JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of an ELISA for the Detection of the Residues of the Fungicide Iprovalicarb

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A competitive indirect enzyme-linked immunosorbent assay was developed for the fungicide iprovalicarb, using a polyclonal antibody produced against a hapten conjugated through the carboxyl group on the benzene ring to keyhole limpet hemocyanin. Under an optimized condition using a heterologous format, an IC_{50} of 3.51 ng/mL and the lowest detection limit of 0.065 ng/mL were obtained. When the isopropoxy group was removed from the iprovalicarb structure for the synthesis of a hapten, the resulting hapten was not successful as an immunogen, indicating that the isopropyl moiety was an important epitope, as evidenced by the cross-reactivities of some structurally related compounds. When applied to the real crop and water samples, the recoveries were in the range of 80.52−144.70% ($n = 4$) and 72.11−100.43% ($n = 4$), respectively. Accordingly, this ELISA can be used as a useful method for monitoring iprovalicarb residues in crop and water samples.

KEYWORDS: Iprovalicarb; polyclonal antibody; ELISA; monitoring; residues

INTRODUCTION

Iprovalicarb, isopropyl 2-methyl-1-[(1-*p*-tolylethyl)carbamoyl]-(*S*)-propylcarbamate, is a protective, curative, and eradicative systemic fungicide introduced by Bayer AG in 1999. The product is a mixture of (*SS*)-and (*SR*)-diastereoisomers. It belongs to the new amino acid amidocarbamate class of compounds (*1*) and is currently in wide use for the control of anthracnose, downy mildew, and black shank in many crops such as grape, cucumber, onion, tomato, potato, and watermelon in Korea. Accordingly, residue problems will arise in agricultural products and in the environment, even if its half-life is not very long.

Brennecke (*2*) used liquid chromatography with mass spectroscopic detection for residue analysis. Ansuman and Irani (*3*) also reported liquid chromatographic determination of iprovalicarb in cabbage and soil.

The present investigation is aimed at developing a fast, simple, sensitive, and cost-effective ELISA for the detection of iprovalicarb residues in agricultural and environmental samples, because no immunoassay for iprovalicarb has been reported as yet.

MATERIALS AND METHODS

Chemicals. Iprovalicarb and *p*-methylphenylethylamine, of analytical grade, were provided by Bayer AG (Leverkusen, Germany). Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), goat anti-rabbit IgG peroxidase conjugate as a second antibody, and Freund's complete and incomplete adjuvants were all purchased from Sigma Chemical Co. (St. Louis, MO).

Instrument. ^{1}H NMR (nuclear magnetic resonance) spectra of haptens were obtained on a 300 MHz NMR spectrometer, DPX 300 (Bruker, Germany).

FAB-MS (fast atom bombardment mass spectra) using 3-nitrobenzyl alcohol as a matrix were obtained on a JEOL four sector tandem mass spectrometer, JMS-HX/HX 110A (JEOL, Japan), at Korea Basic Science Institute. ELISA was performed on 96-well microtiter plates (Nunc-Immunoplate, MaxiSorp surface, Roskilde, Denmark) and read spectrophotometrically with a microplate reader, Bio-Rad model 550 (Hercules, CA).

Buffer Solutions. Buffer solutions used for the ELISA were normal strength phosphate-buffered saline (1 × PBS, 0.001 M KH_2PO_4, 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 (pH 9.6), and 0.05 M borate buffer (pH 8). Each buffer was prepared according to the method of Gee et al. (*4*).

Hapten Synthesis and Verification. Compound 2, [1-(1-*p*-tolylethylcarbamoyl)-2-methylpropyl]carbamic acid. One millimole (320.4 mg) of iprovalicarb was put into a 300 mL round-bottomed flask and dissolved in 10 mL of ethanol. To this ethanol solution of iprovalicarb was added 5 mL of 5 N NaOH, and the solution was refluxed at 80 °C for 7 h 40 min in an oil-bath to hydrolyze the isopropyl ester moiety in the iprovalicarb structure. After hydrolysis, ethanol was removed from the reaction mixture on a rotary evaporator and a small amount of distilled water was added to it. Then the alkaline solution was acidified down to pH 2 by adding dropwise 6 N HCl, and the resulting precipitate was collected by suction-filtration, washed with cold distilled water, and dried to obtain 234 mg of compound **2** (yield: 84.1%). ^{1}H NMR (CD_3OD): δ 7.19 (d, 2H, $J = 3$ Hz, ar), 7.10 (d, 2H, $J = 6$ Hz,

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10.1021/jf048840a CCC: $27.50
Published on Web 10/05/2004

ar) 4.78 (q, 1H, J = 6 Hz, HN−C*H*(CH_3)ar), 4.18 (t, 1H, HNC*H* isopropyl(CO)), 2.28(s, 3H, Ph−CH_3), 2.12 (m, 1H, C*H*(CH_3)$_2$, 1.18 (d, 3H, CHCH_3), 0.93 (d, J = 6.66 Hz, 6H, (CH_3)$_2$CH). FAB-MS: m/z 279 $[M + H]^+$, 301 $[M + Na]^+$, 323 $[M + 2Na - H]^+$.

Ammonium 6-Aminohexanoate. 6-Aminohexanoic acid was dissolved in NH_4OH in an evaporating dish, and the dissolved solution was heated in a water bath of 80 °C until dryness to obtain the ammonium salt.

