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Development of ELISAs for the Class-Specific Determination of Organophosphorus Pesticides

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Enzyme-linked immunosorbent assays (ELISAs) for the class-specific determination of organophosphorus (OP) pesticides were developed from monoclonal antibodies raised against haptens with the functional group common to OP pesticides. To develop antigen-coated, indirect, competitive ELISAs, four haptens with different spacer arm structures were used to prepare antibodies, while eight haptens were tested for use as coating antigens. A total of 32 ELISAs were developed with one selected as the most suitable one based on average IC_{50} and % CV values. The chosen ELISA showed class-selective response to *O,O*-diethyl phosphorothioate and phosphorodithioate OP pesticides with negligible cross-reactivity to other types of pesticides. Average IC_{50} and % CV values of this ELISA for the 12 OP pesticides were 89 ng/mL and 96%, respectively. Compared to ELISAs previously developed with the same objective, the current ELISA demonstrates better sensitivity based on much lower mean IC_{50} values in addition to improved class-selective determination based on considerably lower % CV values as well as precise discrimination against other types of pesticides.

KEYWORDS: Organophosphorus pesticide; class-specific determination; immunoassay; enzyme-linked immunosorbent assay

INTRODUCTION

OP pesticides persist only briefly in the environment yet are acutely toxic after administration inhibiting acetylcholinesterase activity (*1*). Other toxicological effects proposed for OP pesticides include disrupting the endocrine system (*2*). Their extensive use has made them widely present in water and food (*3*), posing a potential serious hazard to human health.

Increasing public concern over pesticide contamination of food and the environment has increased demands for broader and stricter pesticide monitoring. Agencies responsible for regulation are criticized for not monitoring enough samples, because of the expensive, sophisticated, and time-consuming sample-preparation procedures involved in current chromatographic methods. Furthermore, detection of every pesticide from a single sample is difficult using a single multiresidue procedure, thereby requiring multiple workups. These problems demand additional search into low-cost and rapid residue detection methods. Recently, some alternative methods such as immunoassays (*4*, *5*) and biosensors (*6*, *7*) were thought to circumvent these obstacles, yet only a few of them have gained official approval.

Class-specific determination of pesticides prior to chromatographic determination could be an attractive approach for broader pesticide monitoring. If a class of pesticides in a sample has a total quantity less than the maximum residue limits of the pesticides, the sample does not require further inspection for those pesticides. Since a vast majority of food and environmental samples usually fall under pesticide maximum residue limits, the time and cost saved by this approach could be substantial, especially in major class pesticides such as OP and carbamate pesticides.

The class-specific (class-selective) determination of pesticides has been developed in numerous studies such as the use of biosensors and enzymatic analysis based on cholinesterases for the class-specific determination of OP and carbamate pesticides (*8*−*11*). The principle of this methodology is that OP and carbamate pesticides inhibit cholinesterases, the degree of which depends on the pesticide concentration. Many similar studies have appeared, yet none was quantitative because of the variable degree of enzyme inhibition among different pesticides (*12*).

Some investigators tried to develop class-selective immunoassays (enzyme-linked immunosorbent assays, ELISAs) for OP pesticides using polyclonal antibodies raised against the functional group [$(RO)_2P(=S)$- or $(RO)_2P(=O)$-] common to OP pesticides, but none was quantitative because of the vastly different responses antibodies have to different pesticides (*13*−*17*).

In order to resolve this problem in our previous study, we used an immunizing hapten containing a benzene ring, which provides a rigid bridge structure promoting high response of the antibody to pesticides, with a substituent at the ortho position of the benzene ring to suppress possible effects of the ring on the immune system (*17*). Two monoclonal antibodies raised against this hapten exhibited quite uniform and strong responses to many

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diethyl OP pesticides versus an extraordinarily low response to diazinon. Therefore, the strategy adopted in this current study was to use immunizing haptens similar to the previous structures but different in that a substituent is placed at the meta position of the benzene ring. This would enhance the response of the antibody to pesticides with a bulky substituent at the meta position, such as diazinon. The strategy was effective, allowing a more quantitative class-selective determination of OP pesticides.

MATERIALS AND METHODS

Chemicals and Instruments. OP and carbamate pesticides were purchased from Dr. Ehrenstorfer (Augsburg, Germany). 4-Methoxy-2-methylbenzoic acid, methyl 3-hydroxybenzoate, methyl 4-hydroxybenzoate, 2,4-dihydroxybenzoic acid, *N*-hydroxysuccinimide, *N*,*N*-dicyclohexylcarbodiimide, and 4-dimethylaminopyridine were obtained from Aldrich (Milwaukee, WI). 4-Fluoro-3-hydroxybenzoic acid was purchased from Apollo (Derbyshire, U.K.). β-Alanine methyl ester was obtained from TCI (Tokyo, Japan). Ovalbumin (OVA), gelatin, horseradish peroxidase (HRP), HRP-labeled goat antimouse IgG, and polyoxyethylene (20) sorbitan monolaurate (Tween 20) were purchased from Sigma (St. Louis, MO). The 3,3′,5,5′-tetramethylbenzidine (TMB) used was a product of Boehringer Mannheim (Mannheim, Germany). Keyhole limpet hemocyanin (KLH) was obtained from CalBiochem (La Jolla, CA). Analytical (silica gel F254) and preparative TLC plates (silica gel, 1 mm) were purchased from Merck (Darmstadt, Germany). Microtiter plates (Maxisorp, 442404 and 439454) were purchased from Nunc (Roskilde, Denmark).

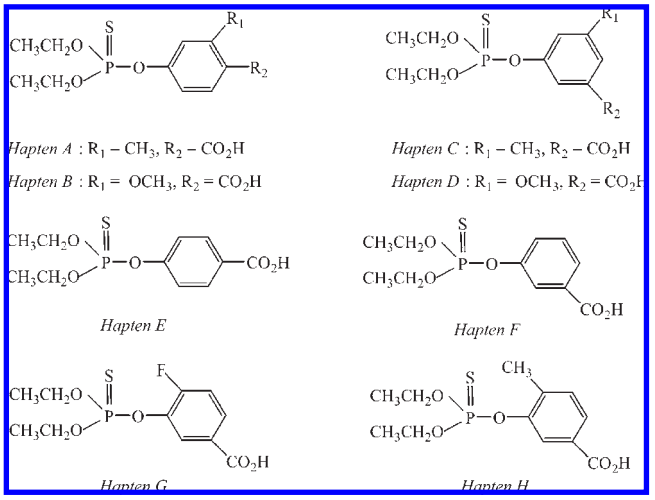

Figure 1. Structures of the haptens used to prepare the immunogens and the competitors (coating antigen).

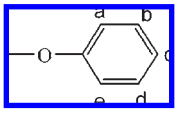

Figure 2. The designation of the benzene ring protons of the intermediates and products in the synthesis of haptens.

ELISA plates were washed with a Model 1575 ImmunoWash from Bio-Rad (Hercules, CA), and well absorbances were read with a Vmax microplate reader from Molecular Devices (Menlo Park, CA). NMR spectra were obtained with a Bruker (Rheinstetten, Germany) ARX spectrometer (300 MHz).

Synthesis of Haptens. The haptens used to prepare the immunogens for obtaining antibodies and the competitors in the immunoassay (coating antigen) are presented in **Figure 1**. Of these haptens, *Hapten H* was synthesized as described in our previous paper (*17*). *Hapten G* was used in our previous study (*17*) but was synthesized in this study following a different method. The remaining haptens were synthesized by the following procedures. Chemical shift values in the NMR data presentation are given relative to internal tetramethylsilane. Coupling constants are expressed in Hz, and the abbreviations s, d, t, q, qn, m, and ar represent singlet, doublet, triplet, quartet, quintet, multiplet, and aromatic, respectively. H_a−H_e in the NMR data interpretation indicate the benzene ring protons as indicated in **Figure 2**.

Hapten A. The synthetic route for *Hapten A* is illustrated in **Figure 3**. The procedure for the synthesis of *Hapten A* was as follows.

4-Hydroxy-2-methylbenzoic Acid (***1***). To a magnetically stirred mixture of 4-methoxy-2-methylbenzoic acid (1.79 g, 10.8 mmol) and red phosphorus (4.9 g) was added dropwise 26 mL of acetic anhydride/hydriodic acid (1:1), and the mixture was refluxed for 3 h. The reaction mixture was filtered through Celite followed by extraction with ethyl acetate (50 mL × 3). The separated organic layer was washed with brine and then dried over anhydrous magnesium sulfate. After evaporation of the solvent, the residue was subjected to column chromatography (silica gel, hexane/ethyl acetate/acetic acid, 200:100:8; TLC R_f under the same condition was 0.42) to provide 1.4 g (85%) of the product as a powder. ^{1}H NMR ($CDCl_3$): δ 8.01 (1H, d, J = 9.3, H_d), 6.74−6.68 (2H, H_a and H_e), 2.60 (3H, s, CH_3-ar).

