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Article

Development of an ELISA for the Detection of Fenazaquin Residues in Fruits

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To develop an enzyme-linked immunosorbent assay (ELISA) for the detection of the residues of the acaricide fenazaquin, five haptens were synthesized and assessed. A competitive indirect format was used with polyclonal antibodies. Under an optimized condition using the selected rabbit C antiserum, an IC_{50} of 96.97 ng · ml^{-1}, the detection range of 14.9~631 ng · ml^{-1}, and the lowest detection limit of 8 ng · ml^{-1} were obtained. Some structurally related compounds of practical use showed low cross-reactivities to the antibody. Highest cross-reactivity observed with hapten IV indicates that the antiserum C recognizes very well quinazoline ring, 4-tert-butylphenyl, and an adequate length of spacer arm. The length of spacer arm affected recognition of quinazoline ring and 4-tert-butylphenyl moieties. When applied to apple and pear, recoveries were within acceptable ranges of 93.18~104.77% (n = 4) and 79.40~111.95% (n = 4), respectively.

Key words: *ELISA, polyclonal antibody, fenazaquin, cross-reactivity, monitoring*

Fenazaquin, 4-tert-butylphenethyl quinazolin-4-yl ether, is a contact acaricide/insecticide with good knockdown activity on motile forms, as well as true ovicidal activity.[1)] The quinazolines including fenazaquin are one of the new classes of NADH: ubiquinone oxidoreductase inhibitors.[2,3)] Ever since fenazaquin was developed by Dow Elanco in 1983 and introduced to Korea in 1995, it has been in wide use for the control of two-spotted spider mite in apple, pear, grape, watermelon, and perilla, European red mite in apple, and citrus red mite in citrus. For the residue analysis of fenazaquin in tea, high performance liquid chromatography (HPLC) was used.[4,5)] The instrumental analysis requires extensive work for clean-up, expensive equipment, and lengthy time. Although immunoassays are useful for determining a wide variety of compounds due to their selectivity, sensitivity, and simplicity,[6)] none has been performed on fenazaquin. Therefore, this investigation was aimed at developing an enzyme-linked immunosorbent assay (ELISA) for the detection of fenazaquin residues in agricultural and environmental samples.

Materials and Methods

Chemicals. Fenazaquin of analytical and working grade was provided by Kyung Nong Corporation (Seoul, Korea). Bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), goat anti-rabbit IgG peroxidase conjugate, Freunds complete and incomplete adjuvants were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All compounds used for hapten synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The silica gel 60 (0.063-0.200 mm; Merck, Germany) was used for flash column chromatography.

Instruments. ^{1}H nuclear magnetic resonance (NMR) spectra of the synthesized compounds were obtained on a 300-MHz NMR spectrometer (DPX 300; Bruker, Germany) using tetramethylsilane (TMS) as an internal standard. Fast atom bombardment mass spectra (FAB MS) were obtained using 3-nitrobenzyl alcohol as a matrix on a JEOL four sector tandem mass spectrometer, JMS-HX/HX 110A (JEOL, Japan). Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254}, precoated plates (Merck, Germany) with visualization under exposure to either ultraviolet light (254 nm) or iodine vapor. HPLC analysis was carried out using a Hewlett-Packard 1100 series HPLC equipped with a diode array detector(DAD). 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, USA).

Synthesis of hapten I. 7-Nitroquinazolin-4-ol, compound **2**. To 12 m*l* of conc. HNO_3 (70%) in a 100-m*l* round-bottomed flask was added 12 m*l* of conc. H_2SO_4 (97%) and the mixed acid was kept at 0-1°C in an ice-bath. 4-Hydroxyquinazoline (8 mmol, 1169.2 mg) was added to the acid mixture dropwise, while stirring with a magnetic stirrer. The residual 4-hydroxyquinazoline was rinsed into the round-bottomed flask with 5 m*l* $CHCl_3$, and the reaction was allowed to continue for 22 h at room temperature. After the reaction was terminated, the reaction mixture was adjusted to pH 7 with 12.5 N-NaOH and evaporated to dryness in an evaporating dish in a water-bath at 70°C. The residue was dissolved in ethyl acetate-

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methanol (5 : 1, v/v) and centrifuged. The supernatant was collected, concentrated, and weighed (yield: 85.5%).