Hapten-I (Compound 4), 6-{3-[2-Methyl-1-(1-*p*-tolylethylcarbamoyl)propyl]ureido}hexanoic Acid. To compound **2** (0.2 mmol, 55.7 mg) dissolved in 10 mL of DMF were added ammonium 6-aminohexanoate (0.23 mmol, 34.1 mg), DMAP (1 mg), and DCC (1 mmol, 122.17 mg). The mixture was reacted at 0 °C for 10 min and at room temperature for 6 days and 3 h. After the reaction was completed, DMF was removed completely on a rotary evaporator and a small amount of distilled water was added to the resulting residue. The solution was acidified slowly with 2 N HCl to get compound **4** (hapten-I). ^{1}H NMR ($CDCl_3$): δ 7.18 (d, 2H, J = 6.3 Hz, ar), 7.13 (d, 2H, J = 6 Hz, ar), 5.63 (m, 1H, HNC*H*(CH_3)ar), 4.76 (m, 1H, isopropyl−C*H*(NH)CO), 3.18 (t, 2H, J = 6 Hz, CH_2C*H*$_2$NHC(O)), 2.36 (s, 3H, Ph−CH_3), 2.31 (m, 2H, CH_2C*H*$_2$COOH), 1.69 (qn, 2H, J = 3 Hz, CH_2C*H*$_2$CH$_2$COOH), 1.38 (qn, 2H, J = 6 Hz, CH_2C*H*$_2$CH$_2$CH$_2$COOH), 0.83 (d, 6H, J = 6 Hz, (CH_3)$_2$CH. FAB-MS: m/z 414 $[M + Na]^+$.

Hapten-II (Compound 5), 4-[1-(2-Isopropoxycarbonylamino-3-methylbutyrylamino)ethyl]benzoic Acid. To iprovalicarb (compound **I**, 1 mmol, 320.5 mg) dissolved in 30 mL of acetic acid in a 200 mL round-bottomed flask were added cobalt(II) acetate tetrahydrate (52 mg), manganese(II) acetate tetrahydrate (16 mg), and sodium bromide (44 mg).The mixture was refluxed at 60 °C in an oil-bath in a stream of oxygen for 27 h. After the reaction was finished, acetic acid was removed from the reaction mixture on a rotary evaporator and a small amount of distilled water was added to the residue. The resulting solution was extracted with CH_2Cl_2 and ethyl acetate consecutively. The combined organic solvent portions were dehydrated over anhydrous sodium sulfate and concentrated on a rotary evaporator to obtain compound **5** (hapten-II).

The product was subjected to flash column chromatography using CH_2Cl_2, CH_2Cl_2−MeOH (76:4, v/v), CH_2Cl_2−MeOH (72:8, v/v), CH_2Cl_2−MeOH (68:12, v/v), CH_2Cl_2−MeOH (64:16, v/v), CH_2Cl_2−MeOH (60:20, v/v), and MeOH sequentially as eluents to obtain the product (68 mg; yield, 19.4%). ^{1}H NMR (300 MHz, CD_3OD): δ 7.31 (d, 2H, J = 3 Hz, ar), 7.09 (d, 2H, J = 9 Hz, ar), 4.92 (q, 1H, J = 23.6 Hz, HNC*H*(CH_3)ar), 3.87 (d, 1H, J = 6 Hz, HNC*H*(CH)C(O)), 1.46 (d, 3H, J = 6.9 Hz, HNCH(CH_3)ar), 1.20 (d, 6H, J = 6 Hz, (CH_3)$_2$-CHOCO), 0.94 (d, 6H, J = 6 Hz, (CH_3)$_2$CHCH); FAB-MS: m/z 351 $[M + H]^+$. IR (ν_{max}, KBr): 3860, 3300, 2980, 1700, 1110 cm^{-1}.

Conjugation of the Haptens to Carrier Proteins. The two haptens were conjugated to carrier proteins (KLH and BSA) using the activated ester method (*5*). That is, each hapten was dissolved in 0.2 mL of DMF with equimolar *N*-hydroxysuccinimide (NHS) and a 10% molar excess of 1,3-dicyclohexylcarbodiimide (DCC). After 5 h of stirring at 22 °C, the precipitated dicyclohexylurea was discarded through filtration, and the resulting activated ester was added slowly to each 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,followed by an exhaustive dialysis against normal strength phosphate-buffered saline (1 × PBS) which was changed twice a day for 5 days. The collected conjugates were divided into 2 mL cryogenic vials and stored at −80 °C until use.

Determination of Proteins. The protein contents of the hapten−protein conjugates were determined according to the Bio-Rad protein assay based on the method of Bradford (*6*).

Determination of the Coupling Density. The free amino groups of each hapten−protein conjugate were determined by a modification of the trinitrobenzenesulfonic acid (TNBSA) method (*7*).

Immunization. Six female New Zealand white rabbits weighing 3.0 kg were used to produce polyclonal antibodies. Each hapten−KLH conjugate (100 μg: protein equivalent) 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. Rabbits A−C were immunized against hapten-I−KLH and D−F against hapten-II−KLH.

Freund's complete adjuvant was used for the first injection, and Freund's incomplete adjuvant was used for the subsequent boost injections, which were given at every 3-week interval by the same method. Blood samples were taken 7 days after each boost injection from the jugular vein of the ear to check the titer of each antiserum. The blood samples were placed at room temperature for about 2 h for coagulation and kept in a refrigerator overnight. The serum was decanted and centrifuged at 800*g*, the supernatant being collected, dispensed into cryogenic vials, and stored at −80 °C. Boost injections and subsequent bleedings were done five times.

Checkerboard Titration and Indirect ELISA. To obtain an optimum combination of coating antigen concentration and antiserum dilution for the highest sensitivity in the ELISA (*8*), a checkerboard titration on all the antisera was performed by the homologous indirect ELISA. The coating antigen concentration ranged from 0.01 to 1 μg/mL, and the antiserum dilution was between 1:16000 and 1:256000. The optimum combination was selected on the basis of the absorbance in the range of 0.5−1.0. Microtiter plates were coated with 100 μL/well of the hapten−BSA conjugates in a carbonate buffer (pH 9.6) and allowed to stand at 4 °C overnight. On the following day, the plates were washed 5 times with 0.1 × PBS containing 0.05% Tween 20 (0.1 × PBST) to remove the nonadsorbed antigen. 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 as described previously. Then 100 μL/well of each antiserum diluted with 1 × PBS was added to the plate, which was then reacted at 24 °C for 1 h and washed. Again, 100 μL/well of a secondary antibody, goat antirabbit IgG conjugated to horseradish peroxidase diluted 1:10000 with 1 × PBST, was added and the plates were incubated at room temperature for 1 h. The plate was washed, and 100 μL/well of a substrate solution (0.1 mL of 1% hydrogen peroxide and 0.4 mL of 0.6% 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide (DMSO) added to 25 mL of citrate−acetate buffer, pH 5.5) was added to each well. After reaction at room temperature for 15 min, the reaction was stopped by addition of 50 μL/well of 4 N sulfuric acid. The yellow-colored plate was read in a dual wavelength mode (450−655 nm).