Methyl 4-Hydroxy-2-methylbenzoate (***2***). A solution of 1.35 g (8.9 mmol) of **1** in 3.5 mL of H_2SO_4/MeOH (1:10, v/v) was refluxed for 24 h. After evaporation of the solvent, the residue was subjected to column chromatography (silica gel, hexane/ethyl acetate, 2:1; TLC R_f 0.54) to provide 1.16 g (79%) of the product. ^{1}H NMR ($CDCl_3$): δ 7.89 (1H, d, J = 9.1, H_d), 6.69 (1H, s, H_a), 6.67 (1H, d × d, J = 2.6, H_e), 3.85 (3H, s, OCH_3), 2.58 (3H, s, CH_3-ar).

O,O-Diethyl O-[3-Methyl-4-(methyloxycarbonyl)phenyl]-phosphorothioate (***3***). To a solution of 0.26 g (1.57 mmol) of **2** in butanone (3 mL) were added diethyl chlorothiophosphate (0.50 g,

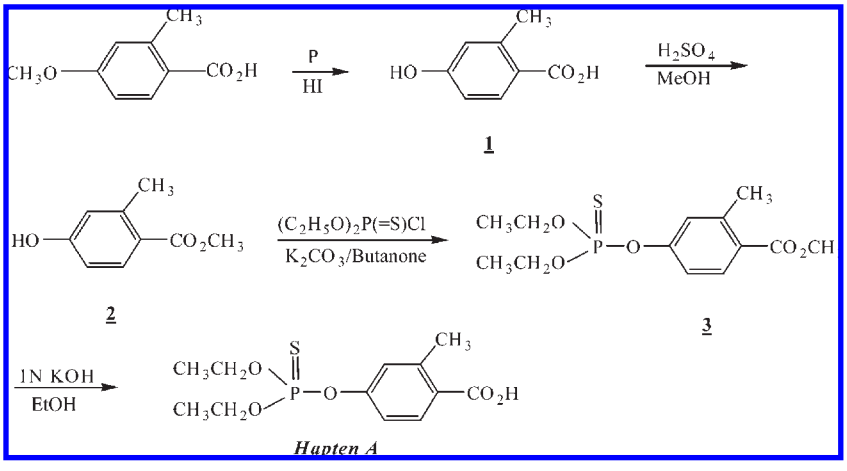

Figure 3. Synthetic route for *Hapten A*.

2.65 mmol) and finely ground K_2CO_3 (5 g). After stirring for 24 h at 80 °C, the reaction mixture was filtered through Celite, then concentrated. The residue was next subjected to column chromatography (silica gel, hexane/ethyl acetate, 5:1; TLC R_f 0.48) to provide 0.362 g (72%) of the product. ^{1}H NMR ($CDCl_3$): δ 7.93 (1H, d, J = 9.5, H_d), 7.08 (1H, d, H_e), 7.06 (1H, s, H_a), 4.25 (4H, d × q, J = 10.0 and 7.0, $CH_3C\underline{H}_2O$), 3.88 (3H, s, CO_2CH_3), 2.60 (3H, s, CH_3-ar), 1.38 (6H, t × d, J = 7.1 and 0.85, $C\underline{H}_3CH_2O$).

O,O-Diethyl O-(4-Carboxy-3-methylphenyl)phosphorothioate (*Hapten A*). To a magnetically stirred solution of 0.318 g (1.0 mmol) of **3** in 60 mL of ethanol was added 25 mL of 1 M KOH and stirring was continued at room temperature. After stirring for 30 min, 30 mL of 1 N HCl was added, and the reaction mixture was extracted with ethyl acetate (50 mL). The separated organic layer was washed with 1 N HCl (10 mL × 2) and then dried over anhydrous magnesium sulfate. After evaporation of the solvent, the residue was subjected to column chromatography (silica gel, hexane/ethyl acetate/acetic acid, 200:100:8; TLC R_f 0.57) to provide 70 mg (23%) of the product. ^{1}H NMR ($CDCl_3$): δ 8.07 (1H, d, J = 8.3, H_d), 7.11 (1H, d, H_e), 7.09 (1H, s, H_a), 4.26 (4H, d × q, J = 10.0 and 7.1, $CH_3C\underline{H}_2O$), 2.65 (3H, s, CH_3-ar), 1.38 (6H, t × d, J = 7.1 and 0.8, $C\underline{H}_3CH_2O$).

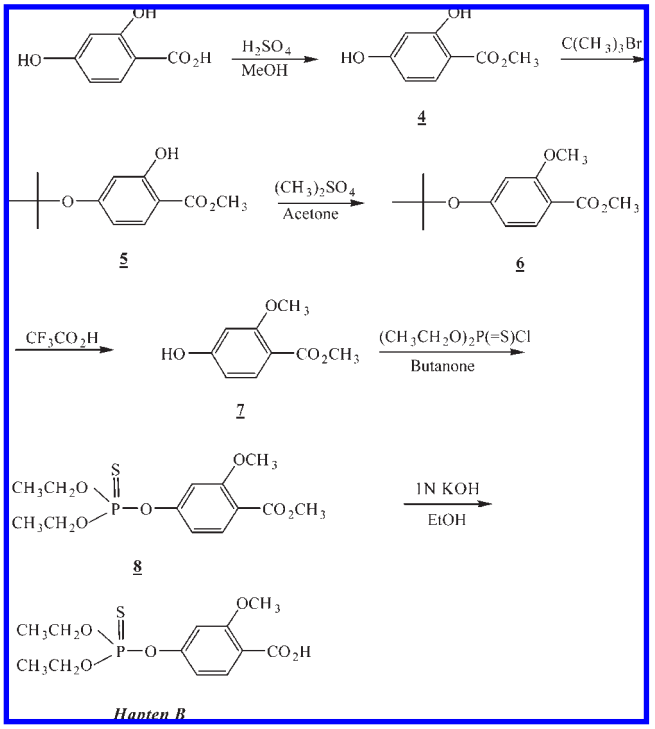

Figure 4. Synthetic route for *Hapten B*.

Hapten B. The synthetic route for *Hapten B* is illustrated in **Figure 4**.

Methyl 2,4-Dihydoxybenzoate (***4***). This compound was prepared from 2,4-dihydroxybenzoic acid (4.04 g, 26.2 mmol) by the same procedure as that used for compound **2**. Yield: 61%. TLC R_f (silica gel, hexane/ethyl acetate, 2:1): 0.52. ^{1}H NMR ($CDCl_3$): δ 7.72 (1H, d, J = 8.6, H_d), 6.42 (1H, d, J = 2.1, H_a), 6.39 (1H, d × d, J = 8.6 and 2.4, H_e), 3.91(3H, s, CO_2CH_3).

Methyl 4-(t-Butyloxy)-2-hydroxybenzoate (***5***). This compound was synthesized according to a published procedure (*18*) using 1.34 g (7.98 mmol) of **4**. Yield: 85%. TLC R_f (silica gel, hexane/ethyl acetate 8:1): 0.64. ^{1}H NMR ($CDCl_3$): δ 7.69 (1H, d, J = 8.9, H_d), 6.69(1H, s, H_a), 6.46 (1H, d, J = 8.7, H_e), 3.89 (3H, s, CO_2CH_3). 1.41 (9H, s, *t*-butyl).

Methyl 4-(t-Butyloxy)-2-methoxybenzoate (***6***). To a solution of 76 mg (0.34 mmol) of **5** in acetone (3 mL) were added dimethyl sulfate (172 mg, 1.36 mmol) and finely ground K_2CO_3 (1 g). After refluxing for 12 h, the reaction mixture was filtered through Celite. After evaporation of the solvent, the residue was subjected to column chromatography (silica gel, hexane/ethyl acetate, 4:1; TLC R_f 0.52) to provide 78 mg (97%) of the product. ^{1}H NMR ($CDCl_3$): δ 7.75 (1H, d, J = 8.6, H_d), 6.59 (1H, d × d, J = 8.6 and 2.2, H_e), 6.53 (1H, d, J = 2.1, H_a), 3.85 (3H, s, CH_3O-ar or CO_2CH_3), 3.84 (3H, s, CH_3O-ar or CO_2CH_3), 1.40 (9H, s, *t*-butyl).

