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Development of Enzyme-Linked Immunosorbent Assays for the Organophosphorus Insecticide EPN

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Competitive enzyme-linked immunosorbent assays (ELISAs) in indirect and direct format were developed for the quantitative detection of the organophosphorus insecticide EPN. Five EPN derivatives (haptens) were synthesized and coupled to carrier proteins to use as an immunogen or as a competitor. Rabbits were immunized with two of the five haptens coupled to KLH for production of polyclonal antibodies, and the sera were screened against one of the haptens coupled to ovalbumin (OVA). Using the serum with the highest specificity and a coating antigen (hapten−OVA conjugate), an indirect (antigen-coated) ELISA was developed, which showed an IC_{50} of 5.6 ng/mL with a detection limit of 0.2 ng/mL (20% inhibition). A direct (antibody-coated) ELISA using an enzyme tracer (hapten−enzyme conjugate) was also developed, which showed an IC_{50} of 8.4 ng/mL with a detection limit of 0.9 ng/mL (20% inhibition). The antibodies showed negligible cross-reactivity with other organophosphorus pesticides except with the insecticide parathion-ethyl only in the direct ELISA. The recoveries of EPN from spiked samples determined by the indirect ELISAs were between 37 and 164%.

KEYWORDS: EPN; insecticide; immunoassay; enzyme-linked immunosorbent assay; ELISA

INTRODUCTION

The current analytical methods involving gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been used successfully for the analysis of many pesticides (*1*). However, these classical methods require a high cost, skilled analysts, and time-consuming sample preparation steps. Therefore, there is a growing demand for more rapid and economical methods for determining pesticide residues. Immunoassays began recently to gain acceptance as an alternative to the traditional methods that can meet such demands because they are fast, sensitive, and cost-effective tools for detecting trace amounts of chemicals such as pesticides (*2*).

EPN (*O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate) is an organophosphorus insecticide, which is effective against a wide range of insects (*3*). The most sensitive and toxicologically relevant effect after administration of EPN is inhibition of acetylcholinesterase activity (*4*). EPN is also reported to be an endocrine-disrupting chemical with estrogenic and antiandrogenic activity (*5*). Analysis of EPN is carried out by multiresidue methods using gas chromatography (*6*). An ELISA for this pesticide has not yet been reported.

The reason for the nondevelopment of an EPN ELISA seems to be the difficulty in synthesizing haptens for EPN. The development of an immunoassay requires the production of antibodies to the analyte. Because 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 the thiophosphate group, which has been used successfully in the development of ELISAs for several organophosphorus pesticides (*7, 8*). We developed a novel method for the synthesis of such haptens, which is much easier than the previous one (*9*), and developed ELISAs using the haptens (*10*−*15*). We found in this study that this method can be successfully applied to the synthesis of EPN haptens. The synthesized haptens allowed production of antibodies specific to EPN, from which sensitive and selective ELISAs for EPN were developed.

MATERIALS AND METHODS

Chemicals and Instruments. Organophosphorus pesticides including EPN were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The reagents needed for the synthesis of EPN haptens were obtained from Aldrich (Milwaukee, WI). Ovalbumin (OVA), horseradish peroxidase (HRP), phosphate-buffered saline (PBS), polyethylene sorbitan monolaurate (Tween 20), gelatin, Freund's incomplete and complete adjuvants, peroxidase-labeled goat anti-rabbit IgG, 3,3′,5,5′-tetramethylbenzidine (TMB), and Sephadex G-25 were purchased from Sigma (St. Louis, MO). Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem (La Jolla, CA). The dialysis membrane (MW cutoff 12000−14000) was obtained from Spectrum Laboratories (Rancho

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Figure 1. Structures of haptens for EPN.

Figure 2. Synthetic route for hapten A.

Dominguez, CA). Microtiter plates (Maxisorp, 442404) were purchased from Nunc (Roskilde, Denmark). ELISA plates were washed with a model 1575 ImmunoWash, and well absorbances were read with a model 550 plate reader, both from Bio-Rad (Hercules, CA). 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 hertz, and the abbreviations d, t, q, qn, sp, m, ph, and nph represent doublet, triplet, quartet, quintet, septet, multiplet, phenyl, and *p*-nitrophenyl, respectively. The gas chromatography was performed on an Agilent 6890N instrument (Agilent, Palo Alto, CA).

Synthesis of Haptens. The haptens used for immunization and preparation of competitors (coating antigen and enzyme tracer) are presented in **Figure 1**. Haptens A and B were used for immunization and haptens A−E as competitors. All of the haptens were purified by column chromatography and/or preparative TLC to give a single spot on an analytical TLC plate. The synthetic route for hapten A is illustrated in **Figure 2**. The procedures involved in the synthetic routes were as follows.

1. A solution of phenylthiophosphonic dichloride (6.32 g, 30 mmol) in 20 mL of acetonitrile was added dropwise to a solution of 4-nitrophenol (2.77 g, 20 mmol) in 20 mL of acetonitrile containing 30 g of finely ground K_2CO_3. After stirring for 18 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, hexane/ethyl acetate (5:1)] to give 3.69 g (59%) of the product as a yellow syrup: ^{1}H NMR ($CDCl_3$), δ 8.31 (2H, d, J = 8.8, nph), 8.15 (2H, d × d, J = 17.2 and 7.7, ph), 7.73−7.58 (3H, m, ph), 7.49 (2H, d × d, J = 9.1 and 1.9, nph).

