JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of an Enzyme-Linked Immunosorbent Assay for the Detection of the Fungicide Fenarimol

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To develop an enzyme-linked immunosorbent assay for the fungicide fenarimol, two synthesized haptens, haptens-1 and -2, and the purchased 4,4′-DDA were conjugated to carrier proteins (BSA, KLH, and OVA). Polyclonal antibodies raised against hapten-1,2−KLH conjugates in rabbits and the coating antigens of hapten-1,2−BSA conjugates, hapten-2−OVA conjugate, and 4,4′-DDA−BSA conjugate were screened and selected for the homologous and/or heterologous ELISA formats. Two competitive indirect ELISAs were selected: assays I and II. The optimized ciELISAs of assays I and II showed average IC_{50} values of fenarimol of 5.4 and 9.4 ng/mL, detection ranges of 1.1−25.9 and 1.1−82.7 ng/mL, and lowest detection limits of 0.3 and 0.3 ng/mL, respectively. The cross-reactivities with several structurally related compounds indicated the importance of the steric fitness in the antigen−antibody interaction. Recoveries of fenarimol from apple and pear samples spiked with the analyte by assay I were in the range of 93−113% by simple extraction, concentration, and dilution. This assay could be a convenient and supplemental analytical tool for monitoring fenarimol residues in environmental and agricultural samples.

KEYWORDS: Fenarimol; ELISA; polyclonal antibody; homologous; heterologous; residue analysis

INTRODUCTION

The fungicide fenarimol, (±)-α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidinemethanol, is extensively used for the control of powdery mildew infesting apple, pear, cucumber, and strawberry, anthracnose infesting red pepper, and downy mildew infesting onion. Fenarimol is known to be unstable photochemically and immobile in soil and to have a very low toxicity to mammals orally and dermally (*1*).

In the conventional analysis of fenarimol residues, the matrix ground and extracted with a mixture of acetone/dichloromethane (1:1, v/v) is filtered, and the filtrate is cleaned up and concentrated (*2*). The analysis is mainly carried out by HPLC equipped with a diode array detector (DAD), gas chromatography with an electron capture detector (GC-ECD), gas chromatography−mass spectrometry (GC-MS), and high-performance liquid chromatography−mass spectrometry (HPLC-MS). As these instrumental methods require many cleanup procedures prior to determination, they are laborious and time-consuming and require sophisticated equipment available only in well-equipped laboratories. In contrast, immunoassays are simple, sensitive, fast, and cost-effective analytical methods with high throughput for detection of pesticides and toxins (*3*−*10*). The purpose of this investigation is to develop an enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies to detect the fenarimol residues in agricultural and environmental samples simply and conveniently.

MATERIALS AND METHODS

Chemicals. Fenarimol and nuarimol of analytical grade were a gift from Kyung Nong Corp. (Seoul, Korea). Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), ovalbumin (OVA), goat anti-rabbit immunoglobulin G (IgG) peroxidase conjugate as a second antibody, and Freund's complete and incomplete adjuvants were all purchased from Sigma Chemical Co. (St. Louis, MO). All organic compounds used for hapten syntheses were purchased from Aldrich Chemical Co. (Milwaukee, WI). DDT and its analogues were obtained from Rural Development Administration (Suwon, Korea). Bis(4-chlorophenyl)acetic acid (4,4′-DDA) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Flurprimidol and flutriafol of analytical grade were purchased from Chem Service (West Chester, PA).

Instrument. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of hapten-1 were obtained on a 600 MHz NMR spectrometer, DMX 600 (Bruker). To simplify the interpretation of NMR spectra, two-dimensional (2D) NMR techniques, $^{1}H-^{1}H$ correlation spectroscopy (COSY), and heteronuclear multiple quantum coherence (HMQC) were used. For the ^{1}H NMR spectrum of hapten-2, a 300 MHz NMR spectrometer, DPX 300 (Bruker), was used. Fast atom bombardment mass (FAB-MS) spectra using 3-nitrobenzyl alcohol as a matrix were obtained on a JEOL four sector tandem mass spectrometer, JMS-HX/HX110A (JEOL), at Korea Basic Science Institute. ELISA was

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10.1021/jf049425l CCC: $27.50 © 2004 American Chemical Society
Published on Web 11/05/2004