Methyl 4-Hydoxy-2-methoxybenzoate (***7***). A solution of 68 mg (0.288 mmol) of **6** in 0.2 mL of trifluoroacetic acid was stirred for 5 min. After evaporation of the solvent, the residue was subjected to column chromatography (silica gel, hexane/ethyl acetate, 1:1; TLC R_f 0.42) to provide 51 mg (97%) of the product. ^{1}H NMR ($CDCl_3$): δ 7.73 (1H, d × d, J = 7.8 and 1.5, H_d), 6.45 (1H, s, H_a), 6.43 (1H, d × d, J = 2.5, H_e), 3.91 (3H, s, CH_3O-ar or CO_2CH_3). 3.82 (3H, s, CH_3O-ar or CO_2CH_3).

O,O-Diethyl O-[3-Methoxy-4-(methyloxycarbonyl)phenyl]-phosphorothioate (***8***). This compound was prepared from **7** (51 mg, 0.28 mmol) according to the same procedure as that used for compound **3**. Yield: 56%. TLC R_f (silica gel, hexane/ethyl acetate, 4:1): 0.53. ^{1}H NMR ($CDCl_3$): δ 7.80 (1H, d, J = 8.4, H_d), 6.82 (1H, t, J = 1.6, H_a), 6.80 (1H, d × t, J = 8.5 and 1.6, H_e), 4.22 (4H, d × q, J = 9.9 and 7.1, $CH_3C\underline{H}_2O$), 3.87 (3H, s, CH_3O-ar or CO_2CH_3), 3.85 (3H, s, CH_3O-ar or CO_2CH_3), 1.34 (6H, t × d, J = 7.1 and 0.7, $C\underline{H}_3CH_2O$).

O,O-Diethyl O-(4-Carboxy-3-methoxyphenyl)phosphorothioate (*Hapten B*). *Hapten B* was obtained by hydrolysis of **8** (51 mg, 0.153 mmol) according to the same procedure as that for the hydrolysis of compound **3**. Yield: 71%. TLC R_f (silica gel, hexane/ethyl acetate/acetic acid, 50:25:1): 0.46. ^{1}H NMR ($CDCl_3$): δ 8.15 (1H, d, J = 9.2, H_d), 6.97−6.91 (2H, m, H_a and H_e), 4.24 (4H, d × q, J = 10.1 and 7.1, $CH_3C\underline{H}_2O$), 4.05 (3H, s, CH_3O-ar), 1.35 (6H, t × d, J = 7.1 and 0.7, $C\underline{H}_3CH_2O$).

Haptens C and D. The synthetic route for *Hapten C* and *D* are illustrated in **Figure 5**.

Methyl 3-Hydroxy-5-methylbenzoate (***9***). This compound was synthesized by a published procedure (*19*). ^{1}H NMR ($CDCl_3$): δ 7.44 (1H, d, J = 0.51, H_c), 7.33 (1H, s, H_e), 6.88 (1H, s, H_a), 3.90 (3H, s, CO_2CH_3), 2.35 (3H, s, CH_3-ar).

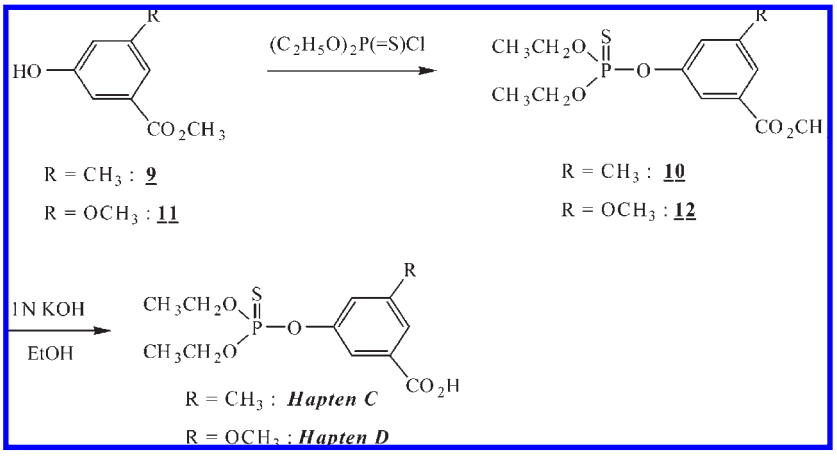

Figure 5. Synthetic route for *Haptens C and D*.

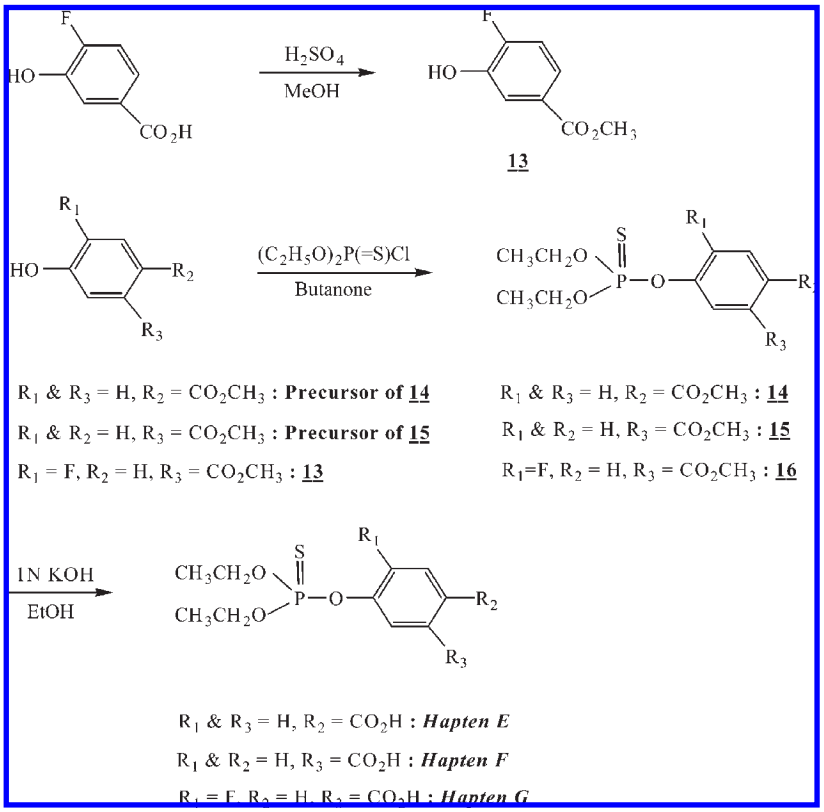

Figure 6. Synthetic route for *Haptens E*, *F*, and *G*.

O,O-Diethyl O-[3-Methyl-5-(methyloxycarbonyl)phenyl]-phosphorothioate (***10***). This compound was prepared from **9** (0.299 g, 1.80 mmol) by the same procedure as that used for compound **3**. Yield: 93%. TLC R_f (silica gel, hexane/ethyl acetate, 5:1): 0.49. ^{1}H NMR ($CDCl_3$): δ 7.70 (1H, s, H_c), 7.63 (1H, d, J = 1.2, H_e), 7.20 (1H, d, J = 0.9, H_a), 4.25 (4H, d × q, J = 9.9 and 7.1, $CH_3\underline{CH_2}$), 3.91 (3H, s, $CO_2\underline{CH_3}$), 2.40 (3H, s, CH_3-ar), 1.38 (6H, t × d, J = 7.1 and 0.32, $\underline{CH_3}CH_2$).

O,O-Diethyl O-(3-Carboxy-5-methylphenyl)phosphorothioate (*Hapten C*). *Hapten C* was obtained by hydrolysis of **10** (0.53 g, 1.67 mmol) according to the same procedure as that used for the hydrolysis of compound **3**. Yield: 60%. TLC R_f (silica gel, hexane/ethyl acetate/acetic acid, 200:100:8): 0.50. ^{1}H NMR ($CDCl_3$): δ 7.74 (1H, s, H_c), 7.68 (1H, s, H_e), 7.23 (1H, s, H_a), 4.25 (4H, d × q, J = 9.9 and 7.1, $CH_3\underline{CH_2}$), 2.40 (3H, s, CH_3), 1.38 (6H, t, J = 7.1, $\underline{CH_3}CH_2$).

Methyl 3-Hydroxy-5-methoxybenzoate (***11***). This compound was synthesized by a published procedure (*20*). ^{1}H NMR ($CDCl_3$): δ 7.16 (1H, d × d, J = 2.3 and 1.3, H_c), 7.12 (1H, d × d, J = 2.3 and 1.3, H_e), 6.61 (1H, d × d, J = 2.4 and 2.4, H_a), 5.26 (1H, s, OH), 3.90 (3H, s, $CO_2\underline{CH_3}$), 3.83 (3H, s, CH_3O-ar).