Hapten A. To a stirred solution of 250 mg (0.8 mmol) of **1** in 1 mL of methanol cooled in an ice−water bath was added dropwise a solution of 122 mg (2.2 mmol) of KOH and 129 mg (0.98 mmol) of 6-aminocaproic acid in 1 mL of methanol. After stirring for 30 min, the reaction mixture was filtered and extracted with 1 N HCl−ethyl acetate. The organic extract was dried over $MgSO_4$, and the solvent was evaporated. Column chromatography [silica gel, hexane/ethyl acetate/acetic acid (50:25:2)] of the residue gave 195 mg (60%) of a yellow syrup: ^{1}H NMR ($CDCl_3$), δ 8.23 (2H, d, J = 9.0, nph), 7.96 (2H, d × d, J = 14.3 and 7.4, ph), 7.65−7.43 (3H, m, ph), 7.39 (2H, dd, J = 9.1 and 1.3, nph), 3.42 (1H, m, NH), 3.02 (2H, m, NCH_2), 2.30 (2H, t, J = 7.3, CH_2CO), 1.56 (2H, qn, J = 7.4, $NHCH_2CH_2$), 1.46 (2H, qn, J = 7.2, CH_2CH_2CO), 1.30 (2H, qn, J = 7.2, $(CH_2)_2CH_2(CH_2)_2$).

The synthetic route for hapten B is illustrated in **Figure 3**. The procedures involved in the synthetic route were as follows.

2. A mixture of γ-butyrolactone (20 mL, 260 mmol) and NaOH (11.5 g, 287 mmol) in 20 mL of distilled water was refluxed for 6 h. After cooling in an ice−water bath, the water was evaporated and the mixture dissolved in hot ethanol. After filtration through filter paper, the insoluble residue in hot ethanol was removed. The mixture was added to diethyl ether for crystallization. A white solid (16.8 g, 52%) was obtained from the crystallization.

3. A solution of benzyl bromide (3.4 g, 20 mmol) in 50 mL of dichloromethane was added dropwise to a solution of sodium-4-hydroxybutanoate (5.0 g, 40 mmol) and 600 mg of tetrabutylammonium hydrogen sulfate in 20 mL of distilled water. After stirring violently for 4 days, the reaction mixture was extracted with chloroform, and the extract was dried over Na_2SO_4. Evaporation of the solvent and column chromatography [silica gel, hexane/ethyl acetate (1:1)] of the residue gave 780 mg (20%) of the product as a colorless oil: ^{1}H NMR ($CDCl_3$), δ 7.27 (5H, s, ph), 5.04 (2H, s, $C_6H_5CH_2$), 4.25 (2H, t, J = 7.0 CH_2O) 2.53 (2H, t, J = 7.1, CH_2CO_2), 1.82 (2H, qn, J = 6.3, $CH_2CH_2CH_2$).

4. A solution of **1** (103 mg, 0.33 mmol) in 500 μL of acetonitrile was added dropwise to a solution of **3** (74 mg, 0.38 mmol) in 500 μL of acetonitrile containing 0.5 g of finely ground K_2CO_3. After 2 days of stirring at room temperature, the solvent was removed under reduced pressure. The residue was subjected to preparative TLC [silica gel, hexane/ethyl acetate (5:1)] to give 31 mg (20%) of the product as a yellow oil: ^{1}H NMR ($CDCl_3$), δ 8.10 (2H, d, J = 9.1, nph), 7.92 (2H, d × d, J = 15.0 and 7.8, ph), 7.63−7.44 (3H, m, ph), 7.28 (5H, m, ph), 7.12 (2H, d, J = 9.1 and 1.5, nph), 5.04 (2H, s, $C_6H_5CH_2$), 4.20 (2H, m, CH_2O) 2.44 (2H, t, J = 7.1, CH_2CO_2), 2.02 (2H, m, $CH_2CH_2CH_2$).

Figure 3. Synthetic route for hapten B.

Hapten B. A solution of **4** (26 mg, 0.055 mmol) in 250 μL of HBr−acetic acid was stirred for 2 h, and the solvent was removed under reduced pressure. The residue was subjected to preparative TLC [silica gel, hexane/ethyl acetate/acetic acid (70:40:1)] to give 18 mg (88%) of the product as a yellow oil: ^{1}H NMR ($CDCl_3$), δ 8.18 (2H, d, J = 9.0, nph), 7.97 (2H, d × d, J = 15.0 and 7.7, ph), 7.66−7.46 (3H, m, ph), 7.19 (2H, d × d, J = 9.1 and 1.5, nph), 4.28 (4H, m, CH_2O) 2.51 (2H, t, J = 7.1, CH_2CO_2), 2.07 (2H, qn, J = 6.6, $CH_2CH_2CH_2$).

The synthetic routes for haptens C and D were similar to that of hapten A.

Hapten C. A reaction of **1** (75 mg, 0.24 mmol) with KOH (63 mg, 1.16 mol) and 4-(methylamino)butyric acid (47 mg, 0.31 mmol) followed by preparative TLC gave 39 mg (44%) of the product as a yellow oil: ^{1}H NMR ($CDCl_3$), δ 8.23 (2H, d, J = 9.1, nph), 7.88 (2H, d × d, J = 14.1 and 7.8, ph), 7.60−7.47 (3H, m, ph), 7.34 (2H, d × d, J = 9.1 and 1.2, nph), 3.34 (2H, m, NCH_2), 2.76 (3H, d, J = 11.6, CH_3N), 2.31 (2H, t, J = 7.3, CH_2CO), 1.83 (2H, m, $CH_2CH_2CH_2$).

Hapten D. A reaction of **1** (210 mg, 0.67 mmol) with KOH (102 mg, 1.82 mmol) and β-alanine (73.5 mg, 0.82 mmol) followed by preparative TLC gave 98 mg (38%) of the product as a yellow oil: ^{1}H NMR ($CDCl_3$), δ 8.22 (2H, d, J = 8.9, nph), 7.98 (2H, d × d, J = 14.4 and 7.8, ph), 7.64−7.42 (3H, m, ph), 7.36 (2H, d × d, J = 9.2 and 1.4, nph), 3.34 (2H, sp, J = 6.6, NCH_2), 3.18 (1H, qn, J = 5.7, NH), 2.50 (2H, t, J = 7.4, CH_2CO).

The synthetic route for hapten E is illustrated in **Figure 4**. The procedures involved in the synthetic route were as follows.