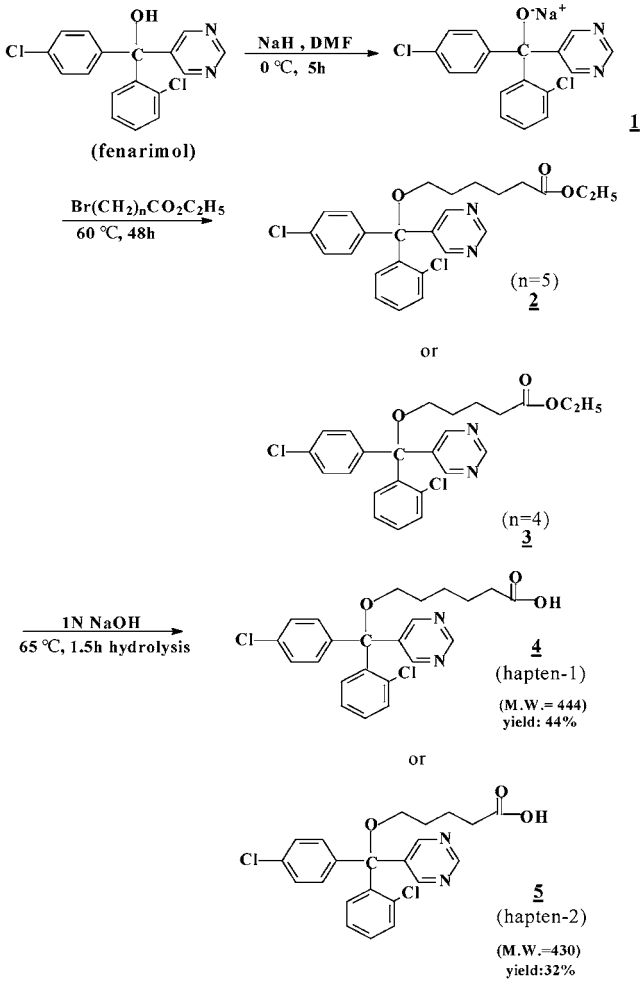


Figure 1. Procedure for the synthesis of haptens.

performed on 96-well microtiter plates (Nunc-Immuno plate, MaxiSorp surface, Roskilde, Denmark) and read spectrophotometrically with a microplate reader, Bio-Rad model 550 (Hercules, CA).

Buffer Solutions. Buffer solutions used for the immunoassay include normal strength phosphate-buffered saline (1× PBS; 8 g/L NaCl, 0.2 g/L KH_2PO_4, 1.2 g/L Na_2HPO_4, and 0.2 g/L KCl, pH 7.5), PBST (1× PBS containing 0.05% Tween 20, pH 7.5), 0.1× PBST (0.1× PBS containing 0.05% Tween 20), carbonate buffer (1.59 g/L Na_2CO_3, 2.93 g/L $NaHCO_3$, pH 9.6), and 0.05 M borate buffer (19.1 g/L $Na_2B_4O_7 \cdot 10H_2O$, pH 8).

Hapten Synthesis and Verification. *Hapten-1 (Compound **4**), (±)-6-[(2-Chlorophenyl)-(4′-chlorophenyl)pyrimidin-5-yl-methoxy]hexanoic Acid.* Sodium hydride (NaH) was added slowly to fenarimol (99.36 mg, 0.3 mmol) dissolved in 5 mL of dry dimethylformamide (DMF) at 0 °C until no more hydrogen gas was generated. The mixture was stirred at room temperature for 5 h to form the sodium salt (compound **1**). Then, ethyl 6-bromohexanoate (167.34 mg, 0.75 mmol) in 2 mL of DMF was reacted with the resulting oxygen nucleophile. After 48 h of stirring at 60 °C, DMF was removed on a rotary evaporator under reduced pressure to obtain compound **2**, which was hydrolyzed with 1.5 mL of 1 N NaOH at 65 °C for 1.5 h. The reaction mixture was cooled and poured into 50 mL of distilled water. The aqueous solution was washed with 50 mL of ethyl acetate to remove some impurities, acidified to pH 2 with 6 N hydrochloric acid, and then extracted with ethyl acetate (2 × 50 mL). The combined organic layer was dried over anhydrous sodium sulfate and concentrated on a rotary evaporator under reduced pressure. The residue was purified by silica gel flash chromatography using ethyl acetate/*n*-hexane as an eluent to obtain hapten-1 (compound **4**), a yellow solid (**Figure 1**): ^{1}H NMR (DMSO-d_6) δ 9.06 (s, 1H, pyrimidine H), 8.76 (s, 2H, pyrimidine H), 7.38−7.75 (m, 8H, 2Ar), 2.94 (m, 2H, $O\underline{CH_2}CH_2CH_2CH_2CH_2COOH$), 2.03 (t, 2H, $OCH_2CH_2CH_2CH_2\underline{CH_2}COOH$), 1.57 (2H, $OCH_2\underline{CH_2}CH_2CH_2CH_2COOH$), 1.41 ($OCH_2CH_2CH_2\underline{CH_2}CH_2COOH$), 1.27 (2H, $OCH_2CH_2\underline{CH_2}CH_2CH_2COOH$); ^{13}C NMR (600 MHz, DMSO-d_6) δ 177.568, 157.094, 156.062, 139.572, 138.073, 136.658, 132.975, 132.797, 132.219, 130.849, 130.769, 129.844, 128.768, 127.804, 83.216, 64.115, 29.279, 25.818, 25.395; low FAB (+)-MS, *m*/*z* 445 $[M + H]^+$.

*Hapten-2 (Compound **5**), (±)-5-[(2-Chlorophenyl)-(4′-chlorophenyl)-pyrimidin-5-yl-methoxy]pentanoic Acid.* NaH was added slowly to fenarimol (99.36 mg, 0.3 mmol) dissolved in 5 mL of dry DMF at 0 °C until no more hydrogen gas was generated. The mixture was stirred at room temperature for 5 h to form the sodium salt (compound **1**). Ethyl 5-bromovalerate (156.8 mg, 0.75 mmol) in 2 mL of DMF was reacted with the resulting oxygen nucleophile. After 48 h of stirring at 60 °C, DMF was removed on a rotary evaporator under reduced pressure (compound **3**). The subsequent hydrolysis and purification procedures of compound **3** were the same as those for hapten-1 (**Figure 1**): ^{1}H NMR ($CDCl_3$) δ 9.11 (s, 1H, pyrimidine H), 8.84 (s, 2H, pyrimidine H), 7.18−7.37 (m, 8H, 2Ar), 3.06 (t-like, 2H, $O\underline{CH_2}CH_2CH_2CH_2COOH$), 2.22 (m, 2H, $OCH_2CH_2CH_2\underline{CH_2}COOH$), 1.74 (m, 4H, $OCH_2\underline{CH_2}\underline{CH_2}CH_2COOH$); low FAB (+)-MS, *m*/*z* 431 $[M + H]^+$.

