.2, CH_3OP), 3.16 & 3.45 (2H, m, J = 5.1, NCH_2), 2.90 (3H, d, J = 11.4, CH_3N), 2.43 (2H, t x d, J = 7.4 & 2.6, CH_2CO_2), 2.28 (3H, d, J = 1.7, CH_3-ar), 1.92 (2H, qn, J = 7.3, $NHCH_2C\underline{H}_2$).

Hapten 2 was synthesized by the same procedure as that for Hapten 1 using **1** and 4-aminobutyric acid. Yield 73%. ^{1}H NMR ($CDCl_3$): δ 7.15 (2H, s, ar), 3.91 (3H, d, J = 14.3, CH_3OP), 3.47 (1H, qn, J = 7.2, NH), 3.16 (2H, d x q, J = 6.6 & 11, $NHC\underline{H}_2$), 2.75 (2H, t, J = 7.2, CH_2CO_2), 2.35 (3H, s, CH_3-ar), 1.99 (2H, d x qn, J = 6.7 & 1.8, $NHCH_2C\underline{H}_2$).

Preparation of hapten-protein conjugates. Hapten 1 was covalently attached to BSA to use as the immunogen. Hapten 2 was conjugated to OVA to use as a coating antigen and also conjugated to HRP to use as an enzyme tracer. The method of conjugation used was the active ester method.[6] The procedure for the synthesis of the active ester of Hapten 1 is described below. The procedure for the synthesis of the other active ester was similar. Hapten 1 (100 mg, 0.24 mmol) was dissolved in CH_2Cl_2 (0.2 mL) to which *N*-hydroxy-succinimide (30 mg, 0.26 mmol) dissolved in 5 mL of CH_2Cl_2 followed by *N*,*N*-dicyclohexylcarbodiimide (68 mg, 0.26 mmol) dissolved in 0.4 mL of CH_2Cl_2 and 4-dimethyl-aminopyridine (3 mg) were added. After stirring for 1 h, the mixture was filtered and the solvent was removed. Preparative TLC of the resultant oil on silica gel using 29 : 9 : 1 $CHCl_3$-ethyl acetate-acetic acid gave the active ester as a syrup (95 mg, 76%). ^{1}H NMR ($CDCl_3$): δ 7.13 (2H, s, ar), 3.87 (3H, d, J = 13.8, CH_3OP), 3.18 (2H, m, J = 4.97, NCH_2), 2.91 (3H, d, J = 11.2, CH_3N), 2.76 (4H, s, succinyl), 2.43 (2H, sx, J = 7.35, CH_2CO_2), 2.29 (3H, d, J = 1.67, CH_3-ar), 1.92 (2H, qn, J = 7.2, $NHCH_2C\underline{H}_2$).

The yield of active ester of Hapten 2 was 88%. ^{1}H NMR ($CDCl_3$): δ 7.15 (2H, s, ar), 3.92 (3H, d, J = 14.2, CH_3OP), 3.28 (2H, sx, J = 6.4, $NHC\underline{H}_2$), 2.85 (4H, s, succinyl), 2.49 (2H, t, J = 7.3, CH_2CO_2), 2.29 (3H, s, CH_3-ar), 2.03 (2H, qn, J = 7.0, $NHCH_2C\underline{H}_2$).

The procedure for the conjugation was as follows. To prepare BSA conjugate (immunogen), BSA (20 mg, 0.3 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 (16 mg, 0.03 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 conjugate (coating antigen) was prepared by the same procedure. Conjugates were purified by gel filtration (Sephadex G-25) using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4) followed by dialysis in water at 4 °C overnight. Purified conjugate solutions were then freeze-dried. HRP conjugates (enzyme tracers) were prepared similarly using the hapten-HRP molar ratios of 1/10 and 1/50. 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 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 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 aliquoted and stored at -70 °C.

Screening of antisera. Checkerboard assays, in which sera were titrated against varying amounts of the coating antigen were used to measure reactivity of antibodies and to select appropriate antigen coating and antibody dilutions for competitive indirect assays. The assay procedure was as follows. Microtiter plates were coated with Hapten 2-OVA (200-1000 ng/mL, 100 μL/well) in Tris buffer (50 mM, pH 7.8) by overnight incubation at 4 °C. The plates were washed five times with PBST (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 (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 or 1/3000) goat anti-rabbit IgG-HRP 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 competitive 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 by dissolving the analyte in a mixture of 10 mM PBS and methanol in various proportions (10, 20 and 40% methanol, which became 5, 10 and 20% after combining with equal volume of a diluted antiserum) by serial dilutions from a stock solution. The effect of phosphate ion concentration on ELISA performance was studied using different concentrations of PBST used to dissolve the antisera (10, 90, 190 and 390 mM phosphate which became 10, 50, 100 and 200 mM, respectively, after combining with equal volume of pesticide standard in 10 mM PBS). The procedure of the competitive 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,[15] from which IC_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) are determined.