5. Phenylthiophosphate dichloride (2.37 g, 11.3 mmol) was added dropwise to ethanol (5.2 mL, 113 mmol) over 10 min. After stirring for 20 min in ice−water, the reaction mixture was extracted using distilled water/ethyl acetate. The organic extract was dried over $MgSO_4$, and the solvent was evaporated. Column chromatography [silica gel, hexane/ethyl acetate (40:1)] of the residue gave 800 mg (59%) of the product as a colorless oil. Further purification by preparative TLC [silica gel, hexane/ethyl acetate/acetic acid (50:25:2)] gave 39 mg (44%) of the product as a yellow oil: ^{1}H NMR ($CDCl_3$), δ 8.05 (2H, d × d, J = 16.6 and 7.7, ph), 7.66−7.49 (3H, m, ph), 4.58−4.32 (2H, m, CH_3CH_2), 1.48 (2H, t, J = 7.1, CH_3CH_2).

6. A solution of 2-amino-4-nitrophenol (800 mg, 5.0 mmol) and **5** (368 mg, 3.1 mmol) in 75 mL of butanone was added 857 mg of Na_2CO_3. The mixture was refluxed for 20 h. After cooling in ice−water bath, the reaction mixture was extracted using distilled water/ethyl acetate. The solvent was removed from the organic extract under reduced pressure, and the residue was subjected to column chromatography [silica gel, hexane/ethyl acetate (5:1)] to give 507 mg (69%) of the product as a yellow oil: ^{1}H NMR ($CDCl_3$), δ 7.94 (2H, d × d, J = 14.9 and 7.7, ph), 7.57−7.42 (5H, m, ph and nph), 7.05 (1H, d × d, J = 8.8 and 1.7, nph), 4.30−4.11 (3H, m, CH_3CH_2), 1.29 (2H, t, J = 7.1, CH_3CH_2).

7. A solution of **6** (350 mg, 0.91 mmol) in 5 mL of toluene was added to a solution of adipoyl chloride (1.0 g, 0.54 mmol) in 5 mL of toluene and 2 mL of 1 M Na_2CO_3 (0.07 mmol) over 3 h at 90 °C. After cooling, the reaction mixture was extracted using 1 N HCl/ethyl acetate. The organic phase was dried over $MgSO_4$, and the solvent was evaporated to give 200 mg (57%) of the product as a yellow syrup.

Hapten E. A solution of **7** (100 mg, 0.26 mmol) in 10 mL of THF was added to 1 N HCl (2.5 mL) and stirred for 3 h at 60 °C. The reaction mixture was added to a solution of saturated NaCl. The organic phase was separated and dried over Na_2SO_4. The solvent was evaporated, and the residue was subjected to preparative TLC [silica gel, hexane/ethyl acetate/acetic acid (200:100:8)] to give 30 mg (33%) of the product as a yellow syrup: ^{1}H NMR ($CDCl_3$), δ 9.15 (1H, d, J = 2.4, NH), 7.99−7.84 (3H, m, ph and nph), 7.82 (1H, d × d, J = 8.9 and 2.7, nph), 7.64−7.46 (3H, m, ph), 7.11 (1H, d × d, J = 9.0 and 1.8, nph), 4.21 (2H, d × q × d, J = 10.1, 7.1 and 1.6, CH_2CH_3), 2.33 (2H, t, J = 6.9, $NHCOCH_2$), 2.28 (2H, t, J = 7.0, CH_2CO_2), 1.74−1.57 (4H, m, $CH_2(CH_2)_2CH_2$), 1.30 (2H, t, J = 7.1, CH_3CH_2).

Preparation of Hapten−Protein Conjugates. Haptens A and B were covalently attached to KLH to be used as immunogens. Haptens A−E were attached to OVA to be used as the coating antigens for serum screening and competitive indirect assay. Haptens A−D were conjugated to HRP to be used as enzyme tracers in direct assay. The method of conjugation used was the active ester method (*10−15*). The procedure for the synthesis of the active ester of hapten A is described below. Other active esters were synthesized according to the same procedure.

Active Ester of Hapten A. Hapten A (115 mg, 0.28 mmol) was dissolved in dichloromethane (5 mL) to which *N*-hydroxysuccinimide (36 mg, 0.3 mmol), *N,N*-dicyclohexylcarbodiimide (63.8 mg, 0.3 mmol), and 4-dimethylaminopyridine (3.4 mg, 0.028 mmol) were added. The mixture was stirred for 3 h and filtered, and the solvent was removed. Chromatography of the resultant oil on silica gel using hexane/ethyl acetate/acetic acid (200:100:8) followed by preparative TLC using the same solvent gave the active ester as a syrup (105 mg, 74%): ^{1}H NMR ($CDCl_3$), δ 8.23 (2H, d, J = 9.1, nph), 7.97 (2H, d × d, J = 14.3 and 7.4, ph), 7.62−7.48 (3H, m, ph), 7.39 (2H, d × d, J = 9.0 and 1.0, nph), 3.44 (1H, m, NH), 3.01 (2H, m, NCH_2), 2.84 (4H, s, succinyl), 2.55 (2H, t, J = 7.1, CH_2CO), 1.67 (2H, qn, J = 7.2, $NHCH_2CH_2$), 1.53−1.34 (4H, m, $(CH_2)_2CH_2CO$).

Active Ester of Hapten B. Yield 42%. ^{1}H NMR ($CDCl_3$), δ 8.28 (2H, d, J = 9.0, nph), 8.00 (2H, d × d, J = 15.0 and 7.7, ph), 7.67−7.48 (3H, m, ph), 7.23 (2H, d × d, J = 9.1 and 1.5, nph), 4.32 (2H, m, CH_2O), 2.85 (4H, s, succinyl), 2.77 (2H, t × d, J = 7.4 and 1.8, CH_2CO_2), 2.16 (2H, qn, J = 6.7, $CH_2CH_2CH_2$).

Active Ester of Hapten C. Yield 40%. ^{1}H NMR ($CDCl_3$), δ 8.25 (2H, d, J = 9.1, nph), 7.89 (2H, d × d, J = 14.1 and 7.1, ph), 7.62−7.48 (3H, m, ph), 7.36 (2H, d × d, J = 8.8 and 1.2, nph), 3.40 (2H, m, NCH_2), 2.85 (4H, s, succinyl), 2.76 (3H, d, J = 11.4, CH_3N), 2.56 (2H, t, J = 7.4, CH_2CO), 1.93 (2H, m, $CH_2CH_2CH_2$).