O,O-Diethyl O-[3-Methoxy-(5-methyloxycarbonyl)phenyl]-phosphorothioate (***12***). This compound was prepared from **11** (0.23 g, 1.26 mmol) by the same procedure as that used for compound **3**. Yield: 84%. TLC R_f (silica gel, hexane/ethyl acetate, 5:1): 0.43. ^{1}H NMR ($CDCl_3$): δ 7.44 (1H, d × d, J = 3.6 and 1.5, H_c), 7.41 (1H, d × d × d, J = 2.3, 1.2 and 1.2, H_e), 6.96 (1H, d × d, J = 4.1 and 2.5, H_a), 4.25 (4H, d × q, J = 10.0 and 7.1, $CH_3\underline{CH_2}$,), 3.91 (3H, s, $CO_2\underline{CH_3}$), 3.85 (3H, s, CH_3O-ar), 1.38 (6H, t × d, J = 7.1 and 0.8, $\underline{CH_3}CH_2$).

O,O-Diethyl O-(3-Carboxy-5-methoxylphenyl)phosphorothioate (*Hapten D*). *Hapten D* was obtained by the hydrolysis of **12** (0.355 g, 1.06 mmol) according to the same procedure as that used for the hydrolysis of compound **3**. Yield: 55%. TLC R_f (silica gel, hexane/ethyl acetate/acetic acid, 200:100:8): 0.42. ^{1}H NMR ($CDCl_3$): δ 7.51 (1H, s, H_c), 7.47 (1H, s, H_e), 7.01 (1H, d × d, J = 3.7 and 2.1, H_a), 4.26 (4H, d × q, J = 10.0 and 7.1, $CH_3\underline{CH_2}$), 3.86 (3H, s, CH_3O-ar), 1.38 (6H, t × d, J = 7.1 × 0.8, $\underline{CH_3}CH_2$).

***Haptens E, F,* and *G*.** The synthetic routes for *Haptens E*, *F*, and *G* are illustrated in **Figure 6**.

Methyl 4-Fluoro-3-hydroxybenzoate (***13***). This compound was prepared from 4-fluoro-3-hydroxybenzoic acid (0.865 g, 5.5 mmol) by the same procedure as that used for compound **2**. Yield: 63%. TLC R_f (silica gel, hexane/ethyl acetate, 2:1): 0.45. ^{1}H NMR ($CDCl_3$): δ 7.69 (1H, d × d, J = 8.5 and 2.1, H_e), 7.56 (1H, d × d × d, J = 8.5, 4.7 and 2.1, H_c), 7.09 (1H, d × d, J = 10.1 and 8.5, H_b), 3.88 (3H, s, $CO_2\underline{CH_3}$).

O,O-Diethyl O-[(4-Methyloxycarbonyl)phenyl]phosphorothioate (***14***). This compound was prepared from methyl *p*-hydroxybenzoate (0.147 g, 0.97 mmol) by the same procedure as that for compound **3**. Yield: 33%. TLC R_f (silica gel, hexane/ethyl acetate, 4:1): 0.67. ^{1}H NMR ($CDCl_3$): δ 8.04 (2H, d, J = 8.7, H_b and H_d), 7.25 (2H, d × d, J = 1.5, H_a and H_e), 4.25 (4H, d × q, J = 10.0 and 7.1, $CH_3\underline{CH_2}$), 3.91 (3H, s, $CO_2\underline{CH_3}$), 1.37 (6H, t, J = 7.1, $\underline{CH_3}CH_2$).

O,O-Diethyl O-[(3-Methyloxycarbonyl)phenyl]phosphorothioate (***15***). This compound was prepared from methyl *m*-hydroxybenzoate (0.4 g, 2.63 mmol) by the same procedure as that used for compound **3**.Yield: 95%. TLC R_f (silica gel, hexane/ethyl acetate, 8:1): 0.55. ^{1}H NMR ($CDCl_3$): δ 7.89−7.83 (1H, m, H_c), 7.82−7.79 (1H, m, H_e), 7.41−7.37 (2H, m, H_a and H_b), 4.23 (4H, d × q, J = 10.0 and 7.1, $CH_3\underline{CH_2}$), 3.90 (3H, s, $CO_2\underline{CH_3}$), 1.35 (6H, t × d, J = 7.0 and 0.6, $\underline{CH_3}CH_2$).

O,O-Diethyl O-[2-Fluoro-(5-methyloxycarbonyl)phenyl]-phosphorothioate (***16***). This compound was prepared from compound **13** (0.27 g, 1.59 mmol) by the same procedure as that used for compound **3**. Yield: 69%. TLC R_f (silica gel, hexane/ethyl acetate, 4:1): 0.49. ^{1}H NMR ($CDCl_3$): δ 7.94 (1H, d × d × d, J = 7.6, 1.9 and 1.9, H_e), 7.86 (1H, d × d × d, J = 8.5, 4.4 and 1.8, H_c), 7.18 (1H, d × d, J = 9.1 and 9.1, H_b), 4.27 (4H, d × q, J = 9.9 and 7.1, $CH_3\underline{CH_2}$), 3.89 (3H, s, $CO_2\underline{CH_3}$), 1.35 (6H, t, J = 7.0, $\underline{CH_3}CH_2$).

Haptens E, F, and G. These haptens were obtained by hydrolysis of **14** (0.097 g, 0.319 mmol), **15** (0.304 g, 1 mmol), and **16** (0.283 g, 0.88 mmol), respectively, according to the same procedure as that used for the hydrolysis of compound **3**.

O,O-Diethyl O-(4-Carboxyphenyl)phosphorothioate (*Hapten E*). Yield: 82%. TLC R_f (silica gel, hexane/ethyl acetate/acetic acid, 50:25:2): 0.56. ^{1}H NMR ($CDCl_3$): δ 8.12 (2H, d, J = 8.5, H_b and H_d), 7.29 (2H, d × d, J = 1.4, H_a and H_e), 4.28 (4H, d × q, J = 10.0 and 7.1, $CH_3\underline{CH_2}$), 1.39 (6H, t × d, J = 7.1& 0.6, $\underline{CH_3}CH_2$).

O,O-Diethyl O-(3-Carboxyphenyl)phosphorothioate (*Hapten F*). Yield: 79%. TLC R_f (silica gel, hexane/ethyl acetate/acetic acid, 50:25:2): 0.52. ^{1}H NMR ($CDCl_3$): δ 7.97−7.92 (1H, m, H_c), 7.90−7.87 (1H, m, H_e), 7.47−7.42 (2H, m, H_a and H_b), 4.24 (4H, d × q, J = 10.0 and 7.1, $CH_3C\underline{H}_2$), 1.36 (6H, t × d, J = 7.1 and 0.4, $C\underline{H}_3CH_2$).

O,O-Diethyl O-(5-Carboxy-2-fluorophenyl)phosphorothioate (*Hapten G*). Yield: 99%. TLC R_f (silica gel, hexane/ethyl acetate/acetic acid, 50:25:2): 0.49. ^{1}H NMR ($CDCl_3$): 8.01 (1H, d × d × d, J = 7.5, 1.9 and 1.9, H_e), 7.93 (1H, d × d × d, J = 8.5, 4.4 and 1.8, H_c), 7.22 (1H, d × d, J = 9.1 and 9.1, H_b), 4.27 (4H, d × q, J = 9.9 and 7.1, $CH_3C\underline{H}_2$), 1.38 (6H, t × d, J = 7.1 and 0.7, $C\underline{H}_3CH_2$).

Preparation of Hapten−Protein Conjugates. *Haptens A−D* were covalently attached to KLH for use as the immunogen. The haptens presented in **Figure 1** including *Haptens A−D* were attached to OVA for use as the coating antigen for screening of the antiserum and the supernatant after cell fusion as well as in ELISA. The method of conjugation used was the active ester method via the succinimide ester as described in our previous papers (*17*, *21*, *22*). The R_f values of the active esters in TLC using silica gel and hexane/ethyl acetate/acetic acid (200:100:8) were close to 0.4.

Active Ester of Hapten A. Yield: 64% from 52 mg (0.17 mmol) of *Hapten A*. ^{1}H NMR ($CDCl_3$): δ 8.15 (1H, d, J = 9.4, H_d), 7.15 (1H, d, H_e), 7.14 (1H, s, H_a), 4.26 (4H, d × q, J = 10.0 and 7.1, $CH_3C\underline{H}_2$), 2.91 (4H, s, succinyl), 2.62 (3H, s, CH_3-ar), 1.38 (6H, t × d, J = 7.0 and 0.5, $C\underline{H}_3CH_2$).