Competitive direct assay. A checkerboard assay, in which sera were titrated against varying amounts of the enzyme tracer (Hapten 2 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 antibody-coated 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 (1/2000) in PBST for 1 h at room temperature. After another washing step, serial dilutions of the analyte in 20% MeOH-PBS were added (50 μL/well) followed by 50 μL/well of the enzyme tracer previously diluted with PBS. 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 used 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 IC_{20} values (concentration at which binding of the antibody to the coating antigen is inhibited by 20%). The cross-reactivity values were calculated as follows: (IC_{20} of tolclofos-methyl/IC_{20} of compound) × 100.

Results and Discussion

Hapten design and synthesis. The majority of OP pesticides have the thiophosphate group in common and differ only in the structure of aromatic rings. Therefore, to achieve a high selectivity in tolclofos-methyl ELISA, it was desirable to synthesize immunizing haptens having a bridge at the thiophosphate group preserving the aromatic ring unique to tolclofos-methyl. Hapten 1 was chosen as a immunizing hapten on this ground. Heldman *et al.*[7] were the first to synthesize a hapten for OP pesticide with a spacer arm at the thiophosphate group. However, a general method was developed later by Skerritt *et al.*.[16,17] This method was applied to the synthesis of haptens for the development of ELISAs of several OP pesticides.[4-12] 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 general method, which requires only two steps.[4-6] It involves the reaction of *O*-methyl (ethyl) dichlorothiophosphate with a phenol and

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

Methanol (%)[b]	Abs_{max}	Slope	IC_{50} (μg/mL)
5	1.035	0.852	0.345
10	1.111	0.978	0.204
20	1.270	0.911	0.267

[a]ELISA conditions: antiserum to Hapten 1-BSA, diluted 1/2000 with 10 mM PBST; coating antigen, Hapten 2-OVA, 15 ng/well; goat anti-rabbit IgG-HRP diluted 1/3000. Data were obtained from the four-parameter sigmoidal fitting. Each set of data represents the average of three replicates. [b]Final concentration in the competition solution.

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

Concentration (mM)[b]	Incubation time (min)[c]	Abs_{max}	Slope	IC_{50} (μg/mL)
10	12	1.593	0.804	1.241
50	18	1.302	0.838	0.385
100	25	1.018	0.767	0.236
200	30	0.646	0.602	0.284

[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 solution. [c]Time for color development.

K_2CO_3 in acetonitrile and the reaction of the substitution product with an aminocarboxylic acid (not protected) and KOH in MeOH (see Figure 2 for an example). This method was successfully applied to the synthesis of the two haptens for tolclofos-methyl. It worked well for attaching a secondary (Hapten 1) as well as a primary aminocarboxylic acid (Hapten 2). The reactions proceeded facilely with relatively high yield; 67% in the first, and over 70% in the 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 to the synthesis of haptens of several other OP pesticides such as chlorpyrifos,[4] isofenphos[5] and cyanophos,[6] 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.

Indirect ELISA. Of the four antisera obtained, the one that exhibited the most inhibition by the analyte was selected as the assay reagent. In addition to optimizing antigen coating (15 ng/well) and antiserum dilution (1/2000), the concentrations of methanol and buffer in the competition step media were optimized. Since organic solvents are commonly used in the sample preparation for nonpolar pesticides, it is desirable to assess the effects of organic cosolvents on ELISA characteristics. Since several workers reached the same conclusion that methanol causes the least negative effect of the solvents tested,[18-20] methanol was chosen as a cosolvent. Table 1 shows the effect of methanol concentration on ELISA performance. Increasing the concentration of methanol of the standard solutions affected the sensitivity of the assay to a small extent. The optimum

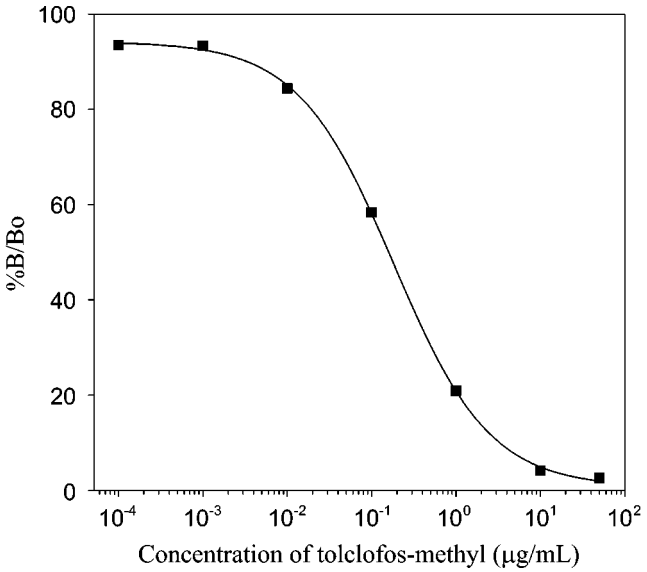


Figure 3. Standard curve for tolclofos-methyl by competitive indirect ELISA using the antiserum against Hapten 1-BSA diluted 1/2000, the coating antigen Hapten 2-OVA (15 ng/well) and gelatin blocking agent (1%). The composition of the assay solution was 10% methanol-PBS (100 mM).