Active Ester of Hapten D. Yield 34%. ^{1}H NMR ($CDCl_3$), δ 8.25 (2H, d, J = 8.7, nph), 8.14 (2H, d × d, J = 14.5 and 7.6, ph), 7.67−7.46 (3H, m, ph), 7.43(2H, d × d, J = 9.0 and 1.1, nph), 3.42 (2H, sp, J = 6.8, NCH_2), 3.25 (1H, qn, J = 5.9, NH), 2.80 (4H, s, succinyl), 2.48 (2H, t, J = 7.2, CH_2CO).

Active Ester of Hapten E. Yield 46%. ^{1}H NMR ($CDCl_3$), δ 9.22 (1H, d, J = 2.7, NH), 8.02 (1H, s, nph), 8.01 (2H, d × d, J = 15.0 and 7.7, ph), 7.90 (1H, d × d, J = 9.0 and 2.8, nph), 7.69−7.52 (3H, m, ph), 7.20 (1H, d × d, J = 9.0 and 1.7, nph), 4.29 (2H, d × q × d, J = 10.0, 7.1 and 1.1, CH_2CH_3), 2.84 (4H, s, succinyl), 2.66 (2H, t, J =

Figure 4. Synthetic route for hapten E.

6.8, NHCOC$\underline{H}_2$), 2.35 (2H, t, J = 6.7, C$\underline{H}_2$CO$_2$), 1.86−1.56 (4H, m, CH$_2$(C$\underline{H}_2$)$_2$CH$_2$), 1.37 (3H, t, J = 7.1, C$\underline{H}_3$CH$_2$).

The procedures for coupling haptens to the carrier proteins were as follows. To prepare hapten−KLH conjugates (immunogens), KLH (20 mg) was dissolved in 2 mL of borate buffer (0.2 M, pH 8.7) to which 0.4 mL of DMF was added. A solution of an active ester dissolved in 0.1 mL of DMF was then added to the stirred protein solution, and stirring was continued overnight at 4 °C. Hapten−OVA conjugates (coating antigens) were prepared according to the same procedure. Hapten−HRP conjugates (enzyme tracers) were prepared according to the same procedure except that two hapten/protein molar ratios (10 and 50) were employed. The conjugates were separated from the uncoupled haptens by gel filtration (Sephadex G-25) using PBS (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Finally, the eluates were dialyzed in water overnight and then freeze-dried.

Immunization of Rabbits. Female New Zealand white rabbits were immunized with hapten A− or hapten B−KLH. 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 2 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 were continued on a monthly basis. Serum was separated from blood cells 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. Several dilutions of each serum were titrated against the coating antigen (hapten A−and hapten B−OVA, 1000 ng/well) to measure the reactivity of antibodies. The procedure was similar to that for checkerboard assays described below.

Competitive Indirect ELISA. Checkerboard assays, in which several dilutions of sera were titrated against various amounts of the coating antigens (haptens A and B conjugated to OVA), were used to select the most suitable antiserum and to have a rough estimate of appropriate antigen coating and antibody concentrations for competitive assays. The procedure for the checkerboard assays was the same as that for competitive assays (see below) except that addition of pesticide standard or sample is omitted at the competition step.

From the results of the checkerboard assays, two antisera were selected as the most suitable ones (A-1 and B-1). Then, to select the most suitable coating antigen, competitive assays were performed under various combinations of immunoreagents at several concentration levels. The concentrations of the antibodies and the chosen coating antigen were further optimized. Additionally, the tolerance of ELISA to various water-miscible organic solvents used to dissolve pesticides was tested for assay optimization. For this test, standard pesticide solutions were prepared in various concentrations of acetone, acetonitrile, or methanol (10, 20, 40, and 80% in PBS, which became 5, 10, 20, and 40%, respectively, after combination with an equal volume of diluted antisera). The effect of buffering capacity of assay solution on ELISA performance was also studied using different concentrations of phosphate ion in 5% methanol−PBS to dissolve the pesticide (10, 90, 190, and 390 mM phosphate, which became 10, 50, 100, and 200 mM, respectively, after combination with an equal volume of diluted antisera). The influence of pH of the assay solution was also studied.

The procedure of the competitive assay was as follows. All incubations except that for antigen coating were carried out at 22 °C. Microtiter plates were coated with 100 μL/well of a coating antigen in carbonate−bicarbonate buffer (50 mM, pH 9.6) by overnight incubation at 4 °C. The plates were washed four 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, 50 μL/well of standard (serial dilutions of the analyte) or sample in methanol−PBS was added, followed by 50 μL/well of a previously determined antiserum dilution. After incubation for 1 h, the plates were washed and 100 μL/well of a diluted (1/6000) goat anti-rabbit IgG−horseradish peroxidase was added. The mixture was allowed to incubate 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 an appropriate time by adding 50 μL of 2 M H_2SO_4, and the absorbance was read at 450 nm. Competition curves were obtained by plotting absorbance against the logarithm of analyte concentration. Sigmoidal curves were fitted to a four-parameter logistic equation, from which IC_{50} values (concentration at which binding of the antibody to the coating antigen is inhibited by 50%) were determined.

Competitive Direct Assay. Checkerboard assays, in which various dilutions of sera were titrated against various amounts of enzyme tracers (haptens A−E conjugated to HRP), were used to select the most suitable antiserum and enzyme tracer and to have a rough estimate of their appropriate concentrations for competitive assays. The procedure for the checkerboard assays was the same as that for competitive assays (see below) except that addition of pesticide standard or sample is omitted at the competition step. After selection of the most suitable antiserum and enzyme tracer from the checkerboard assays, their quantities for the competitive direct assays were optimized. The tolerance of ELISA to various water-miscible organic solvents at the competition step was tested according to the same procedure as that for the indirect assay. The influence of phosphate ion concentration in

methanol−PBS and pH of assay buffer on ELISA performance was also studied using the same procedure as that for the indirect assay.