Screening of Antisera and Coating Antigens. To decide whether the respective polyclonal antibodies raised against the immunogens hapten-I−KLH and hapten-II−KLH conjugates can be used for the iprovalicarb ELISA, competitive inhibition ELISAs were performed by both homologous and heterologous systems in comparison with the control without the analyte. That is, according to the method of Abad and Montoya (*8*), a competition for antibody binding sites takes place between the coating antigen and the analyte iprovalicarb at the concentrations of 10 and 1000 ng/mL. The concentration of the coating antigen was 1 μg/mL, and the dilution ratios of each antiserum and the secondary antibody were 1:16000 and 1:10000, respectively.

The % inhibition was calculated by the following equation:

$$\% \text{ inhibition} = (A_{\text{control}} - A_{\text{iprovalicarb}})/A_{\text{control}} \times 100$$

where the combination which showed the biggest figure was selected for the optimization of the ELISA.

Competitive Inhibition ELISA and Cross-Reactivities. A competitive inhibition ELISA was performed to assess the specificity of the antibody to the analyte iprovalicarb and the cross-reactivities of the related compounds to the antibody. That is, to the plates precoated with the coating antigens (hapten-I−BSA and hapten-II−BSA conjugates) at 4 °C overnight were added 50 μL of the antiserum and 50 μL of the analyte iprovalicarb and the structurally related compounds of various concentrations. After the samples were mixed well and incubated at room temperature for 1 h, the plates were washed as above. The subsequent procedures were the same as in the indirect ELISA. Standard curves were drawn from the raw data using a four-parameter logistic equation (*9*) using a commercial software package (Origin, Microcal). For the examination of the cross-reactivities of the related compounds to the antibody, the optimized ELISA for iprovalicarb was used.

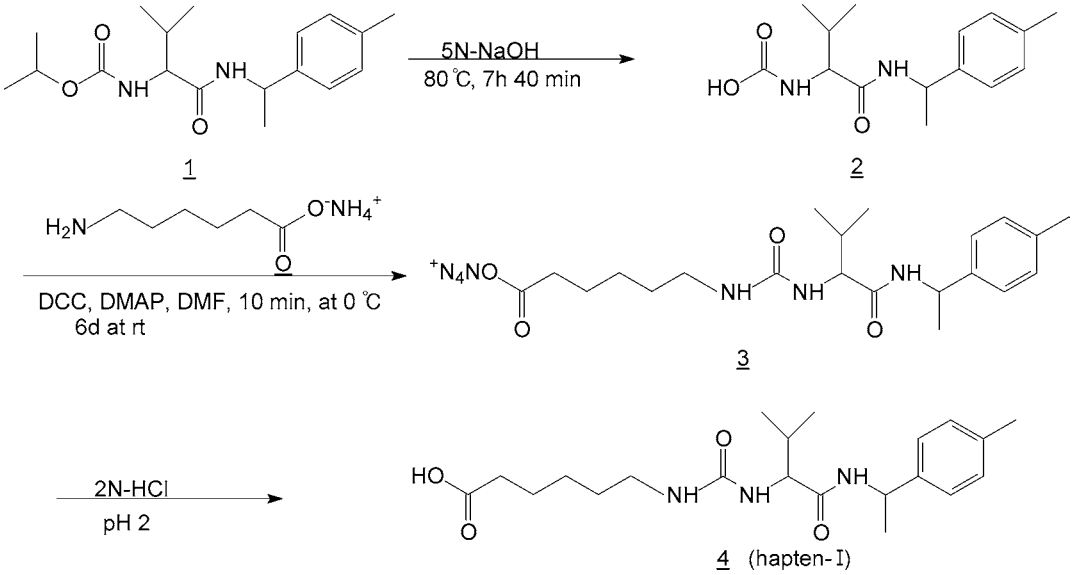


Figure 1. Procedure for the synthesis of hapten-I.

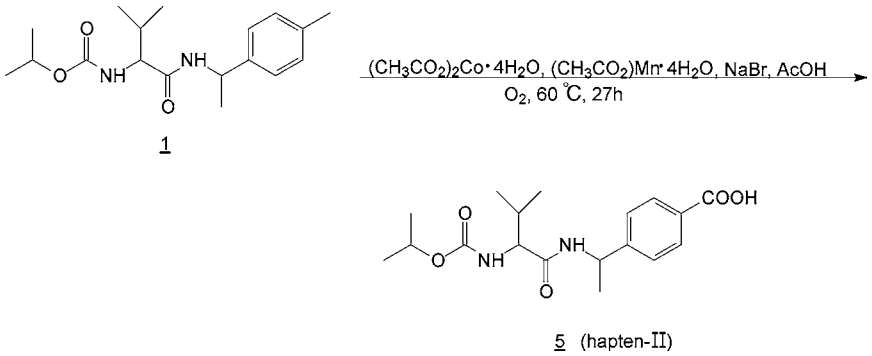


Figure 2. Procedure for the synthesis of hapten-II.