Active Ester of Hapten B. Yield: 43% from 25 mg (0.075 mmol) of *Hapten B*. ^{1}H NMR ($CDCl_3$): δ 8.05 (1H, d, J = 8.4, H_e), 7.00−6.83 (2H, m, H_a and H_e), 4.24 (4H, d × q, J = 10.0 and 7.1, $CH_3C\underline{H}_2O$), 3.90 (3H, s, CH_3O-ar), 2.86 (4H, s, succinyl), 1.36 (6H, t × d, J = 7.1 and 0.7, $C\underline{H}_3CH_2$).

Active Ester of Hapten C. Yield: 49% from 34 mg (0.11 mmol) of *Hapten E*. ^{1}H NMR ($CDCl_3$): δ 7.80 (1H, m, H_c), 7.72 (1H, d × d, H_e), 7.33 (1H, m, J = 0.73, H_a), 4.25 (4H, d × q, J = 10.0 and 7.1, $CH_3C\underline{H}_2$), 2.92 (4H, s, succinyl), 2.43 (3H, s, CH_3-ar), 1.38 (6H, t × d, J = 7.1 × 0.7, $C\underline{H}_3CH_2$).

Active Ester of Hapten D. Yield: 40% from 42 mg (0.14 mmol) of *Hapten F*. ^{1}H NMR ($CDCl_3$): δ 7.53 (1H, d × d, J = 3.6 and 1.5, H_c), 7.47 (1H, d × d × d, J = 2.3, 1.2 and 1.2, H_e), 7.09 (1H, d × d, J = 3.9 and 2.3, H_a), 4.25 (4H, d × q, J = 10.0 and 7.1, $CH_3C\underline{H}_2$), 3.86 (3H, s, CH_3O-ar), 2.91 (4H, s, succinyl), 1.38 (6H, t × d, J = 7.1 × 0.8, $C\underline{H}_3CH_2$).

Active Ester of Hapten E. Yield 81% from 73 mg (0.25 mmol) of *Hapten I*. ^{1}H NMR ($CDCl_3$): δ 8.15 (2H, d, J = 8.5, H_b and H_d), 7.33 (2H, d × d, J = 8.7 and 1.4, H_a and H_e), 4.26 (4H, d × q, J = 10.0 and 7.1, $CH_3C\underline{H}_2$), 2.92 (4H, s, succinyl), 1.38 (6H, t × d, J = 7.1 and 0.5, $C\underline{H}_3CH_2$).

Active Ester of Hapten F. Yield: 81% from 101 mg (0.35 mmol) of *Hapten J*. ^{1}H NMR ($CDCl_3$): δ 7.99−7.93 (1H, m, H_c), 7.92−7.88 (1H, m, H_e), 7.52−7.47 (2H, m, H_a and H_b), 4.23 (4H, d × q, J = 10.0 and 7.1, $CH_3C\underline{H}_2$), 2.89 (4H, s, succinyl), 1.35 (6H, t, J = 7.1, $C\underline{H}_3CH_2$).

Active Ester of Hapten G. Yield: 90% from 154 mg (0.50 mmol) of *Hapten K*. ^{1}H NMR ($CDCl_3$): δ 8.04 (1H, d × d × d, J = 7.4, 1.9 and 1.9, H_e), 7.99 (1H, d × d × d, J = 8.5, 4.3 and 1.4, H_c), 7.27 (1H, d × d, J = 8.8 and 8.8, H_b), 4.27 (4H, d × q, J = 9.9 and 7.1, $CH_3C\underline{H}_2$), 2.89 (4H, s, succinyl), 1.37 (6H, t × d, J = 7.1 and 0.7, $C\underline{H}_3CH_2$).

Production of Monoclonal Antibodies. Procedures for the production of monoclonal antibodies against the hapten−KLH conjugates, i.e., immunization, cell fusion, hybridoma selection, and cloning, were similar to those previously described in our papers (*17*, *22*). Procedures performed differently were as follows. Homologous as well as several heterologous coating antigens were used in noncompetitive indirect ELISAs for hybridoma screening. Competitive indirect ELISAs performed to select suitable coating antigens utilized three OP pesticides (chloropyrifos, diazinon, and parathion) as analytes. Ascites rather than the culture supernatants were used as the antibody reagent. To obtain ascites, female BALB/c mice were given a priming intraperitoneal injection of 0.5 mL of tetramethylpentadecane (pristine oil). Ten days later, the mice were given an intraperitoneal injection of approximately 10^7 of hybridoma cells. Ascite fluids were harvested by peritoneal tap with an 18-gauge needle on the seventh day after the cells were introduced, followed by purification by ammonium sulfate precipitation. Immunoglobulin subclass was determined by using a commercial kit from Boehringer Mannheim (Mannheim, Germany).

Competitive Indirect ELISA. Suitable antibody and coating antigen concentrations for ELISA were determined as described in our previous papers (*17*, *21*, *22*). *Haptens A−H* were tested for plate coating. The concentration of MeOH used to dissolve pesticides and the concentration of phosphate ion in the assay mixture were optimized as described in our previous papers (*17*, *21*, *22*) using chlorpyrifos, diazinon, and parathion as analytes. After optimization of the ELISA procedure, 18 organophosphorus pesticides and metabolites, and two carbamate pesticides were used as the analytes. The assay procedure was as follows. All incubations except that for antigen coating were carried out at room temperature followed by washing five times with PBST (10 mM PBS containing 0.05% Tween 20 at pH 7.4). Microtiter plates were coated with hapten−OVA in carbonate−bicarbonate buffer (50 mM, pH 9.6) by incubating overnight at 4 °C. Plates were blocked by incubation with 1% gelatin in PBS (200 μL/well) for 1 h. Fifty microliters/well of analyte dissolved in 10% MeOH−PBS (10 mM, pH 7.4) and 50 μL/well of the ascites diluted with PBS (90 mM, pH 7.4) were added to the blocked plate. After incubation for 1 h, 100 μL/well of diluted (1/3000) goat antimouse IgG-HRP was added. The mixture was incubated for 1 h, followed by the addition of 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 at pH 5.5). After incubation for an appropriate time (typically 10 min), the reaction was stopped by adding 50 μL of 2 M H_2SO_4, and the absorbance was read at 450 nm.

Analysis of Spiked Samples. Three representative OP pesticides (chlorpyrifos, diazinon, and parathion-ethyl) were prepared with MeOH in 100, 200, 500, and 1000 ng/mL solutions for the spiking of lettuce and kale samples. One milliliter of a spiking solution was added to 1 g of pesticide-free lettuce or kale leaves that had been chopped in fine pieces. After setting this aside for 24 h, the leaves were incubated in 5 mL of MeOH for 10 min with four vigorous shakes and then filtered through a Whatman No. 1 filter paper. The container and the residues were rinsed with 5 mL of MeOH and filtered, and the filtrate was combined with the previous filtrate. MeOH was evaporated to dryness under reduced pressure, and the residue was extracted with 10 or 100 mL of a 10% MeOH−PBS (50 mM). The extract was analyzed using the most suitable ELISA chosen earlier (ELISA using the antibody raised against *Hapten A* and the coating antigen containing *Hapten D*).

RESULTS AND DISCUSSION

Hapten Selection and Synthesis. Most of OP pesticides have a $(RO)_2P(=S)$- group (R = methyl or ethyl group) in common and differ among their remaining structures usually attached to the phosphorus atom through an oxygen (phosphorothioates) or sulfur atom (phosphorodithioates). Phosphorothioates and phosphorodithioates are the two largest subclasses of OP pesticides comprising most of the OP pesticides currently used. Therefore, to develop an ELISA class-selective for OP pesticides, it was most desirable to use immunizing haptens with a bridge group at the phosphorus atom in order to preserve the common $(RO)_2P(=S)$- group and eliminate the variable part of the molecule.