The direct assays were performed as follows. All incubations except that for precoating the wells with protein A were carried out at 4 °C for 16 h. Microtiter plates were coated with protein A (5 μg/mL, 100 μL/well) in carbonate−bicarbonate buffer (50 mM, pH 9.6) by overnight incubation at 4 °C. The plates were washed four times with PBST and coated with 100 μL/well of the antiserum dilutions in PBST for 1 h. After another washing step, pesticide standard (serial dilutions of the analyte in methanol−PBS) or sample was added (50 μL/well) followed by 50 μL/well of an enzyme tracer previously diluted with PBS. After incubation 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 the absorbance was read at 450 nm. Competition curves were obtained according to the same procedure as that of the indirect assays.

Determination of Cross-Reactivities. Several organophosphorus pesticides were tested for cross-reactivity using both the indirect and direct ELISA procedures described above. The cross-reactivity values were calculated as follows: (IC_{50} of EPN/IC_{50} of compound) × 100.

Analysis of Spiked Samples. *Analysis by ELISA.* Solutions of EPN in methanol for the fortification of vegetable and rice samples were prepared at 100, 200, 500, and 1000 ng/mL. To 1 g of the finely chopped leaves of the vegetables and the grains of rice grown pesticide-free was added 1 mL of a fortification solution. After setting aside for 24 h, the vegetable leaves were incubated in 5 mL of methanol for 10 min with four vigorous shakes and then filtered through Whatman no. 1 filter paper. The container and the residues were rinsed with 5 mL of methanol and filtered, and the filtrate was combined with the previous filtrate. Methanol was evaporated to dryness under reduced pressure, and the residue was resuspended in 10 or 100 mL of 5% methanol−50 mM PBS. Because the indirect ELISA showed much higher reproducibility in recovery values than the direct ELISA, recovery was determined only by the indirect ELISA.

Analysis by GC. The spiked samples were also analyzed by gas chromatography at Korea Advanced Food Research Institute. The sample preparation procedure involved homogenization of spiked samples with acetone/water (4:1), filtration, extraction of the filtrate with petroleum ether/dichloromethane (1:1), concentration of the organic phase, and elution on Florisil SPE with acetone/hexane (1:4). In the case of rice, partition between hexane and acetonitrile was practiced before SPE to eliminate lipid materials. Quantification of EPN was carried out by GC using a DB-17 column (30 m, diameter = 320 μm, film thickness = 0.25 μm) and μ-ECD as the detector. The injection port temperature was 260 °C, and the detector temperature was 280 °C. Oven temperature was programmed from 100 °C (held for 2 min) to 280 °C at a rate of 15 °C/min. He at 1.5 mL/min was used as carrier gas.

RESULTS AND DISCUSSION

Hapten Selection and Synthesis. A suitable hapten for immunization should preserve the structure of the target compound as much as possible. EPN is a member of the phosphonothioate OP pesticides, a rare family of OP pesticides. It has a phenyl, a *p*-nitrophenoxy group, and an ethoxy group attached to a phosphorus atom (**Figure 1**). Because the phenyl and *p*-nitrophenoxy group are unique to EPN and the ethoxy group is common among many OP pesticides, to achieve a high specificity in EPN ELISA, it was desirable to synthesize haptens that have a bridge at the phosphorus atom in replacement of the ethoxy group and preserve the aromatic ring structures. Haptens A and B used as the immunizing haptens have such structural features. Haptens to be used as competitors (coating antigens in indirect ELISAs and enzyme tracers in direct ELISAs) should also be similar in structure to the target compound; however, structural similarity does not necessarily guarantee high sensitivity in ELISA (*11, 16*). Therefore, it is desirable to use as many haptens as possible and to select the most suitable one. Haptens C−E were synthesized for that reason.

McAdam et al. (*17, 18*) developed a method for synthesizing haptens for phosphorothioate OP pesticides with a bridge at the thiophosphate group, which was applied to the synthesis of haptens for the development of ELISAs of several OP pesticides (*7, 8*). This method requires a synthetic route involving seven steps including protection and deprotection of both amino and carboxyl groups of the bridge reagent. Recently, we developed a simpler general method, which requires only two steps (*9*). It involves the reaction of *O*- alkyldichlorothiophosphate with a phenol and K_2CO_3 in acetonitrile and the reaction of the substitution product with an aminocarboxylic

Table 1. Standard Curve Characteristics[a] of the Indirect ELISA[b] with Different Combinations of Antisera and Coating Antigens

antiserum	dilution	coating Ag (ng/well)	*A*	*B*	*C* (ng/mL)	*D*
A-1	1/40000	hapten A (25)	1.027	0.658	887	0.613
	1/10000	hapten B (50)	1.196	0.569	1933	0.172
	1/5000	hapten C (100)	0.974	0.548	1926	0.090
	1/5000	hapten D (50)	0.965	0.630	393	0.311
	1/5000	hapten E (400)	1.048	0.444	429	0.516
B-1	1/1000	hapten A (100)	0.901	0.383	50	0.107
	1/2000	hapten B (25)	1.109	0.588	80	0.130
	1/1000	hapten C (50)	1.019	0.575	15	0.122
	1/1000	hapten C (200)	1.102	0.435	21	0.160

[a] Maximal absorbance (*A*), slope (*B*), IC_{50} (*C*), and minimal absorbance (*D*) are values from the four-parameter curve fit program. Data are the means of duplicate. [b] Assay conditions: goat anti-rabbit IgG-HRP diluted 1/6000.