Analysis of Crop and Water Samples. Crop Samples. The crop samples used for the ELISA were potato, onion, cucumber, and tomato. In order to examine the matrix effect of the extract of each crop sample, each sample was extracted with 50 mL of CH_3CN for about 20 min. To the extract was added an appropriate amount of Celite 545, and the mixture was filtered through a Büchner funnel by suction. The remaining residues in the beaker were rinsed with 20 mL of CH_3CN and filtered likewise, the filtrate being combined with the previous one. The combined filtrate was concentrated at 60 °C on a rotary evaporator. In the case of onion extract, to get rid of the mucous substances formed after concentration, a coagulant was used. The coagulant consists of 2 g of NH_4Cl and 4 mL of phosphoric acid dissolved in distilled water to 400 mL. That is, a mixture of acetone (10 mL) and the 10-fold-diluted coagulant (50 mL) was added to the onion concentrate, and the resulting mixture was allowed to stand at room temperature for 30 min. Then, two spoonfuls of Celite 545 were added to the onion extract and the mixture was shaken and filtered in suction. The remaining residues in the beaker were rinsed with 20 mL of coagulant, filtered, and combined with the previous filtrate. The combined filtrate was concentrated at 60 °C on a rotary evaporator to remove the acetone. The concentrated extract transferred to a 1 L separatory funnel added with 100 mL of distilled water and 50 mL of saturated saline was partitioned twice with 50 mL of CH_2Cl_2 each. After drying over anhydrous Na_2SO_4, the CH_2Cl_2 solution was concentrated to dryness on a rotary evaporator. The obtained extract of each crop was diluted 10-fold with 1 × PBS. The diluted extract was filtered through a syringe filter (Cameo 25AS, acetate pore size: 0.45, Osmonics). This final filtrate was diluted to various levels to find the optimum dilution which shows the lowest matrix effect. The recovery test was performed with this determined optimum dilution. That is, potato samples were fortified with the stock solution of iprovalicarb dissolved in CH_3CN to 0.5, 1, 1.5, and 2 ppm; onion samples fortified to 0.3, 0.6, 0.9, and 1.2 ppm; cucumber samples to 0.4, 0.8, 1.2, and 1.6 ppm; and tomato samples to 0.4, 0.8, 1.2, and 1.6 ppm, respectively (**Table 5**). The subsequent procedure was the same as that for the matrix effect.

Water Samples. The iprovalicarb-free water samples were collected from the experimental rice-paddy of Chungbuk National University (pH, 7.24; EC, 0.187 mS/cm; color, pale yellow), a nearby pond (pH, 7.77; EC, 0.216 mS/cm; color, pale yellow), the Mushimcheon stream (pH, 7.88; EC, 0.175 mS/cm; color, colorless), and groundwater for drinking (pH, 7.26; EC, 0.148 mS/cm; color, colorless). The suspended matter in water samples was removed by filtration using Whatman filter paper. The water samples were filtered through a syringe filter (Cameo 25AS, acetate pore size: 0.45, Osmonics). For the recovery test, all the water samples were fortified with iprovalicarb dissolved in CH_3CN to 15, 45, 80, and 120 ppb (**Table 6**). After fortification, the water samples were analyzed directly by the ELISA without any pretreatment.

RESULTS AND DISCUSSION

Synthesis of Haptens. Hapten-I. Since iprovalicarb is a carbamate containing an isopropyl group replacing a hydrogen atom in the carbamic acid, an alkaline hydrolysis was carried out to get a carboxylic compound. That is, iprovalicarb was hydrolyzed with 5 N NaOH at 80 °C for more than 7 h to get a starting compound with a carboxyl group. Ammonium 6-aminohexanoate reacted with the carboxyl group. The resulting ammonium salt was transformed to a carboxylic compound by acidifying with 2 N HCl to pH 2 (hapten-I, compound **4**) as seen in **Figure 1**. Schlaeppi et al. (*10*) produced monoclonal antibodies specific to the sulfonylurea herbicide triasulfuron using a simple hapten corresponding only to the chloroethoxy sulfonamide with an additional succinic acid spacer. On the basis

Table 1. The Protein Contents and Conjugation Ratios in the Hapten–Protein Conjugates

hapten–protein conjugate	use	protein content (mg/mL)	conjugation ratio TNBSA[a] (%)
hapten-I–BSA	coating antigen	3.60	54.51
hapten-I–KLH	immunogen	3.02	38.46
hapten-II–BSA	coating antigen	6.45	43.45
hapten-II–KLH	immunogen	3.70	44.23

[a] TNBSA stands for trinitrobenzenesulfonic acid.

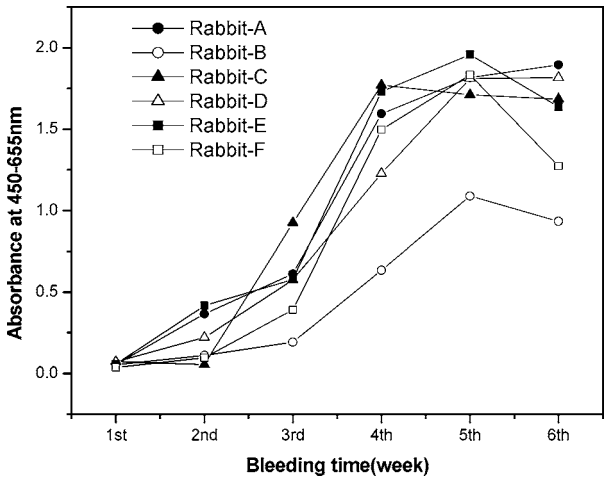


Figure 3. Titration of the antisera produced by six rabbits. Rabbits A–C were immunized against hapten-I–KLH and rabbits D–F against hapten-II–KLH. Boosts were given every three weeks, and bleeding was done a week after each boost to check the titers of the antisera by a homologous indirect ELISA. Each antiserum was diluted 16000-fold. Each well was coated with the corresponding plate-coating antigen at the concentration of 1 μg/mL.

of this idea, the alkaline hydrolysis of iprovalicarb was undertaken, leaving the possible epitope phenyl group unscathed.