The haptens previously used for the ELISAs class-selective for OP pesticides are presented in **Figure 7**. *Haptens I* (*13*, *14*) and *II* (*13*) have a phosphonate and phosphsphate group, respectively (instead of a thiophosphate group). As expected, these haptens produced antibodies (polyclonal) with poor response to many of the OP pesticides leading to low assay sensitivity. *Hapten III* yielded antibodies (polyclonal) with a higher response to OP pesticides, but the range of response was large possibly because of the strong recognition of the aromatic ring of the hapten molecule by the animal immune system which would make the antibody differentiate between pesticides with and without the aromatic ring (*15*). *Hapten IV* may be considered a suitable hapten for dimethyl OP pesticides (R = methyl) (*16*). However, the response of the ELISA using the polyclonal antibodies against this hapten was too low for any sensitive detection of the pesticides (typical IC_{50} value = 1−10 μg/mL) despite being fairly uniform. The low sensitivity of this ELISA may be due to the flexible bridge group

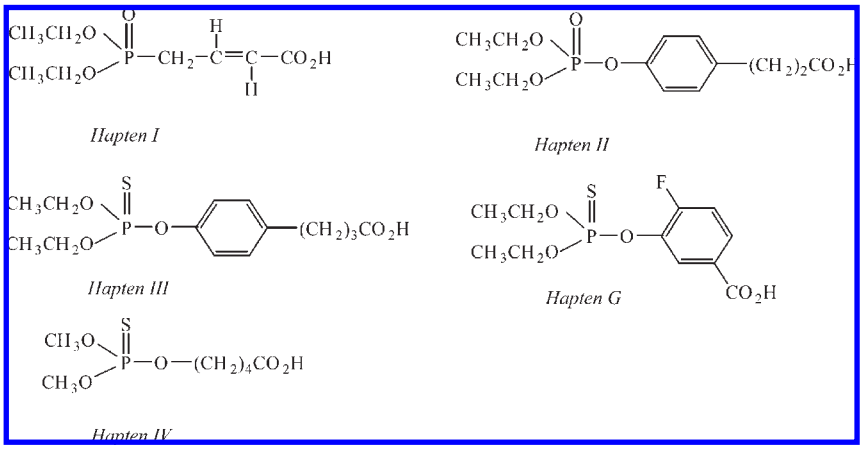

Figure 7. Haptens used previously for the class-selective determination of OP pesticides by ELISA.

containing a long alkyl chain, as a rigid hapten structure is considered an important factor in obtaining a good antibody (*23*). This hypothesis is in agreement with the observation that the ELISA using the antibody to *Hapten II* is generally more sensitive compared to that using the antibody to *Hapten I*.

In order to resolve the problems associated with these haptens in our previous study for the development of an ELISA for the class-selective determination of *O*,*O*-diethyl OP pesticides, we used *Hapten G* (**Figures 6** and **7**) with a bridge structure containing a benzene ring with a substituent at the ortho position (*17*). The two monoclonal antibodies against this hapten exhibited quite uniform and strong responses to many diethyl OP pesticides, except diazinon.

The strategy adopted in this study to resolve the previous problem was to use haptens (*Haptens A*−*D* in **Figure 1**) with structures similar to that of *Hapten G* yet different in that a substituent is placed at the meta position of the benzene ring. Similar to our previous study, a rigid benzene ring was inserted in the bridge to promote a high antibody response to pesticides, along with a substituent attached to the benzene ring for suppression of the influence of the benzene ring on the immune system. This substituent was attached to the benzene ring at the meta position to enhance the antibody response to pesticides containing a bulky substituent at the meta position, such as diazinon. The strategy was effective and is discussed in the next section.

Development of Indirect ELISA. Mice injected with each of the four immunogens all produced sera exhibiting high titer values after the third injection (second boost). Screening of hybridoma cells by competitive indirect ELISA, using several hapten−OVA conjugates as coating antigens and three OP pesticides as analytes, allowed us to select the most suitable antibody for ELISA. Criteria for antibody selection were intensity and uniformity in response to the three pesticides. A total of four high-response monoclonal antibodies against *Haptens A*−*D* were obtained. The isotype of all of the antibodies was IgG and the type of light chain was κ.

The antibodies and coating antigens for the antigen-coated assays were optimized for concentration by running the assays under various combinations of antibody and coating antigen dilutions. The concentration of MeOH added to the assay solution to dissolve the pesticides was also optimized. Increasing the concentration of MeOH generally decreased and then increased the IC_{50} value. The organic solvent chosen for the optimum condition was 5% MeOH, giving the lowest IC_{50} value. Increasing the concentration of the phosphate ion in the assay solution generally caused a continuous decrease in the IC_{50} value but a continuous increase in the time for color development. The optimum concentration selected was 0.05 M, where IC_{50}/A_{max} was the lowest.

Table 1. IC_{50} Values[a] of Pesticides Produced by Indirect ELISAs Using the Antibody C5 (Antibody against *Hapten A*) and Different Coating Antigens

		CoAg[b]							
	pesticides	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
1	chlorpyrifos	10	44	20	7	79	16	15	6
2	diazinon	45	191	85	21	318	58	51	35
3	parathion	5	7	7	2	14	3	6	3
4	pirimiphos-ethyl	132	218	150	78	486	119	133	160
5	bromophos-ethyl	56	159	54	29	258	36	35	35
6	dichlorofenthion	4	10	7	6	24	6	8	15
7	azinphos-ethyl	330	901	657	132	3220	344	182	168
8	phosalone	147	159	222	127	852	156	104	82
9	phorate	1270	2000	405	191	5700	278	352	284
10	disulfoton	149	444	291	97	821	159	105	123
11	ethion	512	ni	2220	282	ni	2470	514	629
12	EPN	135	320	179	97	857	118	96	69
13	paraoxon-ethyl	37000	12600	19200	8710	36400	11100	6030	8130
14	TEPP	ni	ni	46400	19600	ni	10800	25200	26100
15	parathion-methyl	2610	8390	4270	1010	10600	3150	1250	1870
16	fenitrothion	470	1830	662	272	4700	512	523	750
17	azinphos-methyl	16600	13600	13600	6870	ni	11200	6970	6550
18	malathion	10400	ni	ni	ni	ni	ni	ni	ni
19	carbaryl	ni	ni	ni	ni	ni	ni	ni	ni
20	carbofuran	ni	ni	ni	ni	ni	ni	ni	ni
	mean for 1−12[c]	233	*405	358	89	*1150	314	133	134
	% CV for 1−12[c]	154	*145	172	96	*153	219	115	132

[a] ni means no inhibition by a pesticide. [b] CoAg means coating antigen. Coating antigens *A*, *B*, *C*, etc. mean *Hapten A*-, *Hapten B*-, and *Hapten C*-OVA, etc., respectively. [c] Average IC_{50} and % CV values labeled with * were those with a single target analyte exhibiting no inhibition and were calculated excluding that pesticide. The exact values including such pesticides may be considerably higher.

Performance of the Developed ELISAs. The results of the 32 ELISAs using the 4 antibodies, 8 coating antigens, and 20 pesticides (analytes) are presented in **Tables 1**, **2**, **3**, and **4**. The more sensitive ELISAs with lower average IC_{50} values (below 150 ng/mL) for the *O*,*O*-diethyl phosphorothioates (1−6), *O*,*O*-diethyl phosphorodithioates (7−11), and *O*-ethyl phosphonate (12) used the antibodies from *Haptens A* and *B* (**Tables 1** and **2**). The antibody-coating antigen combinations of five more sensitive ELISAs with lower IC_{50} values are *A*−*D* (89), *A*−*G* (133), *A*−*H* (134), *B*−*D* (140), and *B*−*H* (141) (average IC_{50} values are presented in the parentheses in ng/mL). The results of the selected ELISAs are presented separately in **Table 5**. Unlike our previously developed ELISA for the class-selective

Table 2. IC_{50} Values[a] of Pesticides Produced by Indirect ELISAs Using the Antibody H10 (Antibody against *Hapten B*) and Different Coating Antigens

		CoAg[b]							
	pesticides	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
1	chlorpyrifos	48	105	29	14	162	20	20	20
2	diazinon	50	122	72	28	210	53	73	67
3	parathion	13	14	6	8	37	3	10	4
4	pirimiphos-ethyl	134	169	161	101	416	125	147	96
5	bromophos-ethyl	91	199	53	43	363	27	33	44
6	dichlorofenthion	12	19	8	7	34	5	12	9
7	azinphos-ethyl	569	1510	727	209	3480	374	271	263
8	phosalone	200	319	263	195	1430	228	183	86
9	phorate	1100	3400	586	593	7040	389	639	480
10	disulfoton	185	627	337	108	1310	198	241	214
11	ethion	449	475	1310	328	11000	11400	573	297
12	EPN	169	346	104	46	653	101	47	107
13	paraoxon-ethyl	ni	ni	ni	8150	ni	ni	9590	13200
14	TEPP	2180	ni	ni	17400	ni	ni	19300	ni
15	parathion-methyl	6900	15400	4630	958	ni	2730	2210	2740
16	fenitrothion	605	881	583	505	4230	390	818	431
17	azinphos-methyl	11700	13400	11500	7460	ni	10100	8840	10500
18	malathion	ni	ni	ni	ni	ni	nii	ni	ni
19	carbaryl	ni	ni	ni	ni	ni	ni	ni	ni
20	carbofuran	ni	ni	ni	ni	ni	ni	ni	ni
	mean for 1−12	252	609	305	140	2180	1080	187	141
	% CV for 1−12	126	159	129	124	158	302	115	103

[a] ni means no inhibition by a pesticide. [b] CoAg means coating antigen. Coating antigens *A*, *B*, *C*, etc. mean *Hapten A*-, *Hapten B*-, and *Hapten C*-OVA, etc., respectively.