Table 2. Influence of Organic Cosolvent, PBS, and pH of the Assay Solution on Indirect Competitive ELISA[a]

variables		*A*	*B*	*C* (ng/mL)	*D*	time (min)
methanol (%)	5	1.110	0.672	13.1	0.028	10
	10	1.159	0.451	10.5	0.001	10
	20	1.138	0.378	7.0	0.008	12
	40	1.194	0.315	5.6	0.234	12
acetone (%)	5	1.027	0.364	17.1	0.070	12
	10	0.962	0.518	41.4	0.084	13
	20	1.238	0.124	36.0	0.132	13
	40[b]					
acetonitrile (%)	5	1.005	0.267	29.4	0.013	14
	10	1.068	0.206	11.3	0.050	15
	20	1.016	0.247	0.04	0.127	18
	40[b]					
PBS[c] (mM)	10	1.306	0.417	10.1	0.063	12
	50	1.307	0.422	7.3	0.076	12
	100	1.317	0.322	15.9	0.068	13
	200	1.227	0.294	31.2	0.105	14
	500	1.069	0.396	56.9	0.021	14
pH	6.0	0.865	0.303	16.2	0.009	10
	6.5	0.852	0.356	13.9	0.008	10
	7.0	0.836	0.427	8.5	0.021	10
	7.4	1.018	0.345	7.7	0.003	12
	8.0	0.959	0.365	11.2	0.007	12
	8.5	0.944	0.373	13.6	0.012	12

[a] Assay conditions: antiserum to hapten B−KLH, diluted 1/1000 with 10 mM PBST; coating antigen, hapten C−OVA, 50 ng/well; goat anti-rabbit IgG-HRP diluted 1/6000. Maximal absorbance (*A*), slope (*B*), IC_{50} (*C*), and minimal absorbance (*D*) are values from the four-parameter sigmoidal fitting. Data are the means of triplicate. [b] Data fitting was impossible due to poor color development. [c] Final concentration of phosphate ions of the competition buffer containing 138 mM NaCl and 2.7 mM KCl.

Table 3. Standard Curve Characteristics[a] of the Indirect ELISA[b] with Different Combinations of Antisera and Enzyme Tracers

antibody (dilution)	dilution	enzyme tracer	molar ratio	dilution	*A*	*B*	*C* (ng/mL)	*D*	time (min)
A-1	1:5000 (10:1, 1/10000)	hapten A	10:1	1:10000	1.168	0.523	8.8	0.026	8
	1:5000 (10:1, 1/20000)	hapten A	10:1	1:20000	1.123	0.427	11.0	0.011	10
	1:5000 (50:1, 1/20000)	hapten A	50:1	1:20000	1.202	0.572	41.2	0.143	5
	1:10000 (50:1, 1/20000)	hapten A	50:1	1:20000	1.011	0.447	29.2	0.045	8
	1:5000 (10:1, 1/100)	hapten B	10:1	1:100	0.898	0.382	98.7	0.095	15
	1:5000 (50:1, 1/200)	hapten B	50:1	1:200	1.097	0.500	38.7	0.085	20
	1:1000 (10:1, 1/100)	hapten C	10:1	1:100	1.101	0.615	33.3	0.098	15
	1:1000 (50:1, 1/100)	hapten C	50:1	1:100	1.194	0.537	72.9	0.090	12
B-1	1:500 (10:1, 1/50)	hapten A	10:1	1:50	0.838	0.797	97.2	0.166	17
	1:500 (50:1, 1/50)	hapten A	50:1	1:50	0.704	0.549	95.6	0.032	10
	1:500 (10:1, 1/50)	hapten B	10:1	1:50	1.000	0.434	25.8	0.481	15
	1:500 (50:1, 1/50)	hapten B	50:1	1:50	1.111	0.579	56.2	0.472	15

[a] Maximal absorbance (*A*), slope (*B*), IC_{50} (*C*), and minimal absorbance (*D*) are values from the four-parameter curve fit program. Data are the means of duplicates. [b] Assay conditions: precoating with protein A (0.5 μg/well); blocking with 1% gelatin; antiserum diluted with 50 mM carbonate−bicarbonate buffer (pH 9.6); enzyme tracer diluted with 10 mM PBS.

Table 4. Influence of Organic Cosolvent, PBS, and pH of the Assay Solution on Direct Competitive ELISA[a]

variables		*A*	*B*	*C* (ng/mL)	*D*	time (min)
methanol (%)	5	1.036	0.679	14.1	0.087	10
	10	0.978	0.675	21.0	0.067	10
	20	1.019	0.450	16.6	0.058	15
	40	0.961	0.404	20.8	0.028	17
acetone (%)	5	1.131	0.486	87.3	0.040	15
	10	1.033	0.603	153.0	0.094	20
	20[b]					
	40[b]					
acetonitrile (%)	5	0.999	0.653	96.0	0.088	15
	10	0.936	0.588	98.3	0.093	21
	20[b]					
	40[b]					
PBS[c] (mM)	10	1.022	0.485	16.4	0.058	8
	50	1.035	0.606	14.3	0.087	10
	100	1.061	0.599	20.4	0.082	10
	200	0.980	0.480	17.6	0.055	15
	500	0.974	0.568	22.1	0.056	25
pH	5.0	1.003	0.527	16.1	0.048	10
	6.0	0.974	0.556	19.8	0.042	10
	7.4	1.027	0.604	17.8	0.087	10
	8.0	0.966	0.572	18.6	0.052	11
	9.0	0.951	0.539	16.2	0.049	12
	10.0	0.907	0.474	19.7	0.043	14

[a] Assay conditions: precoating with protein A (0.5 μg/well); antiserum to hapten A−KLH, diluted 1/5000 with 50 mM carbonate−bicarbonate buffer (pH 9.6); enzyme tracer, hapten A−HRP (prepared at molar ratio 10:1, diluted 1:10000). Maximal absorbance (*A*), slope (*B*), IC_{50} (*C*), and minimal absorbance (*D*) are values from the four-parameter sigmoidal fitting. Data are the means of triplicates. [b] Data fitting was impossible due to poor color development. [c] Final concentration of phosphate ions of the competition buffer containing 138 mM NaCl and 2.7 mM KCl.

acid (not protected) and KOH in methanol (see **Figure 2** for an example). In addition to the advantages of reduction in cost and time, this method allows attachment of secondary as well as primary amino acids as the spacer arm (hapten C). We found that this method can be successfully applied to the synthesis of haptens of EPN, which is a phosphonothioate OP pesticide. This method worked well for the synthesis of haptens A, C, and D, which have an aminocarboxylic acid as the bridge. The reaction time was relatively short: 20 min and a few minutes for the first and second reactions, respectively. Hapten B could be synthesized by adopting the synthetic pathway similar to that developed by Manclus et al. (*8*). Hapten E was synthesized by a modification of the synthetic pathway we developed previously (*14*). All of the carboxylic acid haptens could be converted to the succinimide esters, which are active esters for coupling haptens to carrier proteins.