Hapten-II. In order to synthesize another hapten, the methyl group on the benzene ring in the iprovalicarb structure was subjected to oxidation. In general, aromatic hydrocarbons containing side chains may be oxidized by alkaline permanganate to the corresponding acids (*11*). Nevertheless, the methyl group in the benzene ring present in iprovalicarb was not vulnerable to this oxidizing agent. Tanaka et al. (*12*) reported that, in the presence of catalytic amounts of cobalt(II) acetate, manganese(II) acetate, and bromide ion, 3,5-dichloro-1-methylpyrazole-4-carboxylic acid was formed from 3,5-dichloro-1,4-dimethylpyrazole by the selective oxidation with molecular oxygen. This oxidation method was applied to iprovalicarb (**Figure 2**). In purifying the product by flash column chromatography, most of the unreacted iprovalicarb could be removed by eluting with CH_2Cl_2 at first, because iprovalicarb is very soluble in CH_2Cl_2.

Conjugation of Haptens to Carrier Proteins. Hapten-I and hapten-II were coupled to carrier proteins (KLH and BSA) by using the *N*-hydroxysuccinimide ester method (*5*).

Determination of Protein Contents and Coupling Densities. The protein contents and coupling densities of the two haptens are presented in **Table 1**.

Checkerboard Titration of the Antisera. One week after the first immunizing injection, the first bleeding was done for checkerboard titration. The subsequent bleedings for titration continued 7 days after each boost injection in the same way.

Table 2. Screening of the Antisera from Rabbits and Coating Antigens in a Competitive Indirect ELISA by Percent Inhibition[a] of Color Development

		coating antigen			
		hapten-I–BSA		hapten-II–BSA	
immunogen	rabbit	10[b]	1000[b]	10[b]	1000[b]
hapten-I–KLH	A			0	3.29
	B		5.10		25
	C				16.37
hapten-II–KLH	D	65.22	84.06	10.53	64.01
	E	63.64	97.73	14.68	58.48
	F	26.32	63.16	4.59	19.46

[a] Percent inhibition $= [(A - B)/A] \times 100$, where A is the absorbance of negative control and B is the absorbance of iprovalicarb. An antiserum dilution of 1:16000. [b] Concentration of iprovalicarb (ng/mL).

Table 3. Effect of Various Factors on the Sensitivity of the ELISA

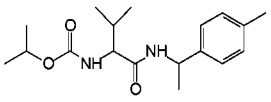
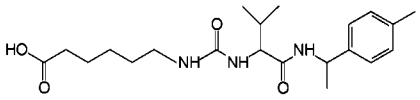
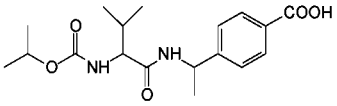
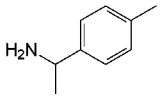
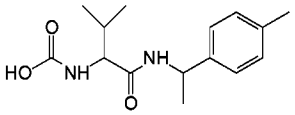
factor	A_{max} (A)	slope (B)	IC_{50} (ng/mL) (C)	A_{min} (D)	A/D
Coating Method					
4 °C, overnight	0.14	0.78	5.36	0.02	7
37 °C, 2 h	0.09	0.79	5.48	0.00	
Solvent					
0% CH_3CN	0.19	0.71	8.13	0.02	9.5
4% CH_3CN	0.20	0.58	8.64	0.06	3.3
10% CH_3CN	0.17	0.77	14.74	0.02	8.5
20% CH_3CN	0.12	0.90	35.23	0.02	6
Dilution of Antisera (Rabbit E)					
1:16000	0.08	0.69	6.13	0.00	
1:32000	0.05	0.73	3.41	0.00	
1:64000	0.02	1.05	4.20	0.00	
Concentration of the Assay Buffer (Ionic Strength)					
0.5 × PBS	0.09	0.67	4.83	0.00	
1.0 × PBS	0.16	0.82	9.87	0.00	
1.5 × PBS	0.06	0.73	4.88	0.00	
Detergent					
0% Tween 20	0.09	0.71	6.01	0.00	
0.05% Tween 20	0.10	0.84	8.07	0.00	
Dilution of the Second Antibody					
1:10000	0.14	0.78	5.36	0.02	7
1:20000	0.06	0.72	5.51	0.00	
1:30000	0.02	0.67	6.70	0.00	
Blocking Agent					
none	0.19	0.62	7.15	0.06	3.17
1% gelatin	0.05	0.66	7.98	0.00	
3% OVA	0.07	0.84	6.92	0.01	7
3% skim milk	0.12	0.78	5.92	0.02	6
pH					
4.5	0.09	0.65	6.68	0.00	
5.5	0.12	0.81	6.06	0.00	
6.5	0.07	0.76	4.61	0.00	
7.5	0.10	0.61	3.51	0.00	
8.5	0.04	1.04	4.81	0.00	

Figure 3 shows that the titers of most of the antisera reduced at the sixth bleeding.

Selection of Coating Antigens and Antisera. To establish a sensitive ELISA, all combinations between coating antigens and antisera were tested by homologous and heterologous formats in the competitive indirect ELISA. The inhibition was examined at two different concentrations (10 and 1000 ng/mL) of the analyte iprovalicarb dissolved in the assay buffer (**Table 2**). The decreased affinity of heterologous antigen−antibody interaction improves the sensitivity of ELISAs (*13*−*16*).

Optimization of the ELISA. To develop a sensitive ELISA, the effects of the coating method of coating antigens, blocking

Table 4. Cross-Reactivity of Some Structurally Related Compounds to the Rabbit E Antiserum in the ELISA

compound	chemical structure	IC_{50} (ng/mL)	CR (%)[a]
iprovalicarb		6.72	100
hapten- I		2614	0.26
hapten- II		10.34	65.00
p-methyl phenylethylamine		190.6	3.53
hydrolysis product of iprovalicarb		3206	0.21

[a] % cross-reactivity (CR) = (IC_{50} of iprovalicarb/IC_{50} of test compound) × 100.

agents, organic solvents, dilution of antisera, ionic strength of the buffer, detergents, and dilution of the second antibody were evaluated.