4-(4-Tert-butylbenzyloxy)-7-nitroquinazoline, compound **4**. Sodium hydride was added to 1.47 mmol (281 mg) 7-nitroquinazolin-4-ol, which was dissolved in 6 m*l* DMF in a 100-m*l* round-bottomed flask and kept at 0°C in an ice-bath, until no hydrogen gas evolved. To the resulting sodium salt was added 1.47 mmol (284 μ*l*) 4-(tert-butyl)benzyl chloride. After 40 min at 0°C, the reaction was continued for 19 h 10 min at room temperature. After the reaction was terminated, DMF was removed from the reaction mixture on a rotary evaporator. The desired product was purified by flash column chromatography with a mixture of methylene chloride-ethyl acetate (10 : 1, v/v) as an eluent. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.199 (s, 1H, quinazoline ring, C-2,), 9.195 (s, 1H, quinazoline ring, C-8), 8.543 (d, 1H, quinazoline ring, C-6), 8.525 (d, 1H, quinazoline ring, C-5), 7.407 (d, 2H, *J* = 2 Hz, Ar), 7.394 (d, 2H, *J* = 2 Hz, Ar), 5.196 (s, 2H, methylene), 1.30 (s, 9H, tert-butyl); CI-MS: *m/z* 338 $(M+H)^+$.

4-(4-Tert-butylbenzyloxy)quinazolin-7-amine, compound **5**. In a 100-m*l* round-bottomed flask, iron powder (16.5 mmol, 921.5 mg) and ammonium chloride (4.1 mmol, 219.3 mg) were added to 1.65 mmol (556.6 mg) 4-(4-tert-butylbenzyloxy)-7-nitroquinazoline dissolved in 25 m*l* of 50% aqueous ethanol. The mixture was refluxed at 90°C for 29 h 20 min. After the reaction was completed, the iron powder was removed by centrifugation. The supernatant was concentrated to dryness. The residue was purified by flash column chromatography successively using methylene chloride-ethyl acetate (2 : 1, v/v), methylene chloride-methanol (9 : 1, v/v), and methanol as eluents, obtaining 265.8 mg (yield: 52%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.32 (s, 1H, quinazoline ring, C-2) 7.32 (d, 1H, *J* = 8.5 Hz, quinazoline ring, C-5), 7.21(d, 2H, *J* = 8.4 Hz, Ar), 5.03 (s, 2H, methylene), 1.24 (s, 9H, tert-butyl).

Tert-butyl 5-(4-(4-tert-butylbenzyloxy)quinazolin-7-ylcarbamoyl) pentanoate, compound **6**. To compound **5** (0.56 mmol, 170.4 mg) dissolved in 4 m*l* methylene chloride in an ice-bath (0°C) were added 0.56 mmol (113 mg) adipic acid mono-tert-butyl ester, 0.62 mmol (127 mg) 1,3-dicyclohexylcarbodiimide (DCC), and 10 mg 4-(dimethylamino)pyridine (DMAP). The mixture was allowed to react for 10 min at 0°C, then at room temperature for 6 days, and was centrifuged. The precipitate (dicyclohexylurea, DCU) was then discarded, and the supernatant was combined and concentrated to obtain compound **6** (207. 3 mg; yield, 75.4%).

Synthesis of 5-(4-(4-tert-butylbenzyloxy) quinazolin-7-ylcarbamoyl)pentanoic acid (compound **7**, hapten I). Compound **6** was hydrolyzed with trifluoroacetic acid (TFA, 0.5 m*l*) for 8 min. TFA was stripped off in vacuo, and ethyl acetate was added twice and stripped off to remove the residual TFA. The residue was immediately flash column-chromatographed on silica gel sequentially using methylene chloride-ethyl acetate (9 : 1, v/v) and methylene chloride-methanol (9 : 1, v/v). ^{1}H NMR (500 MHz, CD_3OD) δ 8.5(s, 1H, quinazoline ring, C-2), 8.30 (s, 1H, quinazoline ring C-8), 7.98 (d, 1H, quinazoline ring, C-6), 7.96 (d, 1H, *J* = 2.05 Hz, quinazoline ring, C-5), 7.61 (d, 2H, *J* = 8.8 Hz, phenyl-ortho to tert-butyl), 7.39(d, 2H, *J* = 8.35 Hz, phenyl-meta to tert-butyl), 5.21(s, 2H, O-C$\underline{H_2}$-phenyl), 2.39 (t, 4H, *J* = 6.8 Hz, CH_2 next to carboxyl group and CH_2 next to amide group), 1.38 (qn, 2H, *J* = 16.04 Hz), 1.28 (s, 9H, tert-butyl); ^{13}C NMR (500 MHz, CD_3OD) δ 160.92, 150.94, 146.39, 143.79, 138.07, 133.28, 130.11, 124.34, 122.00, 115.51, 48.12, 47.95, 47.78, 47.61, 47.44, 47.27, 47.10, 36.71, 33.98, 30.29, 25.18. FAB-MS: m/z 458 $(M+Na)^+$. All procedures leading to the synthesis of hapten I are shown in Fig. 1.