Competitive Indirect ELISA. To select the most suitable antiserum, the titer values of sera were measured, and antiserum A-1 raised against hapten A and antiserum B-1 raised against hapten B were selected. To select the most suitable immunoreagents (antiserum and coating antigen) and their appropriate concentrations, competitive assays were performed under various combinations of serum dilutions and various amounts of the coating antigens. **Table 1** presents the results of the competitive assays that showed a relatively high performance. Of the two antisera, antiserum B-1 showed a better performance. It was useless in combinations with coating antigen haptens D and E due to poor color development in the assay, but was better than antiserum A-1 in combinations with coating antigen haptens A−C. Among the three usable coating haptens, hapten C, which is homologous to the immunizing hapten in the position of the bridge group but heterologous in the structure of the bridge group, showed the highest sensitivity. Hapten heterology is commonly used to eliminate problems associated with the strong affinity of the antibodies to the bridge that leads to no or poor inhibition by the target compound (*11, 13*), which appears to work in this indirect ELISA. The optimum condition selected was the combination of antiserum B-1 from hapten B−KLH (second boost) diluted 1:1000 and the coating antigen hapten C−OVA at 50 ng/well.

Because organic solvents are commonly used for extraction and/or solid phase cleanup in the analysis of nonpolar pesticide residues in food and environmental samples, it is desirable to use organic solvent as a cosolvent of assay solution. Then, it is necessary to assess the effect of organic solvents on ELISA performance at the competition step. The effects of solvents (acetone, acetonitrile, and methanol) on the ELISA system were evaluated by preparing standard curves in buffers containing various amounts of organic solvent. The results are presented in **Table 2**. The speed of color development was slower and sensitivity was lower in the presence of acetone and acetonitrile compared with that in the presence of methanol. Accordingly, we selected methanol as the most suitable cosolvent. Several other workers reached the same conclusion in that methanol caused the least negative effect on the performance of ELISA (*19−22*). Among the four concentrations of methanol, 5% methanol was selected as the most suitable on the basis of the dynamic response (slope) that was the highest.

Table 5. Cross-Reactivity of Organophosphorus Pesticides Determined by Indirect and Direct Competitive ELISA[a]

Compound	Structure	Indirect ELISA		Direct ELISA	
		IC_{50} (ng/mL)[b]	CR(%)[c]	IC_{50} (ng/mL)[b]	CR(%)[c]
EPN		6.1	100	8.1	100
Oxon of EPN		1940	0.31	353	2.9
Diazinon		1050	0.5	1570	0.5
Oxon of diazinon		ni	0	ni	0
Pyrimophos-ethyl		ni	0	ni	0
Chlorpyrifos		ni	0	ni	0
Parathion-ethyl		502	1.2	103	5.9
Isofenphos		ni	0	ni	0
Bromophos-ethyl		ni	0	ni	0
Fenthion		ni	0	ni	0
Parathion-methyl		402	1.5	ni	0
4 - Nitrophenol		ni	0	ni	0

[a] Assay conditions were the same as those described in the caption of **Figure 5** (indirect ELISA) or **6** (direct ELISA). [b] "ni" means no inhibition (cross-reactivity lower than 0.5%). [c] CR (cross-reactivity) (%) = (IC_{50} of EPN/IC_{50} of other compound) × 100.

Table 6. Recovery of EPN Spiked into Vegetables and Rice[a]

sample	fortified concn (ng/g)	recoveries by indirect ELISA 10 mL[b]	recoveries by indirect ELISA 100 mL[b]	GC
lettuce	100	54 ± 5.9	117 ± 5.1	91 ± 0.3
	200	84 ± 3.7	93 ± 2.7	89 ± 1.1
	500	56 ± 4.3	60 ± 2.3	103 ± 1.1
	1000	70 ± 7.5	70 ± 4.9	107 ± 2.6
rice	100	118 ± 7.5	164 ± 8.1	107 ± 1.7
	200	100 ± 4.7	134 ± 9.9	107 ± 0.6
	500	80 ± 4.1	103 ± 5.1	99 ± 1.1
	1000	57 ± 3.1	77 ± 5.6	103 ± 1.4
kale	100	37 ± 4.7	113 ± 4.2	90 ± 2.3
	200	56 ± 10	78 ± 4.8	96 ± 0.1
	500	65 ± 7.4	114 ± 9.0	111 ± 1.0
	1000	103 ± 5.1	87 ± 5.2	104 ± 0.3

[a] Assay conditions were the same as those described in the caption of **Figure 5**. ELISA data are the means of triplicates for five samples at each spike level. GC data are the means of triplicates for single samples at each spike level. [b] Volume of final extract.

Table 2 also presents the effect of the phosphate ion concentration on ELISA characteristics at the competition step. Increasing the concentration of phosphate ions caused an initial improvement followed by a decline in assay sensitivity. The optimum concentration selected was 50 mM phosphate, which showed the lowest IC_{50} value.

Table 2 also presents the effect of pH of assay solution on ELISA. The physiological pH, pH 7.4, was selected as the optimum for the assay.

Figure 5 shows a typical inhibition curve obtained under these optimized conditions. The IC_{50} value of the assay was 5.6 ng/mL with a detection limit of 0.2 ng/mL (20% inhibition) (*27*−*29*).