Effect of Coating Methods. Two methods were compared in terms of IC_{50} value. Assay I involves the coating at 4 °C overnight with the value of IC_{50} 5.36 ng/mL. Meanwhile, assay II involves the coating at 37 °C for 2 h with the value of IC_{50} 5.48. There was not much difference between the two methods.

Solvent Effect. Since CH_3CN is used in the conventional residue analysis of iprovalicarb, its matrix effect was investigated at various levels of concentration. Up to 4% CH_3CN, the IC_{50} value did not change remarkably.

Effect of Dilution of Antisera. Three levels of dilution, 1:16000, 1:32000, and 1:64000, did not make much difference in the IC_{50} value. The dilution ratio of 1:32000 was selected for the ELISA.

Effect of Salt Concentration. The 0.5 × PBS solution gave the lowest IC_{50} value of 4.83, but 1.0 × PBS was selected because of the A_{max} value.

Effect of Detergent. The nonionic detergent Tween 20 has been widely used in ELISA to reduce nonspecific interactions and enhance sensitivity (*17*). In the ELISA for iprovalicarb, however, the presence of 0.05% (v/v) Tween 20 did not enhance the sensitivity, as indicated by the IC_{50} value, having a rather negative effect. Some negative effects have also been observed in the ELISAs for permethrin (*18*), endosulfan (*19*), chlorpyrifos (*20*), carbaryl (*8*), polychlorinated dibenzo-*p*-dioxin (*21*), and esfenvalerate (*22*).

Effect of Dilution of the Second Antibody. The dilution ratio of 1:10000 gave the lowest IC_{50} value of 5.36.

Effect of Blocking Agent. 3% skim milk gave the lowest IC_{50} value of 5.92.

Effect of pH. The lowest IC_{50} value of 3.51 was obtained at pH 7.5.

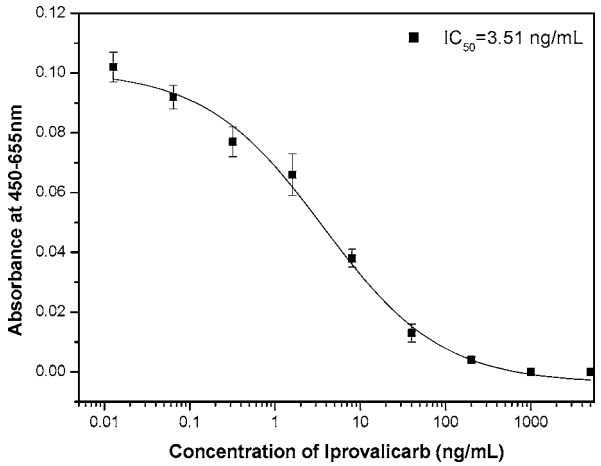


Figure 4. Competitive standard curve of the iprovalicarb ELISA drawn by the optimized conditions. The error bars represent the standard deviations calculated from replicate calibration curves which were obtained with the same set of standards ($n = 4$).

Table 3 presents the effects of these various factors on the sensitivity of the ELISA.

On the basis of these results, the standard curve for the iprovalicarb ELISA was drawn as seen in **Figure 4**.

Cross-Reactivities (CR). The low cross-reactivities of hapten-I, which lacks the isopropoxy moiety of iprovalicarb, and the hydrolysis product of iprovalicarb, (1-(1-*p*-tolylethylcarbamoyl)-2-methylpropyl)carbamic acid, which contains a hydrogen atom instead of the isopropyl group, indicate that the isopropyl group would be an important epitope in the antigen. The high cross-reactivity of hapten-II also supports this argument (**Table 4**). In the carbaryl ELISA, Abad and Montoya (*23*) proposed that the naphthalene ring and the *N*-methylcarbamate group are requisite for effective antibody binding and recogni-

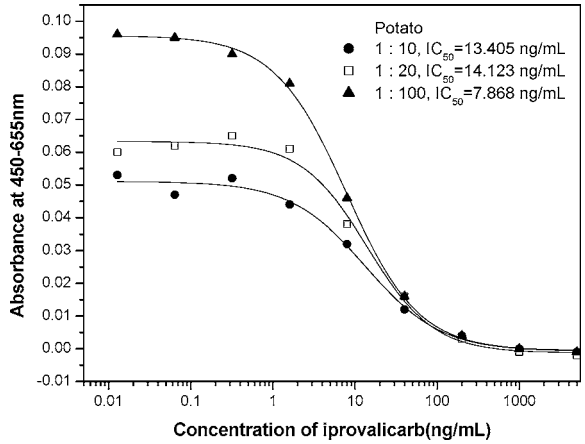


Figure 5. Matrix effect of the extract of potato on the sensitivity of the ELISA, as evidenced by various dilutions.

tion. In the monoclonal antibodies for the organophosphate pesticide azinphos-methyl, Jones et al. (*24*) 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. Lee et al. (*25*), in their ELISA for the sulfonylurea herbicide bensulfuron-methyl, proposed that a phenyl group connected to the sulfonylurea moiety via methylene, oxygen, or NH moieties would be the antigenic determinant. Nevertheless, in the case of iprovalicarb, the phenyl group did not play a role in forming the epitope, presumably because the phenyl group was separated too far from the isopropyl moiety. Dunbar et al. (*26*) developed an ELISA for the herbicide atrazine which contains one isopropylamino group on the *s*-triazine ring in addition to one chloro group. Interestingly enough, propazine with two isopropylamino groups and azidoatrazine with one isopropylamino group were 87% and 58% cross-reactive, respectively. On the contrary, simazine, which contains two ethylamino groups on the *s*-triazine ring instead of one isopropylamino and one ethylamino group, was one-tenth as cross-reactive as atrazine. Furthermore, hydroxyatrazine, deisopropylatrazine, diaminoatrazine, and cyanuric acid were not cross-reactive. All these findings suggest that the isopropyl moiety is important with respect to antibody recognition, as confirmed by our results with iprovalicarb.