Synthesis of hapten II. Compound **1** (3 mmol, 438.45 mg) was dissolved in 8 m*l* DMF in a 100-m*l* round-bottomed flask placed in an ice-bath and stirred magnetically. Sodium hydride was added to the reaction mixture in small portions until no hydrogen gas evolved. After 20 min, 3 mmol (669.36 mg, 534 μ*l*) ethyl 6-bromohexanoate was added, and the mixture was reacted at 60°C for 20 h. After the reaction was terminated, DMF was removed in vacuo, and the remaining mixture was acidified to pH 3 with 1 N-HCl and sequentially extracted with methylene chloride and ethyl acetate. The resulting extracts were combined and flash column-chromatographed to obtain 805.5 mg of the product (compound **9**: yield, 93.2%). The resulting ethyl ester was hydrolyzed with 1 N-NaOH at 65°C for 4 h. After hydrolysis, the mixture was acidified to pH 2-3 with 1 N-HCl. The resulting slurry was collected by suction-filtration, washed with cold distilled water, and dried.

^{1}H NMR (500 MHz, CD_3OD): δ 8.34 (s, 1H, quinazoline ring C-2), 8.25 (d, 1H, *J* = 8.03 Hz, quinazoline ring C-8), 7.83 (t, 1H, *J* = 7.70 Hz, quinazoline ring C-7), 7.69 (d, 1H, *J* = 8.04 Hz, quinazoline ring C-5), 7.57 (t, 1H, *J* = 7.60 Hz, quinazoline ring C-6), 2.23 (t, 2H, J=7.12 Hz, -O$\underline{CH_2}$ $CH_2CH_2CH_2CH_2COOH$), 1.82 (qn, 2H, J=7.53 Hz, -OCH_2 $\underline{CH_2}CH_2CH_2CH_2COOH$), 1.66 (qn, 2H, J=7.58 Hz, -OCH_2 $CH_2CH_2\underline{CH_2}CH_2COOH$), 1.43 (qn, 2H, J=7.76 Hz, -OCH_2 $CH_2\underline{CH_2}CH_2CH_2COOH$), 1.23 (t, 2H, J=7.13 Hz, -OCH_2 $CH_2CH_2CH_2\underline{CH_2}COOH$). FAB-MS: m/z 261 $(M+H)^+$.

Synthesis of hapten III. Compound **1** (3 mmol, 438.45 mg) was dissolved in 8 m*l* DMF in a 100-m*l* round-bottomed flask placed in an ice-bath and stirred magnetically. Sodium hydride was added to the mixture in small portions until no hydrogen gas evolved. After 20 min at ambient temperature, 3 mmol (627.27 mg, 475 μ*l*) ethyl 5-bromovalerate was added to the reaction mixture, which was then allowed to react at 60°C for 24 h 40 min. After the termination of the reaction, DMF was removed in vacuo. To the remaining residue was added a small amount of distilled water, and the pH was adjusted to 2-3 with 2 N-HCl. The residue was then sequentially partitioned twice with each methylene chloride (50 m*l*) and ethyl acetate (50 m*l*). The combined extract was dried over anhydrous sodium sulfate, and the resulting ethyl ester (616.9 mg, yield: 75%) was dissolved in 6 m*l* ethanol and hydrolyzed with 6 m*l* 1 N-NaOH at 65°C for 2 h. After