Conjugation of Carboxylic Acid Haptens to Carrier Proteins. Haptens were conjugated to carrier proteins (BSA, KLH, and OVA) using the activated ester method (*11*). That is, hapten-1 was coupled to KLH and BSA. Meanwhile, hapten-2 was conjugated to KLH, BSA, and OVA. Instead, 4,4′-DDA was conjugated only to BSA. The carboxylic acid haptens (0.04 mmol, each) were dissolved in 0.2 mL of dry DMF with equimolar *N*-hydroxysuccinimide (NHS) and a 10% molar excess of 1,3-dicyclohexylcarbodiimide. After 5 h of stirring at 22 °C, the precipitated dicyclohexylurea was removed by filtration, and the resulting activated ester was added slowly to the protein solution (10 mg of protein in 1 mL of 0.05 M borate buffer of pH 8) with vigorous stirring. The reaction mixture was stirred gently at 4 °C for 24 h to complete the conjugation and then dialyzed exhaustively against normal-strength PBS, which was changed twice a day for 5 days. Finally, the conjugates were dispensed into 2 mL cryogenic vials, stored at −80 °C, and used when needed.

Protein Determination. The protein contents of the hapten−protein conjugates were determined spectrophotometrically at 595 nm according to the Bio-Rad protein assay based on the method of Bradford (*12*).

Determination of the Coupling Density. The free amino groups of the hapten−protein complex were determined according to a modification of the trinitrobenzenesulfonic acid (TNBSA) method (*13*). That is, 0.5 mg/mL of protein or 0.5 mg/mL of protein conjugate solution in 1× PBS, 1.0 mL of carbonate buffer (1.59 g/L Na_2CO_3, 2.93 g/L $NaHCO_3$, pH 9.6), and 1.0 mL of 0.1% TNBSA was reacted at 40 °C for 2 h. After that, 1.0 mL of 10% sodium dodecyl sulfate (SDS) was added, and the reaction was terminated with 0.5 mL of 1 M HCl. After thorough mixing, the absorbance was measured at 335 nm. The coupling density was estimated by comparing the absorbance with the corresponding values of hapten-free proteins.

Immunization. Female New Zealand white rabbits weighing 3 kg were used for raising polyclonal antibodies. Routinely, 100 μg (protein equivalent) of each immunogen (hapten−KLH conjugate) in 0.5 mL of 0.85% saline was thoroughly emulsified with an equal volume of Freund's adjuvant. The emulsion was subcutaneously injected at five different sites on the neck and back of each rabbit. Freund's complete adjuvant was used in the first injection, and Freund's incomplete adjuvant was used for the subsequent boost injections. Boosts were given at 3-week intervals by the same method. On the seventh day after each boost, ∼10 mL blood samples were taken from an ear vein to check the titer of the antisera. The blood samples were allowed to coagulate for ∼2 h at room temperature and then kept in a refrigerator overnight. The serum was decanted and centrifuged at 800*g*, and the supernatant was dispensed into cryogenic vials and stored at −80 °C. Boosting injections and bleedings thereafter were done five times.

Checkerboard Titration. A checkerboard titration (*14*) was performed for the antiserum collected from each rabbit by the homologous indirect ELISA to select the antisera showing an absorbance of 0.5−1.0. The coating antigen concentration ranged from 0.01 to 1 μg/mL, and the antiserum dilution was between 1:16000 and 1:256000. Rabbits A−C were immunized against hapten-1−KLH and rabbits D−F against hapten-2−KLH (**Table 1**). Titers of the antisera raised by six rabbits

Table 1. Screening of Antisera from Rabbits and Coating Antigens in a Competitive Indirect ELISA by Percent Inhibition of Color Development

		concn of fenarimol (ng/mL) for coating antigen					
		hapten-1–BSA[a]		hapten-2–BSA		4,4′-DDA–BSA	
immunogen	rabbit	10	1000	10	1000	10	1000
hapten-1–KLH	A	0.5	21.0	18.0[b]	46.0	13.0	61.0
	B	11.3	33.8	0	29.3	–40.0	–260.0
	C	0	0.4	2.9	20.7	40.0	60.0
hapten-2–KLH	D	3.4	30.0	1.7	19.1	0.0	–25.0
	E	21.8	42.0	4.0	24.9	19.7	83.1
	F	1.3	67.0	4.0	46.7	19.5	91.2

[a] No titer was observed using BSA alone as a coating antigen. [b] % inhibition = $[(A - B)/A] \times 100$, where A is the absorbance of negative control and B is the absorbance of fenarimol.

were evaluated with hapten-1−BSA and hapten-2−BSA conjugates as coating antigens by going through the following process. The 96-well microtiter plates were coated with 100 μL/well of the hapten−BSA conjugates in a carbonate buffer and allowed to stand overnight at 4 °C. After coating, the plates were washed five times with $^1/_{10}$ strength PBST (0.1× PBS with 0.05% Tween 20) and thoroughly tapped dry. Sites not coated with the conjugate were blocked with 200 μL/well of 3% (w/v) skim milk in 1× PBS. After incubation at 37 °C for 1 h, the plates were washed again as described above. To the wells was added 100 μL of an anti-fenarimol antiserum diluted with 1× PBS, and the plates were incubated at room temperature for 1 h. After the plate had been washed, 100 μL of a secondary antibody conjugated to the anti-rabbit IgG−horseradish peroxidase diluted 1:10000 with 1× PBST was added, and the plate was incubated for 1 h at room temperature. The plate was washed, and 100 μL of a substrate solution was added to each well. After 15 min at room temperature, the reaction was stopped by adding 50 μL of 4 N sulfuric acid. The absorbance was read in a dual-wavelength mode at 450 nm using a reference wavelength of 655 nm.