Direct Competitive Assay. To select the most suitable antiserum and enzyme tracer, and their concentrations for direct (antibody coated) ELISA, various combinations of antisera and enzyme tracers at several concentration levels were tested. **Table 3** shows the results of the assays that showed a relatively high performance. The optimum combination selected was a completely homologous one, antiserum A-1 from hapten A−KLH (third boost) and tracer hapten A−HRP prepared at a 10:1 hapten/protein molar ratio. We repetitively observed that direct ELISA with homologous combination shows a better performance compared to those with heterologous ones (*12, 23*). It appears that, unlike the strong attraction between the antibody and homologous coating antigen in the indirect ELISA leading to lower sensitivity, the attraction between the antibody and the homologous enzyme tracer is not particularly strong. The direct

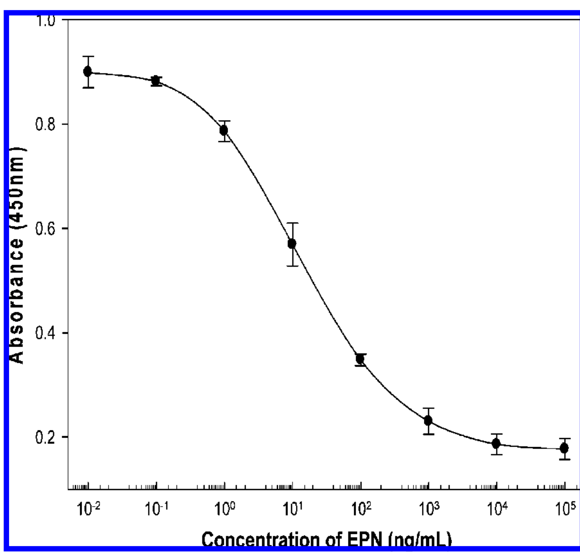

Figure 5. ELISA standard curve for EPN by indirect competitive assay. Assay conditions: antiserum B-1 raised against hapten B–KLH, diluted 1/1000; coating antigen hapten C–OVA, 50 ng/well; goat anti-rabbit IgG-HRP, 1/6000. Each point represents the mean of 16 determinations. Vertical bars indicate ± standard deviation about the mean.

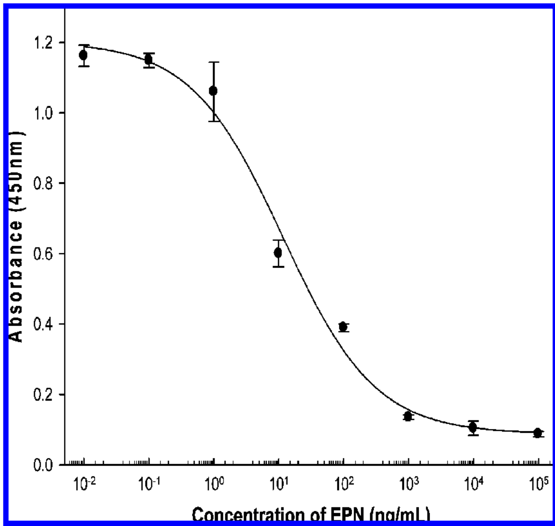

Figure 6. ELISA standard curve for EPN by direct competitive assay. Assay conditions: precoating agent protein A, 0.5 μg/well; antiserum A-1 raised against hapten A–KLH, diluted 1/5000; enzyme tracer hapten A–HRP (prepared at molar ratio of 10:1, diluted 1:10000). Each point represents the mean of 16 determinations. Vertical bars indicate ± standard deviation about the mean.

ELISA with homologous combination was further optimized with regard to the dilution of antiserum and enzyme tracer.

The effects of solvents (acetone, acetonitrile, and methanol) on the direct ELISA system were evaluated according to the same procedure as for the indirect ELISA. The results are presented in **Table 4**. As observed in the indirect assay, the speed of color development at the competition step decreased with increasing concentration of acetone and acetonitrile, causing difficulty in the establishment of standard curves. Such an effect was not observed with methanol. IC_{50} values in the presence of acetone and acetonitrile were much higher than those in the presence of methanol (**Table 4**). Therefore, methanol was the most suitable cosolvent for the assay, in agreement with the results of several other studies (*24–26*). The optimum concentration of methanol selected was 5%, which showed the lowest IC_{50} value and the highest dynamic response.

Table 4 also presents the effect of the concentration of the phosphate ion (buffer) in the competition solution on ELISA characteristics. The optimum concentration selected was 50 mM phosphate, which showed the lowest IC_{50} value and the highest dynamic response.

Table 4 also shows the effect of pH of assay solution on ELISA characteristics. The physiological pH, pH 7.4, was selected as the best one.

Figure 6 shows a typical inhibition curve obtained under the optimized condition. The IC_{50} value of the assay was 8.4 ng/mL with a detection limit of 0.9 ng/mL (20% inhibition) (*27–29*).

Cross-Reactivity Studies. Several organophosphorus pesticides were tested for cross-reactivities. **Table 5** shows the cross-reactivity that was found by both the indirect and direct assays, expressed in percentage of the IC_{50} of EPN. The interference to the assays was negligible except with parathion-ethyl in only the direct assay, which showed a cross-reactivity of 5.9%. The appreciable cross-reactivity of the antibodies for this pesticide is understandable, because EPN and parathion-ethyl have *p*-nitrophenoxy group in common. It is remarkable that neither the oxidized form (oxon) nor the main degradation product of EPN (4-nitrophenol) shows any significant cross-reactivity.

Recovery Studies. Three kinds of crops were spiked with EPN and analyzed by the indirect ELISA. The direct ELISA showed poor reproducibility in recovery values and, thus, was excluded in recovery studies. Samples were prepared by extraction of 1 g of spiked crops with methanol, followed by evaporation of the solvent and dissolution of the residue in 10 or 100 mL of 5% methanol–PBS. Recoveries are presented in **Table 6**. Recoveries of samples prepared in 10 mL of methanol–PBS were generally underestimated. Recoveries from the samples prepared in 100 mL of methanol–PBS were more satisfactory, suggesting that dilution of sample is an effective means to circumvent matrix effects. Recovery values determined by ELISA were less satisfactory in accuracy than those by GC. Because ELISA is a much more rapid method compared to GC, the choice between ELISA and GC would depend on the choice between rapidity and accuracy. In conclusion, the indirect ELISA developed in this study can determine EPN residue in vegetable samples rapidly and easily by following the simple and rapid extraction procedure and the ELISA procedure used in this study.

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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