concentration of methanol in the standard solution was determined to be 20% (10% after combining with equal volume of antiserum), where IC_{50} was lowest. Table 2 presents the effect of the phosphate ion concentration at the competition step on ELISA characteristics. Increasing the concentration of the phosphate caused a large increase of sensitivity up to 100 mM phosphate followed by a decrease of sensitivity. Since increased buffer concentration causes a remarkable retardation of the color development, selecting optimum buffer concentration would depend on the sensitivity as well as speed of color development in assay. The optimum concentration chosen was 100 mM, where IC_{50}

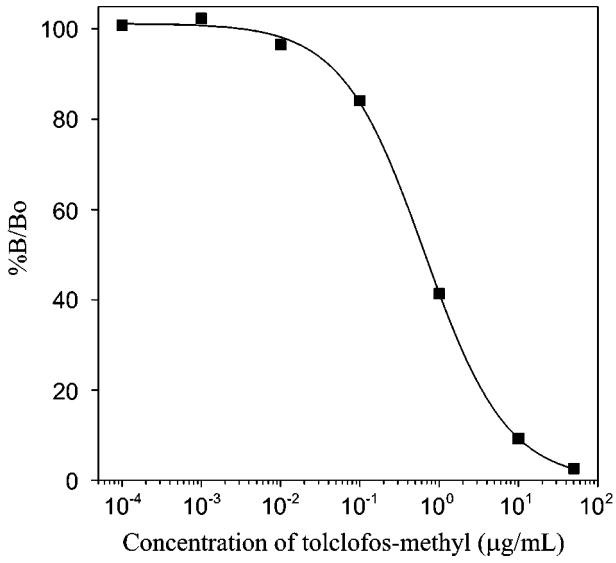
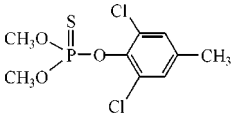
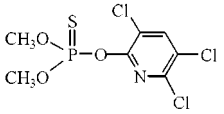
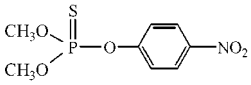
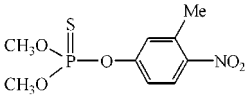
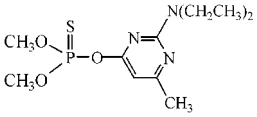
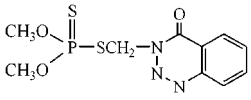
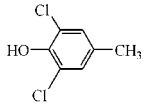
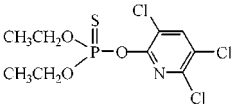
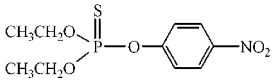


Figure 4. Standard curve for tolclofos-methyl by competitive direct ELISA using the antiserum against Hapten 1-BSA diluted 1/1000, the enzyme tracer Hapten 2-HRP and gelatin blocking agent (1%).

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

Pesticide	Structure	IC_{20} (μg/mL)[b]	CR (%)[c]
Tolclofos-methyl		0.020	100
Chlorpyrifos-methyl		2.2	0.9
Parathion-methyl		62	0.03
Fenitrothion		> 100	< 0.02
Pirimiphos-methyl		> 100	< 0.02
Azinphos-methyl		> 100	< 0.02
2,6-Dichloro-4-methylphenol		> 100	< 0.02
Chlorpyrifos-ethyl		> 100	< 0.02
Parathion-ethyl		> 100	< 0.02

[a]Determined by indirect ELISA under the condition described in Table 1. [b]IC_{20} values of the pesticides below parathion-methyl 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 (%) = (IC_{20} of tolclofos-methyl/I_{20} of other compound) × 100.

value was lowest and the speed of color development was not critically slow. Figure 3 shows a typical inhibition curve obtained after optimization. The lowest IC_{50} value observed was 160 ng/mL with a detection limit of about 20 ng/mL (20% inhibition).