Competitive Inhibition ELISA. A competitive inhibition ELISA was performed to assess the specificity of the antibody to free fenarimol and the cross-reactivities of structurally related compounds to the antibody according to the method of Voller et al. (*15*) as modified by Harrison et al. (*16*). Microplates coated with 100 μL/well of hapten-1−BSA conjugate, hapten-2−BSA conjugate, hapten-2−OVA conjugate, or 4,4′-DDA−BSA conjugate in a carbonate buffer were allowed to stand overnight at 4 °C. After the plates had been washed five times with a washing solution (0.1× PBS with 0.05% Tween 20) and thoroughly tapped dry, sites not coated with the conjugate were blocked with 200 μL/well of 3% (w/v) skim milk in 1× PBS to minimize nonspecific binding of the antibodies to the plates. After incubation at 37 °C for 1 h, the plates were washed as described above. To this plate were added 50 μL/well of serial concentrations of the fenarimol standard from 5000 to 0.0128 ng/mL in 1× PBS and 50 μL/well of each antiserum diluted with 1× PBS. The plate was allowed to stand at room temperature for 1 h, and the subsequent procedures were the same as the above. As an alternative ELISA (assay II), a coating temperature of 37 °C instead of 4 °C and a coating time of 2 h instead of overnight were used. The other procedures were the same as the above-mentioned for assay I.

Cross-Reactivities (CR). The ability of the raised antisera to recognize several structurally related compounds was tested by performing competitive assays and determining their respective IC_{50} values (analyte concentration that reduces the maximum signal of the competitive ELISA to 50%). The CR value was calculated by the equation $[IC_{50}(\text{fenarimol})/IC_{50}(\text{related compound})] \times 100$.

Matrix Effects. To determine the pesticide residues in the fruit samples including apples and pears by ELISA, just as other instrumental analyses, the samples should be ground in a Waring Blendor with appropriate solvents such as acetone and methanol to extract the target chemicals. The effects of acetone and methanol on the sensitivity of the ELISA were examined.

For the analysis of fenarimol residues in apples and pears, acetone was used for the extraction of the target pesticide from the samples. After suction filtration of the homogenate, acetone was evaporated and the residue was redissolved in 1× PBS. The 1× PBS solution was filtered through cotton and an acetate filter (Osmonics, pore size = 0.45 μm) to remove various ingredients interfering with the ELISA. Usually, the matrix effect can be reduced by dilution or cleanup procedures.

Analysis of Fenarimol-Spiked Samples. Apples and pears were collected from an orchard where fenarimol was not applied. Each sample was homogenized in a Waring Blendor and stored at −20 °C until analysis. For the recovery test, three levels of concentrations (0, 100, and 300 ng/mL for apples and 0, 500, and 1000 ng/mL for pears) of fenarimol in the samples were prepared with the fenarimol stock solution in acetone. Fenarimol spiked to the agricultural samples was extracted with acetone. That is, 5 g of homogenized samples was extracted with 50 mL of acetone using an ultrasonicator (JAC Ultrasonic, Jinwoo, Korea) for 20 min. The slurry was filtered through filter paper (Whatman no. 2), and the filtrate was concentrated until acetone was removed completely under reduced pressure at 60 °C using a rotary evaporator. The volume of the concentrate was adjusted to 50 mL with 1× PBS, and the concentrate was again diluted 10-fold for apples and 50-fold for pears with 1× PBS. Each analysis was done in triplicate.

RESULTS AND DISCUSSION

Synthesis of Haptens and Their Conjugation to Carrier Proteins. The hapten synthesis was aimed at preserving the fenarimol structure for electronic and hydrophobic properties and considering the ideal spacer position to elicit antibodies for fenarimol analogues. On the basis of this idea, two analogues of the target analyte were synthesized and an analogue, 4,4′-DDA, was purchased to use as immunogens and coating antigens. The syntheses of the two haptens (hapten-1 and hapten-2) were carried out as outlined in **Figure 1**. The relatively low yields, 44% in hapten-1 and 32% in hapten-2, are thought to be due to the steric hindrance around the hydroxyl group. The two haptens and 4,4′-DDA with a carboxyl group in their structures were conjugated to carrier proteins by using the activated ester method.

Determination of Protein Contents and Coupling Densities. The protein contents of the hapten−protein conjugates of hapten-1−BSA, hapten-1−KLH, hapten-2−BSA, hapten-2−KLH, hapten-2−OVA, and 4,4′-DDA−BSA were 3.02, 4.96, 2.95, 3.24, 10.27, and 2.18 mg/mL, respectively. To the amino groups of the proteins, 62.83% of hapten-1 and 50.88% of hapten-2 were conjugated in the hapten−KLH conjugates, and 57.71% of hapten-1 and 61.69% of hapten-2 were conjugated in the hapten−BSA conjugates. Meanwhile, 18.39% of hapten-2 was conjugated to OVA in the hapten-2−OVA conjugate and 6.24% of 4,4′-DDA was conjugated to BSA in the 4,4′-DDA−BSA conjugate. The extremely low percentage of conjugation to BSA in 4,4′-DDA strongly indicated that the steric hindrance in its chemical structure prevented access of an amino group of the protein.