Recovery of Iprovalicarb from Fortified Crop Samples. As described in Materials and Methods, the extracts from the iprovalicarb-fortified crop samples were diluted with 1 × PBS to various levels to obtain the highest sensitivity. As seen in **Figure 5**, the potato extract exhibited the lowest IC_{50} value (IC_{50}: 7.87 ng/mL) at the dilution of 1:100. In the case of onion, however, the highest sensitivity (IC_{50}: 2.85 ng/mL) was obtained at the dilution of 1:20. Meanwhile, the cucumber extract showed the suitable curve at the dilution of 1:50, giving the IC_{50} value of 6.76 ng/mL. The tomato extract also showed the lowest IC_{50} value of 3.06 ng/mL at the dilution of 1:50. Therefore, for the recovery test by the ELISA, the purified extract of each sample was diluted to 100-, 20-, 50-, and 50-fold with 1 × PBS for potato, onion, cucumber, and tomato, respectively. As seen in **Table 5**, the recovery was in the range of 80.52−144.70% in all the crop samples.

Recovery of Iprovalicarb from Fortified Water Samples. As seen in **Table 6**, the recoveries of iprovalicarb from water samples were in the range of 72.11−100.43% (n = 4). Meanwhile, the four agricultural samples showed recoveries in the range of 80.52−144.70% (n = 4) (**Table 5**). These recoveries are all acceptable in ELISA. Accordingly, this ELISA can be used as a monitoring tool for iprovalicarb residues in agricultural and environmental samples in cooperation with conventional analytical methods such as GC and/or HPLC.

Table 5. Recovery of the Analyte Iprovalicarb Fortified to Agricultural Samples by the ELISA

sample	fortified (ng/mL)	theor concn in the ELISA (ng/mL)	detected (ng/mL)	mean recovery (%, n = 4)	coeff of variation (%)
potato	0	0	0	0	1.00
	500	5	7.235	144.70	2.71
	1000	10	9.201	92.01	5.09
	1500	15	13.766	91.77	7.81
	2000	20	18.720	93.60	3.76
onion	0	0	0	0	1.00
	300	15	12.72	84.80	2.97
	600	30	29.396	97.99	1.86
	900	45	43.131	95.85	7.52
	1200	60	50.406	84.01	1.54
cucumber	0	0	0	0	1.00
	400	8	10.382	129.76	5.37
	800	16	18.700	116.88	7.19
	1200	24	25.068	104.45	2.63
	1600	32	30.865	96.45	5.44
tomato	0	0	0	0	1.00
	400	8	8.832	110.40	4.59
	800	16	17.904	111.90	1.96
	1200	24	19.324	80.52	2.84
	1600	32	27.357	85.49	2.62

Table 6. Recovery of the Analyte Iprovalicarb Fortified to Water Samples by the ELISA

sample	fortified (ng/mL)	theor concn in the ELISA (ng/mL)	detected (ng/mL)	mean recovery (%, n = 4)	coeff of variation (%)
paddy field	0	0	0	0	1.00
	15	15	16.525	110.17	3.34
	45	45	37.774	83.94	4.98
	80	80	79.659	99.57	1.76
	120	120	98.165	81.80	1.80
pond	0	0	0	0	1.00
	15	15	14.00	93.33	10.35
	45	45	42.475	94.39	5.34
	80	80	80.343	100.43	6.10
	120	120	104.925	87.44	0.70
Mushimcheon stream	0	0	0	0	1.00
	15	15	3.601	90.67	4.33
	45	45	32.449	72.11	2.37
	80	80	73.445	91.81	3.66
	120	120	93.101	77.58	2.16
groundwater	0	0	0	0	1.00
	15	15	13.339	82.26	6.20
	45	45	42.315	94.03	4.08
	80	80	74.076	92.60	4.22
	120	120	94.889	79.07	0.91

Conclusion. Iprovalicarb is a new class of fungicide which is used as a mixture of (*SS*)-and (*SR*)-diastereoisomers. Because it is currently in wide use for various crops, residue problems in agricultural and environmental samples will arise. In this respect, a fast, simple, sensitive, and cost-effective analytical tool in addition to the conventional analytical methods is necessary. The developed ELISA would meet the necessity, because the IC_{50} of 3.51 ng/mL and the LOD of 0.065 ng/mL are low enough to cover the MRLs (maximum residue limits) set by Korea for some crop samples (potato, 0.5 ppm; onion, 0.5 ppm; tomato, 1.0 ppm; Chinese cabbage, 2.0 ppm). To enhance the sensitivity, longer spacer arms are under consid-

eration for future work. In this case, the isopropoxy moiety in the structure should be kept intact, because this moiety is thought to be an important epitope in this compound.

ACKNOWLEDGMENT

The authors are grateful to Bayer AG for providing iprovalicarb and its metabolites for this research.

LITERATURE CITED

(1) Tomlin, C. D. S., Ed. Iprovalicarb. In *The Pesticide Manual*, 13th ed.; BCPC: Hampshire, U.K., 2003; pp 580−581.

(2) Brennecke, R., Method for determination of residues of the fungicide iprovalicarb (SZX 0722) in/on various raw agricultural and processed commodities by liquid chromatography using mass spectroscopic detection. *Pflanzenschutz-Nachr. Bayer* **2000**, *53*, 1, 5−71.

(3) Ansuman, M. Irani, M. Liquid chromatographic determination of iprovalicarb in cabbage and soil. *J. AOAC Int.* **2004**, *87*, 157−161.