Table 3. IC_{50} Values[a] of Pesticides Produced by Indirect ELISAs Using the Antibody E6 (Antibody against *Hapten C*) and Different Coating Antigens

		CoAg[b]							
	pesticides	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
1	chlorpyrifos	88	127	272	149	185	249	219	220
2	diazinon	289	400	800	722	361	580	846	919
3	parathion	55	100	252	166	129	164	282	192
4	pirimiphos-ethyl	266	302	299	199	288	330	532	254
5	bromophos-ethyl	2230	1060	2600	2390	1230	1940	34510	2280
6	dichlorofenthion	27	21	212	165	26	182	131	165
7	azinphos-ethyl	11300	ni	2850	3430	ni	2600	10700	3230
8	phosalone	2870	1600	2430	4100	2760	2320	5850	1540
9	phorate	2920	3500	2800	4200	3140	3690	2140	3710
10	disulfoton	6230	5530	1810	1560	7140	1760	3940	1610
11	ethion	ni	33300	1260	ni	ni	17	ni	ni
12	EPN	1550	2130	1200	828	2860	2330	2490	691
13	paraoxon-ethyl	ni	ni	ni	ni	ni	ni	ni	ni
14	TEPP	ni	ni	ni	ni	ni	ni	ni	ni
15	parathion-methyl	ni	ni	21800	11600	ni	ni	ni	ni
16	fenitrothion	ni	ni	ni	ni	ni	16700	ni	ni
17	azinphos-methyl	ni	ni	ni	ni	ni	ni	ni	ni
18	malathion	13000	ni	ni	14400	11600	ni	ni	12100
19	carbaryl	ni	ni	ni	ni	ni	ni	ni	ni
20	carbofuran	ni	ni	ni	ni	ni	ni	ni	ni
	mean for 1−12 [c]	*2530	*4370	1400	*1630	*1810	1350	*5600	*1350
	% CV for 1−12 [c]	*137	*223	76	*100	*124	92	*180	*94

[a] ni means no inhibition by a pesticide. [b] CoAg means coating antigen. Coating antigens *A*, *B*, *C*, etc. mean *Hapten A*-, *Hapten B*-, and *Hapten C*-OVA, etc., respectively. [c] Average IC_{50} and % CV values labeled with * were those with a single target analyte exhibiting no inhibition and were calculated excluding that pesticide. The exact values including such pesticides may be considerably higher.

determination of *O*,*O*-diethyl OP pesticides, current ELISAs show quite a strong response to diazinon. Diazinon has a ring structure containing two nitrogen atoms in addition to a bulky substituent at the meta position, both of which may have an inhibitory effect on the affinity of the antibody we used previously. It may be said that the immunizing haptens used in this study are effective in eliminating such inhibitory effects. The relatively low % CV values as shown in **Table 5** suggest that these ELISAs may be better for the class-selective determination of

Table 4. IC_{50} Values[a] of Pesticides Produced by Indirect ELISAs Using the Antibody H6 (Antibody against *Hapten D*) and Different Coating Antigens

		CoAg[b]							
	pesticides	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
1	chlorpyrifos	203	144	9840	2470	517	912	507	200
2	diazinon	323	326	4890	14100	884	2720	1300	449
3	parathion	6	6	94	1520	15	71	32	7
4	pirimiphos-ethyl	52	53	100	427	73	119	83	49
5	bromophos-ethyl	463	391	2090	20800	616	1040	632	394
6	dichlorofenthion	100	100	177	3290	139	366	146	90
7	azinphos-ethyl	93	95	545	7171	239	461	230	94
8	phosalone	690	817	2440	ni	1550	1010	1150	752
9	phorate	303	303	1285	8820	658	1010	720	264
10	disulfoton	18	15	69	947	35	28	18	13
11	ethion	161	408	150	ni	816	409	559	349
12	EPN	429	439	ni	ni	704	713	703	594
13	paraoxon-ethyl	886	1130	7540	12500	1220	3010	2160	840
14	TEPP	ni	ni	ni	ni	ni	ni	ni	ni
15	parathion-methyl	298	154	591	465	154	276	662	476
16	fenitrothion	1620	1470	ni	ni	3720	2690	2440	1230
17	azinphos-methyl	18800	23500	21500	ni	18700	11500	11300	13800
18	malathion	ni	ni	ni	ni	ni	ni	ni	ni
19	carbaryl	ni	ni	ni	ni	ni	ni	ni	ni
20	carbofuran	ni	ni	ni	ni	ni	ni	ni	ni
	mean for 1−12 [c]	237	258	*1970	-	521	738	507	271
	% CV for 1−12 [c]	89	92	*153	-	87	99	84	89

[a] ni means no inhibition by a pesticide. [b] CoAg means coating antigen. Coating antigens *A*, *B*, *C*, etc. mean *Hapten A*-, *Hapten B*-, and *Hapten C*-OVA, etc., respectively. [c] Average IC_{50} and % CV values labeled with * were those with a single target analyte exhibiting no inhibition and were calculated excluding that pesticide. The exact values including such pesticides may be considerably higher. Average IC_{50} and % CV values in the case of more than two target analytes exhibiting no inhibition were not derived and are indicated as -.

Table 5. IC_{50} Values[a] of Pesticides Produced by Indirect ELISAs that Were Chosen As More Useful on the Basis of Lower IC_{50} and % CV Values

		Ab-CoAg[b]				
	pesticides	*A*−*D*	*A*−*G*	*A*−*H*	*B*−*D*	*B*−*H*
1	chlorpyrifos	7	15	6	14	20
2	diazinon	21	51	35	28	67
3	parathion	2	6	3	8	4
4	pirimiphos-ethyl	78	133	160	101	96
5	bromophos-ethyl	29	35	35	43	44
6	dichlorofenthion	6	8	15	7	9
7	azinphos-ethyl	132	182	168	209	263
8	phosalone	127	104	82	195	86
9	phorate	191	352	284	593	480
10	disulfoton	97	105	123	108	214
11	ethion	282	514	629	328	297
12	EPN	97	96	69	46	107
13	paraoxon-ethyl	8710	6030	8130	8150	13200
14	TEPP	19600	25200	26100	17400	ni
15	parathion-methyl	1010	1250	1870	958	2740
16	fenitrothion	272	523	750	505	431
17	azinphos-methyl	6870	6970	6550	7460	10500
18	malathion	ni	ni	ni	ni	ni
19	carbaryl	ni	ni	ni	ni	ni
20	carbofuran	ni	ni	ni	ni	ni
	mean for 1−12	89	133	134	140	141
	% CV for 1−12	96	115	132	124	103

[a] ni means no inhibition by a pesticide. [b] Antibody and coating antigens *A*, *B*, *C*, etc. mean those derived from *Hapten A*, *Hapten B*, and *Hapten C*, etc., respectively.

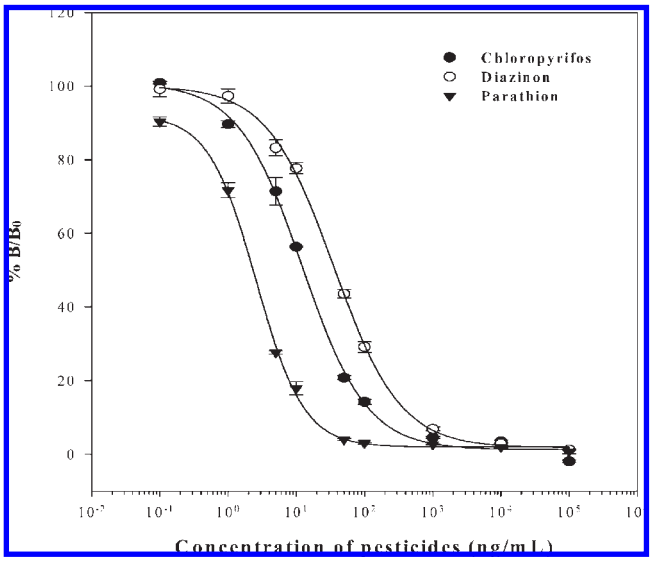

Figure 8. ELISA competition curves produced by indirect ELISA. Assay conditions: monoclonal antibody against Hapten A-KLH, diluted 1/10000; Hapten D-OVA, coating antigen, 100 ng/well; the coated wells were blocked with 1% gelatin; chlorpyrifos, diazinon, and parathion were used as analytes; assay solution of 10% MeOH-PBS (50 mM); goat antimouse IgG-HRP, 1/3000.

pesticides. Interestingly, while the ELISAs using the antibodies from *Haptens C* and *D* exhibit relatively poor sensitivity, many of their responses are quite uniform (low % CV values). The most suitable combination among the 32 ELISAs is *A*−*D*, on the basis of its IC_{50} and % CV values that are the lowest. **Figure 8** shows typical inhibition curves with this combination for the three chosen pesticides.