Screening and Selection of Coating Antigens and Antisera. The titers of the antisera raised against haptens-1 and -2 increased until the third bleeding. After that, the titer was variable. On the whole, the titer of antisera raised against hapten-1 was similar to that against hapten-2. To establish a sensitive ELISA, all combinations between coating antigens and antisera were screened via the inhibition at two different concentrations (10 and 1000 ng/mL) of the analyte dissolved in the assay buffer, using the homologous or heterologous competitive indirect ELISA format (**Table 1**). It is known that the ELISA format and the use of heterologous haptens can

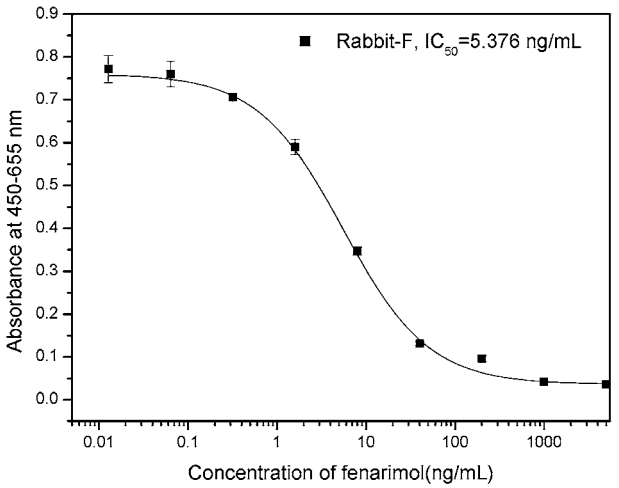


Figure 2. Sensitive heterologous ELISA performed by the combination of antiserum from rabbit F raised against the immunogen hapten-**2**–KLH and coating antigen 4,4′-DDA–BSA.

change the sensitivity of the assay (*17*, *18*). The decreased affinity of heterologous antigen−antibody interaction has been used to improve the sensitivity of immunoassay systems (*19*−*22*).

Interestingly enough, in our ELISA, the heterologous combination between antiserum F raised against hapten-2−KLH and 4,4′-DDA−BSA as a coating antigen resulted in a much higher sensitivity than that between antiserum F and hapten-1−BSA as a coating antigen.

In two ELISAs conducted at nine concentrations of fenarimol, the IC_{50} values in the heterologous ELISAs by the combination of the antisera from rabbits E and F raised against the immunogen hapten-2−KLH and the coating antigen 4,4′-DDA−BSA were 18.05 and 5.38 ng/mL, respectively. The most sensitive heterologous ELISA with the rabbit F antiserum was chosen to optimize the buffer-related factors for the assay (**Figure 2**).

Optimization. The effects of the coating method of coating antigens, blocking agents, and buffer-related factors such as detergent, ionic strength, and pH were evaluated (**Table 2**).

Effect of Coating Methods. There was not much difference between assay I (4 °C, overnight) and assay II (37 °C, 2 h) in the IC_{50} value (6.496 and 9.367 ng/mL, respectively). In terms of rapidity of the ELISA, assay II could be an alternative method to assay I, even if its IC_{50} value is somewhat higher than that of assay I.

Effect of Blocking Agents. Blocking agents were evaluated to prevent nonspecific sorption of an antibody onto the less-coated plate. Because there is no universal blocking buffer for all immunoassays, various blocking agents were tested for each immunoassay. As shown in **Table 2**, all tested blockers except 1% gelatin increased assay sensitivity relative to no blocker. Especially, 3% skim milk showed a lower background signal, a much higher ratio of maximum absorbance (*A*) to minimum absorbance (*D*), and a higher sensitivity for the ELISA relative to other blockers, the IC_{50} value being 3 times lower than no blocking.

Effect of Detergent. The nonionic detergent Tween 20 has been in wide use in ELISA to reduce nonspecific interactions and enhance sensitivity (*23*). However, in this investigation as shown in **Table 2**, the IC_{50} value determined in the buffer containing 0.05% (v/v) Tween 20 was 3.5 times higher than that determined in the buffer without the detergent, indicating that the addition of Tween 20 to the assay buffer did not enhance

Table 2. Effect of Various Factors on the Sensitivity of the ELISA[a]

factor	A_{max} (*A*)	slope (*B*)	IC_{50} (ng/mL) (*C*)	A_{min} (*D*)	*A*/*D*
Blocking Agent					
no blocking	1.04	0.79	13.23	0.31	3.35
1% gelatin	0.94	0.79	15.21	0.18	5.22
3% skim milk	0.26	0.78	4.32	0.01	26
3% OVA	0.36	0.73	7.78	0.05	7.2
Detergent (Tween 20)					
0%	0.32	0.92	13.09	0.01	32
0.05%	0.28	0.92	46.09	0.01	28
Concentration of Assay Buffer (Ionic Strength)					
0.5× PBS	0.26	0.70	29.61	0.03	8.67
1× PBS	0.10	0.90	9.55	0.00	
1.5× PBS	0.08	1.01	10.86	0.01	8
pH					
5.5	0.22	0.96	18.90	0.01	22
6.5	0.29	0.50	12.74	0.00	
7.5	0.28	0.86	6.50	0.02	14
8.5	0.16	0.67	17.45	0.00	
9.5	0.36	0.91	20.19	0.02	18
Preincubation Time					
0 h	0.23	0.80	4.31	0.02	11.5
1 h	0.13	0.71	7.63	0.01	13

[a] Assay conditions: coating antigen, 4,4′-DDA–BSA (1 μg/mL); blocking with 3% skim milk in 1× PBS at 37 °C, 1 h; antiserum, rabbit F diluted by 1:8000 in 1× PBS; standard series of fenarimol was dissolved in 1× PBS; goat anti-rabbit IgG-HRP (1:10000). Data are the means of quadruplicates.