(4) Gee, S. J.; Hammock, B. D.; Van Emon, J. M. *A User's Guide to Environmental Immunochemical Analysis*; EPA/540/R-94/509; U.S. EPA, Office of Research and Development, Environmental Monitoring Systems Laboratory: Las Vegas, NV, 1994.

(5) Lee, J. K.; Ahn, K. C.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Development of an enzyme-linked immunosorbent assay for the detection of the organophosphorus insecticide acephate. *J. Agric. Food Chem.* **2003**, *51*, 3695−3703.

(6) Bradford, M. M. A rapid and sensitive method for the determination of microgramme quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(7) Habeeb, A. F. S. A. Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.* **1966**, *14*, 328−336.

(8) Abad, A.; Montoya, A. Development of an enzyme-linked immunosorbent assay to carbaryl. 2. Assay optimization and application to the analysis of water samples. *J. Agric. Food Chem.* **1997**, *45*, 1495−1501.

(9) Rodbard, D. Mathematics and statistics of ligand assay: an illustrated guide. In *Ligand Assay: Analysis of International Developments on Isotopic and Nonisotopic Immunoassay*; Langan, J., Clapp, J. J., Eds.; Masson Publishing Co.: New York, 1981; pp 45−99.

(10) Schlaeppi, J.-M. A.; Meyer, W.; Ramsteiner, K. A. Determination of triasulfuron in soil by monoclonal antibody-based enzyme immunoassay. *J. Agric. Food Chem.* **1992**, *40*, 1093−1098.

(11) Furniss, B. S., Hannaford, A. J., Smith, P. W. G., Tatchell, A. R., Eds. In *Vogel's Textbook of Practical Organic Chemistry*, 5th ed.; Longman Group UK Limited: 1989; p 1239.

(12) Tanaka, N.; Shinke, S.; Takigawa, S. Synthesis of pyrazole carboxylic acid via cobalt-catalyzed liquid-phase oxidation. *Chem. Lett.* **1991**, *4*, 585−588.

(13) Harrison, R. O.; Brimfield, A. A.; Nelson, J. O. Development of a monoclonal antibody based enzyme immunoassay method for analysis of maleic hydrazide. *J. Agric. Food Chem.* **1989**, *37*, 958−964.

(14) Van Weemen, B. K.; Schuurs, A. H. W. M. The influence of heterologous combinations of antiserum and enzyme labeled estrogen on the characteristics of estrogen enzyme-immunoassays. *Immunochemistry* **1975**, *12*, 667−670.

(15) Wie, S. I.; Hammock, B. D. Comparison of coating and immunizing antigen structure on the sensitivity and specificity of immunoassays for benzoylphenylurea insecticides. *J. Agric. Food Chem.* **1984**, *32*, 1294−1301.

(16) Hinds, J. A.; Pincombe, C. F.; Smith, S.; Duffy, P. The use of a monosaccharide linkage group in a heterologous solid-phase enzyme immunoassay for phenytoin. *J. Immunol. Methods* **1985**, *80*, 239−253.

(17) Vanderlaan, M.; Stanker, L. H.; Watkins, B. E. Improvement and application of an immunoassay for screening environmental samples for dioxin contamination. *Environ. Toxicol. Chem.* **1988**, *7*, 859−870.

(18) Stanker, L. H.; Bigbee, C.; Van Emon, J.; Watkins, B.; Jensen, R. H.; Morris, C.; Vanderlaan, M. An immunoassay for pyrethroids: Detection of permethrin in meat. *J. Agric. Food Chem.* **1989**, *37*, 834−839.

(19) Lee, N.; Skerritt, J. H.; McAdam, D. P. Hapten synthesis and development of ELISAs for detection of endosulfan in water and soil. *J. Agric. Food Chem.* **1995**, *43*, 1730−1739.

(20) Manclús, J. J.; Montoya, A. Development of enzyme-linked immunosorbent assays for the insecticide chlorpyrifos. 2. Assay optimization and application to environmental waters. *J. Agric. Food Chem.* **1996**, *44*, 4063−4070.

(21) Sugawara, Y.; Gee, S. J.; Sanborn, J. R.; Gilman, S. D.; Hammock, B. D. Development of a highly sensitive enzyme-linked immunosorbent assay based on polyclonal antibodies for the detection of polychlorinated dibenzo-*p*-dioxins. *Anal. Chem.* **1998**, *70*, 1092−1099.

(22) Shan, G.; Stoutamire, D. W.; Wengatz, I.; Gee, S. J.; Hammock, B. D. Development of an immunoassay for the pyrethroid insecticide esfenvalerate. *J. Agric. Food Chem.* **1999**, *47*, 2145−2155.

(23) Abad, A.; Montoya, A. Production of monoclonal antibodies for carbaryl from a hapten preserving the carbamate group. *J. Agric. Food Chem.* **1994**, *42*, 1818−1823.

(24) Jones, W. T.; Harvey, D.; Jones, S. D.; Ryan, G. B.; Wynberg, H.; Hoeve, W. T.; Reynolds, P. H. S. Monoclonal antibodies specific for the organophosphate pesticide azinphos-methyl. *Food Agric. Immunol.* **1995**, *7*, 9−19.

(25) Lee, J. K.; Ahn, K. C.; Park, O. S.; Ko, Y. K.; Kim, D. W. Development of an immunoassay for the residues of the herbicide bensulfuron-methyl. *J. Agric. Food Chem.* **2002**, *50*, 1791−1803.

(26) Dunbar, B.; Riggle, B.; Niswender, G. Development of enzyme immunoassay for the detection of triazine herbicides. *J. Agric. Food Chem.* **1990**, *38*, 433−437.

Received for review July 13, 2004. Revised manuscript received August 8, 2004. Accepted August 12, 2004. This work was supported by Korea Research Foundation Grant (KRF-2002-002-F00002).

JF048840A