The five chosen ELISAs show class-selective responses to *O*,*O*-diethyl phosphorothioate and phosphorodithioate OP pesticides (1−11), discriminating among *O*,*O*-diethyl phosphate OP pesticides including an oxon (13 and 14), *O*,*O*-dimethyl OP pesticides (15−18), and carbamate pesticides (19 and 20). The differentiation suggests the antibodies precisely distinguish between P═S and P═O as well as between diethyl and dimethyl groups. One exception is that of fenitrothion, which contains the *O*,*O*-dimethyl group. Its IC_{50} value in all of the chosen ELISAs is close to that of the OP pesticide with the largest IC_{50} value. The relatively high response of the antibody to fenitrothion may be attributed to the structure of the immunizing haptens with a substituent at the meta position of the benzene ring in common with fenitrothion.

It is noteworthy that the antibodies show a strong response to EPN, which is an *O*-ethyl phosphonate OP pesticide. The response is similar to those of other OP pesticides. It requires, however, a further test using some other phosphonate OP pesticides to confirm the applicability of the chosen ELISAs to phosphonate OP pesticides in general.

In order to compare the performance of the finally chosen ELISA (*A*−*D* combination) with those of previously developed ELISAs, IC_{50} and % CV values with the chosen ELISA and those in the literature are presented in **Table 6**. The final selected ELISA may be considered more sensitive than the previously developed ELISAs as evidenced by a much lower mean IC_{50} value.

The current ELISA may be considered more effective in class-selective determination on the basis of their considerably lower % CV values. Class-selective determination must also be evaluated on the basis of discrimination against other types of pesticides. ELISAs in the literature exhibit responses to paraoxon-ethyl that are much stronger than those to many of the *O*,*O*-diethyl OP pesticides. In contrast, the current ELISA exhibits negligible cross-reactivity to paraoxon-ethyl. Two ELISAs from the literature produce a response to three of the *O*,*O*-dimethyl OP pesticides that are higher than that to several *O*,*O*-diethyl OP pesticides (*14*, *15*). In addition, the response of one of them to the *O*,*O*-dimethyl OP pesticide malathion is much higher than that to EPN (*13*). The cross-reactivity of the chosen ELISAs to the *O*,*O*-dimethyl OP pesticides is negligible except to fenitrothion.

Table 6. IC_{50} Values[a] of Pesticides Produced by Indirect ELISAs Presented in This Study and in the Literature

		ELISA				
	pesticides	this study[b] (*A*−*D*)	Südi (*13*)	Johnson (*15*)	Alcocer (*14*)	Jang (*17*)
1	chlorpyrifos	7	55600	1000	67000	111
2	diazinon	21	10100	37	900	8910
3	parathion	2	1010	4.8	600	269
4	pirimiphos-ethyl	78			2500	
5	bromophos-ethyl	29	9900			121
6	dichlofenthion	6				183
7	azinphos-ethyl	132	17700			
8	phosalone	127	17600	3000		
9	phorate	191	3130	90		
10	disulfoton	97	3150	110		
11	ethion	282	3200	28000		
12	EPN	97	17800			
13	paraoxon-ethyl	8710	32	100	37	
14	TEPP	19600	10100	80000		
15	parathion-methyl	1010	178000	200	10500	1060
16	fenitrothion	272			5600	
17	azinphos-methyl	6870	317000	4000		80600
18	malathion	ni	99800	6600	700	
19	carbaryl	ni				
20	carbofuran	ni				
	mean for 1−12[c]	89	(99) 13900	(104) 4610	(27) 17800	(13) 1920
	% CV for 1−12[c]	96	(91) 115	(101) 225	(130) 185	(88) 204

[a] ni means no inhibition by a pesticide. [b] The antibody−coating antigen combination was *A*−*D*. [c] For a precise comparison, the mean IC_{50} and % CV values in the literature were compared with those of the current ELISA that were calculated for the pesticides in common and presented within parentheses on the left of the literature values.

Recovery Studies. For the recovery studies, we used lettuce and kale samples spiked with three representative OP pesticides and analyzed them by the final selected ELISA (*A*−*D* combination). Samples were prepared by extracting 1 g of spiked vegetable with MeOH, followed by evaporation of the solvent, and then dissolving the residue in 10 or 100 mL of 10% MeOH−PBS. Recoveries are presented in **Table 7**. Vegetable samples extracted into 10 mL of MeOH−PBS produced recoveries that were generally underestimated as opposed to generally satisfactory recoveries from the samples extracted into 100 mL of MeOH−PBS. It appeared that the dilution of the sample improved the recovery by reducing the matrix effects.

Underestimation of the recovery from the vegetable samples extracted into 10 mL of solvent is especially remarkable at higher spiked concentrations. The spiked concentrations of 500 and 1000 ng/g correspond to the assay sample concentrations of 50 and 100 ng/mL, respectively, when the volume of the sample is 10 mL. These concentrations are located right of the IC_{50} values and near the right side plateau where a small change in absorbance results in a large change in recovery. Therefore, it is understandable how 10 mL extracts at higher spiked concentrations produce large errors in recovery. Overestimation of recovery

Table 7. Recovery of OP Pesticides (Chlorpyrifos, Diazinon, and Parathion-Ethyl) Spiked into Vegetables

			recovery volume of extract	
sample	pesticide	spiked concentration (ng/g)	10 mL	100 mL
lettuce	chlorpyrifos	100	96 ± 7.3	138 ± 1.9
		200	78 ± 8.9	102 ± 3.0
		500	36 ± 2.4	70 ± 1.8
		1000	50 ± 7.8	82 ± 6.6
	diazinon	100	73 ± 3.6	74 ± 0.5
		200	51 ± 4.4	91 ± 0.8
		500	62 ± 6.4	101 ± 5.6
		1000	65 ± 4.7	93 ± 0.5
	parathion-ethyl	100	102 ± 5.8	124 ± 4.8
		200	70 ± 2.8	104 ± 4.7
		500	97 ± 9.7	98 ± 3.8
		1000	90 ± 7.1	82 ± 5.2
kale	chlorpyrifos	100	76 ± 6.8	104 ± 5.2
		200	48 ± 1.7	82 ± 2.5
		500	30 ± 4.8	96 ± 2.9
		1000	15 ± 4.6	57 ± 4.1
	diazinon	100	210 ± 1.4	167 ± 5.5
		200	103 ± 4.9	114 ± 2.7
		500	84 ± 3.9	85 ± 8.8
		1000	52 ± 5.4	63 ± 4.0
	parathion-ethyl	100	83 ± 5.3	97 ± 5.1
		200	92 ± 5.1	102 ± 1.0
		500	65 ± 4.2	89 ± 0.6
		1000	37 ± 8.6	83 ± 5.3

is observed with samples extracted into 100 mL of solvent at the highest spiked concentration (100 ng/g). The spiked concentration of 100 ng/g corresponds to the assay sample concentration of 1 ng/mL, when the volume of the sample is 100 mL. This concentration is located left of the IC_{50} values and near the left side plateau where a small change in absorbance results in a large change in recovery. Likewise, large errors in recovery are understandable with 100 mL extracts at the spiked concentration of 100 ng/g. Although dilution of the sample is an effective means of reducing the matrix effect, it leads to a reduction of assay sensitivity by moving the detected concentration range to the left side plateau. Therefore, dilution of samples for reducing the matrix effect is applicable only when the detected concentration range remains on the inside of the dynamic range of the calibration curve.

Since the current ELISA shows sensitivity that is considerably lower than that of the currently used chromatographic methods, it cannot be used as a standard official method for pesticide determination. However, it may be utilized for preliminary screening of food and environmental samples for assessing the necessity for further inspection for individual OP pesticides. The maximum residue limit (MRL) of OP pesticides for vegetables is typically between 0.1 and 1.0 ng/g (*24*−*26*). The results of the current recovery study indicate that the final selected ELISA is a potential screening method for the estimation of whether vegetable samples are in violation of MRL of OP pesticides.

ABBREVIATIONS USED

CR, cross-reactivity; DMF, dimethylformamide; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IC_{50}, concentration of analyte giving 50% inhibition of the maximum absorbance; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; NMR, nuclear magnetic resonance; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline−0.05% Tween 20; TLC, thin layer chromatography; TMB, 3,3′,5,5′-tetramethylbenzidine.

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