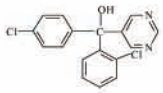
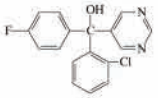
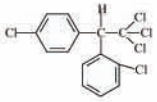
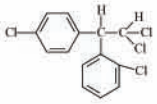
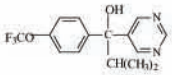
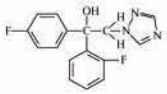
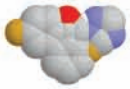
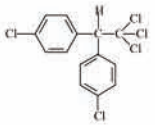
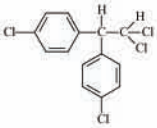
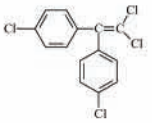
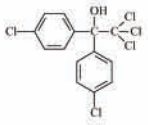
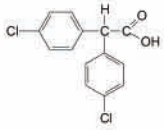
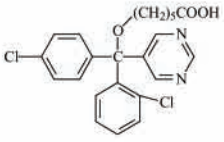
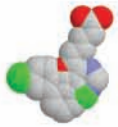
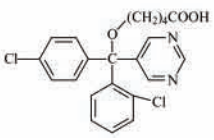
the sensitivity of the ELISA for fenarimol. The negative effect of Tween 20 on ELISA was also reported for permethrin (*24*), endosulfan (*25*), chlorpyrifos (*26*), carbaryl (*27*), polychlorinated dibenzo-*p*-dioxin (*28*), and esfenvalerate (*29*). Manclús and Montoya (*26*) suggested that this negative effect of Tween 20 in these cases would be related with nonspecific hydrophobic interactions between the detergent and nonpolar small organic molecules in an aqueous environment, interfering thereby with the specific analyte−antibody interaction. Accordingly, they proposed that the highly polar compound 3,5,6-trichloro-2-pyridinol (TCP) did not have the negative effect of Tween 20. However, the carbamate insecticide carbaryl (*27*) has the K_{ow} log *P* value of 1.59, which implies a relatively great polarity. Therefore, the explanation based on the polarity of the analyte would not be adequate in this case.

Effect of Ionic Strength and pH. As seen in **Table 2**, the binding of the antibody to the coating antigen showed a tendency to decrease with the increased salt concentration of the assay buffer. The optimum ionic strength of the assay phosphate buffer was selected to be 1× PBS, where the IC_{50} value was the lowest (9.55 ng/mL). Meanwhile, as to the pH effect, pH 7.5 was selected to be optimal, because the IC_{50} value (6.50 ng/mL) at that pH was the lowest. The pH dependence of both signal and sensitivity of ELISAs for pesticides has been reported (*25*, *27*, *30*−*33*).

Effect of the Concentration of Immunoreagents. The concentration of immunoreagents such as the coating antigens selected for the ELISA was determined by checkerboard titration. In addition, an intensive investigation for a more sensitive assay was also carried out at different concentrations of the antiserum for the same amount of the coating antigen. Even if the IC_{50} value at the 1:32000 dilution of the antiserum F was lower than that at the 1:8000 dilution, the latter was adopted on the basis of the criterion of maximum slope (0.93) of the inhibition curve at the lowest fenarimol concentrations (*19*).

Effect of Preincubation. Preincubation of the analyte fenarimol with the antiserum F on the mixing plate before addition

Table 3. Cross-Reactivity of Some Structurally Related Compounds to Antiserum F in the ELISA

compound	chemical structure	space-filling model	CR(%)
fenarimol			100
nuarimol			70.40
o,p'-DDT			39.57
o,p'-DDD			13.77
flurprimidol			9.22
flutriafol			4.07
p,p'-DDT			3.29
p,p'-DDD			1.08
p,p'-DDE			0.24
dicofol			0.21
4,4′-DDA			0.03
hapten-1			599.03
hapten-2			620.30

to the coated plates was tested to check the effect on the assay sensitivity. As seen in **Table 2**, preincubation did not improve the sensitivity but did affect the binding of the antibody to the analyte, decreasing the absorbance.

On the basis of these results, the optimal conditions for the fenarimol ELISA are summarized as follows: A quantity of 1 μg/mL of 4,4′-DDA−BSA conjugate as a coating antigen was coated onto the microwell plate and placed at 4 °C overnight (assay I) or at 37 °C for 2 h (assay II), and then the plate was blocked with 3% skim milk at 37 °C for 1 h. The antiserum F raised against the hapten-2−KLH conjugate as an immunogen was diluted 8000-fold with the assay buffer without the detergent Tween 20 and bound to the target analyte dissolved in 1× PBS (pH 7.5) in competition with the coating antigen. In the heterologous assay optimized under these conditions, the IC_{50} values of the analyte in assays I and II were 5.4 and 9.4 ng/mL, detection ranges (IC_{20-80}) 1.1−25.9 and 1.1−82.7 ng/mL, and the lowest detection limits (LOD, IC_{10}), 0.3 and 0.3 ng/mL, respectively. Because there was not much difference between assays I and II in sensitivity, the latter could be an alternative to the former in terms of rapidity.