Direct ELISA. The enzyme tracer for direct ELISA was prepared by conjugation reaction of Hapten 2 with HRP at 1 : 10 or 1 : 50 molar ratio. Hapten 2-HRP prepared at 1 : 10 molar ratio gave better results than the other one. The antibody-coated ELISA using this enzyme tracer was optimized with regard to the dilution of antiserum and enzyme tracer. Figure 4 shows the inhibition curve obtained after optimization. The assay showed an IC_{50} value of about 410 ng/mL with a detection limit of about 130 ng/mL. The sensitivity of this ELISA is considerably lower than that of the ELISA previously developed (IC_{50} value of 2 ng/mL). Since the structures of the enzyme tracers and assay procedures were similar, the sensitivity difference must have resulted from the difference in antibody characteristics. Since this assay showed a sensitivity inferior to that of the indirect ELISA, no further optimization was attempted.

Cross-reactivity of the assays. Several OP pesticides as well as tolclofos-methyl metabolites were tested for cross-reactivities. Table 3 shows the cross-reactivity that was found by the indirect assay, expressed in percentage of the IC_{20} of tolclofos-methyl. In all cases, the interference to the assay was negligible. It may be concluded that the indirect ELISA that was developed is suitable for the selective detection of tolclofos-methyl.

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References

1. *The Pesticide Manual: A World Compendium*, 11th ed.; Tomlin, C. D. S. Ed.; The British Crop Protection Council: Farnham, Surrey, U.K., 1997; p 238.
2. *Official Methods of Analysis*, 18th Ed.; AOAC International: Arlington, VA, 1995; Section 970.52.
3. Hammock, B. D.; Gee, S. J.; Harrison, R. O.; Jung, F.; Goodrow, M. H.; Li, Q. X.; Lucas, A.; Szekacs, A.; Sundaram, K. M. S. *Immunochemical Methods for Environmental Analysis*; Van Emon, J., Mumma, R., Eds.; ACS Symposium Series 442; American Chemical Society: Washington, DC, USA, 1990; pp 112-139.
4. Cho, Y. A.; Lee, H.-S.; Park, E. Y.; Lee, Y. T.; Hammock, B. D.; Ahn, K. C.; Lee, J. K. *Bull. Korean Chem. Soc*. **2002**, *23*, 481-487.
5. Park, H. J.; Park, W. C.; Jung, T. W.; Rha, C. S.; Lee, Y. T. *Bull. Korean Chem. Soc*. **2002**, *23*, 599-604.
6. Park, J. H.; Park, W. C.; Kim, Y. J.; Lee, Y. T. *Bull. Korean Chem. Soc*. **2002**, *23*, 605-609.
7. Heldman, E.; Balan, A.; Horowitz, O.; Ben-Zion, S.; Torten, M. *FEBS Lett.* **1985**, *180*, 243-248.
8. McAdam, D. P.; Hill, A. S.; Beasley, H. L.; Skerritt, J. H. *J. Agric. Food Chem.* **1992**, *40*, 1466-1470.
9. Manclús, J. J.; Primo, J.; Montoya, A. *J. Agric. Food Chem.* **1996**, *44*, 4052-4062.
10. Hill, A. S.; Skerritt, J. H.; Bushway, R. J.; Perkins, L. B.; Larkin, K.; Thomas, M.; Korth, W.; Bowmer, K. H. *J. Agric. Food Chem.* **1994**, *42*, 2051-2058.
11. Edward, S. L.; Skerritt, J. H.; Hill, A. S.; McAdam, D. P. *Food Agric. Immunol*. **1993**, *5*, 129-144.
12. Skerritt, J. H.; Hill, A. S.; Thwaites, H. L.; Edward, S. L.; McAdam, D. P. *J. AOAC Int*. **1992**, *75*, 519-528.
13. Kawada, M.; Morimune, K.; Kanai, S.; Kagawa, Y.; Watanabe, K. *Japan Patent.* 11-255786, 1999.
14. Tabor, E. J. *Unite States Patent 3,005,005*, 1961.
15. Raab, G. M. *Clin. Chem*. **1983**, *29*, 1757-1761.
16. McAdam, D. P.; Skerritt, J. H. *Aust. J. Chem*. **1993**, *46*, 959-967.
17. Skerritt, J. H.; Lee, N. J. *Residue Analysis in Food Safety: Applications of Immunoassay Methods*.; Beier, R. C., Stanker, L. H., Eds.; ACS Symposium Series 621; American Chemical Society: Washington DC, USA, 1996; pp 124-149.
18. Marco, M.-P.; Gee, S. J.; Cheng, H. M.; Liang, Z. Y.; Hammock, B. D. *J. Agric. Food Chem.* **1993**, *41*, 423-430.
19. Wengatz, I.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. *J. Agric. Food Chem.* **1998**, *46*, 2211-2221.
20. Shan, G.; Leeman, W. R.; Stoutamire, D. W.; Gee, S. J.; Chang, D. P. Y.; Hammock, B. D. *J. Agric. Food Chem*. **2000**, *48*, 4032-4040.