Cross-Reactivities. The specificity of the antiserum F was evaluated with several structurally related compounds by using the optimized assay I format. As seen in **Table 3**, the relatively high CR (70.40%) of nuarimol, compared to that of fenarimol, indicates the similarity between chlorine and fluorine in atomic structure in that they are both halogens and have a similar electronic configuration and/or electron distribution but are different in atomic size (atomic radius of chlorine, 1.01 Å; that of fluorine, 0.71 Å). From a comparison of the CR of nuarimol and that of flurprimidol (9.22.%), it is noticeable that the two *o*- and *p*-halogen-substituted phenyl groups are more important than the hydroxyl and 5-pyrimidinyl groups of the two compounds in the antibody binding. Similarly, Abad and Montoya (*34*) indicated that in the carbaryl ELISA, both the naphthalene aromatic ring and the *N*-methylcarbamate group must be present for effective antibody binding and recognition. Jones et al. (*35*) reported that the high specificity of the monoclonal antibodies for the organophosphate pesticide azinphos-methyl indicated that the specific part of the molecule (heterocyclic or aromatic ring), and possibly the S═P bond, played a major role in forming the epitope. In the ELISA for the sulfonylurea herbicide bensulfuron-methyl [methyl α-(4,6-dimethoxypyrimidin-2-ylcarbamoylsulfamoyl)-*o*-toluate] by Lee et al. (*36*), the antigenic determinant was proposed to be a phenyl group connected to the sulfonylurea moiety via methylene, oxygen, or NH moieties as evidenced by the structures of bensulfuron-methyl and its related compounds. In our ELISA for fenarimol, the big difference in cross-reactivity between o,p′-DDT (39.57%) and p,p′-DDT (3.29%) strongly indicates that the position of the chlorine substituent on the benzene ring is crucial as a part of the epitope in the target fenarimol. Comparison of the CR of flutriafol (4.07%) with that of fenarimol reveals that the atomic sizes of the halogen substituents on the benzene rings are important in the antibody recognition. In addition, the six-membered pyrimidine ring and the five-membered triazole ring would make a great contribution to the big difference in the CR between them. It is interesting that the difference in cross-reactivity between o,p′-DDT and o,p′-DDD was caused by chlorine and hydrogen present in one group of the two compounds. This suggests that the size of each atom constituting a portion of the epitope can affect the steric fitness in the binding. The low CR of dicofol (0.21%), as compared to that of fenarimol, indicates the importance of the position (ortho in fenarimol) of one chlorine atom on one of the two benzene rings in the antibody binding, irrespective of the trichloromethyl and pyrimidinyl groups present in the two compounds. In addition, when the structure of o,p′-DDT is compared with that of dicofol, it is noted that the position of the chlorine atom on the benzene ring affects the CR remarkably. Meanwhile, in the ELISA for TCP (*37*), methylation of the hydroxyl group drastically hindered the antibody binding to the compound, indicating the participation of the charged oxygen in the analyte−antibody interaction. In the case of fenarimol, however, even if the pK_a value of the hydroxyl group is not known (*38*), the high cross-reactivities of haptens-1 and -2 prove that at least the negatively charged oxygen formed by the dissociation of the fenarimol hydroxyl group does not participate in the antibody binding.

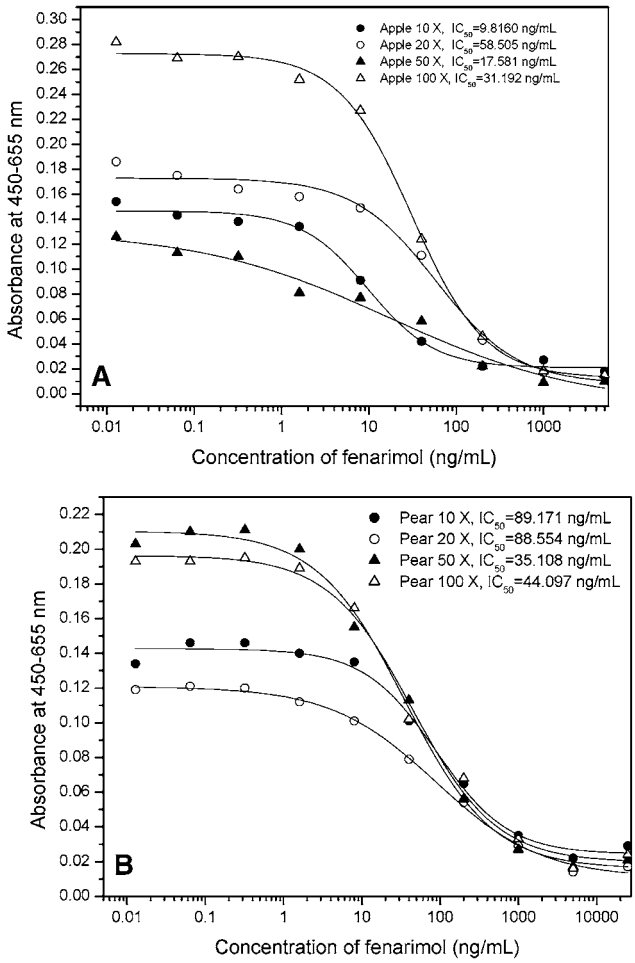


Figure 3. Matrix effect of agricultural samples, as shown by dilution: (**A**) apple; (**B**) pear. The extractant is acetone.

Matrix Effects. The matrix effects from the extracts of apple and pear samples could be reduced to a minimum by diluting 10- and 50-fold with the optimized assay buffer, respectively (**Figure 3**). These results indicate that the ELISA can be used for determining fenarimol residues in apples and pears at the lowest 0.3 ppb level.

With regard to the effect of organic solvents, the presence of both methanol and acetone lowers the sensitivity of the ELISA. The effect of acetone and methanol on the antiserum F was examined because fenarimol residues were extracted with acetone from the fruit samples and methanol is a universal solvent. As can be seen in **Figure 4**, acetone and methanol have a very strong effect on the antibody−antigen binding. That is,

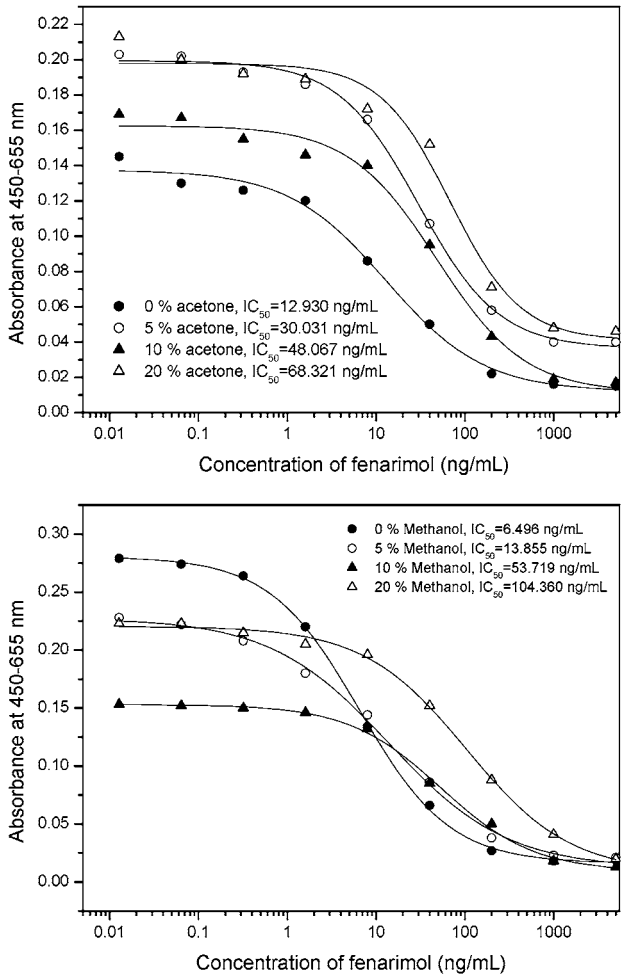


Figure 4. Effect of organic solvent on the sensitivity of the ELISA for the determination of the analyte fenarimol.

the solvents had a negative effect on the assay. Similar effects were also reported elsewhere (*19*, *36*, *39*).

Recovery of Fenarimol Residues from Spiked Agricultural Samples. Apple and pear samples spiked with two different levels of the analyte were analyzed by the optimized ELISA. The extraction of fenarimol from the samples was carried out according to the conventional method. That is, the agricultural samples were ground with acetone to extract the fenarimol residue. The resulting homogenate was filtered, and the filtrate was concentrated to dryness and redissolved in 1× PBS for ELISA.

Recoveries of fenarimol from these agricultural samples (**Table 4**) were in a desirable range of 93−113%. Therefore, this ELISA may be used as a fast and cost-effective alternative and/or supplement to the conventional instrumental methods for determining fenarimol residues in environmental and agricultural samples.

Conclusion. Fenarimol has been in wide use for the control of powdery mildew, anthracnose, and downy mildew in many crops. The ELISA developed for the detection of fenarimol residues in agricultural and environmental samples is very sensitive, as expected. Therefore, this method can be used as a convenient analytical tool for the purpose of monitoring many samples. The relatively high cross-reactivity of nuarimol suggests that the ELISA can be used for this group of pesticides. However, the cross-reactivity of o,p′-DDT will not bring about any problem in practical use because DDT isomers are no longer used in most countries in the world.

Table 4. Recovery of the Analyte Fenarimol from Spiked Samples by the ELISA

sample	spiked to sample (ng/mL)	theor concn in ELISA[a] (ng/mL)	detected (ng/mL)	mean recovery (%, $n = 6$)	coeff of variation[b] (%)	remark
apple	0	0	0	0	6.36	
	100	10	9.27	92.70	4.64	
	300	30	28.82	96.08	7.63	
pear	0	0	<LOD[c]	0	3.61	MRL[d]
	500	10	11.31	113.10	8.77	(0.3 mg/kg)
	1000	20	21.15	105.74	5.55	

[a] Extracts of apple and pear were diluted 10- and 50-fold with 1× PBS, respectively. [b] Coefficient of variation is defined as the standard deviation divided by the mean, expressed as a percentage. [c] Limit of detection. [d] Maximum residue limit; the value for pear was set by the Korea Food and Drug Administration.

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Received for review April 7, 2004. Revised manuscript received September 4, 2004. Accepted September 7, 2004. J.K.L. extends his gratitude to the Korea Science and Engineering Foundation for financial support [R05-2002-000-00531-0 (2003)].

JF049425L