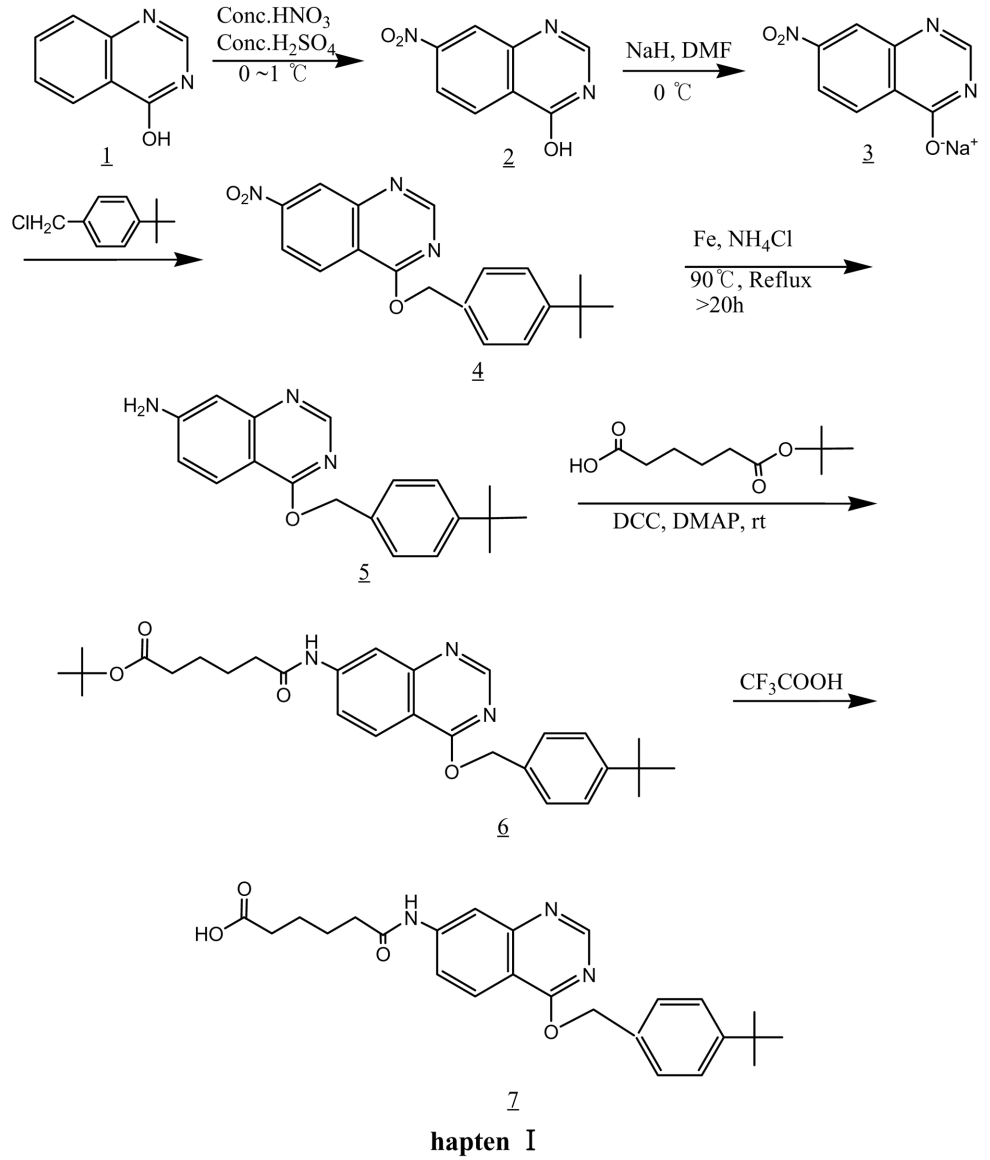


Fig. 1. Procedure for the synthesis of hapten I for fenazaquin ELISA.

hydrolysis, the mixture was acidified to pH 2~3 with 2 N-HCl. The acidified mixture was extracted twice with ethyl acetate, and the extracts were dried over anhydrous sodium sulfate and concentrated to give 314.5 mg (yield: 56.8%) hapten III. ^{1}H NMR (500 MHz, CD_3OD) δ 8.36(s, 1H, quinazoline ring, C-2), 8.25 (d, 1H, J=7.98 Hz, quinazoline ring, C-8), 7.83 (t, 1H, J = 7.69 Hz, quinazoline ring, C-7), 7.70 (d, 1H, J = 8.18 Hz, quinazoline ring, C-5), 7.57 (t, 1H, J = 7.58 Hz, quinazoline ring, C-6), 4.09 (t, 2H, J = 7.30 Hz, -O$\underline{CH_2}CH_2CH_2CH_2COOH$), 2.24 (t, 2H, J = 7.36 Hz, -$OCH_2CH_2CH_2\underline{CH_2}COOH$), 1.83 (qn, 2H, J = 7.49 Hz, -$OCH_2\underline{CH_2}CH_2CH_2COOH$), 1.67 (qn, 2H, J = 7.55 Hz, -$OCH_2CH_2\underline{CH_2}CH_2COOH$). GC-MS (EI), *m/z* 246: GC-MS (CI) 247 $(M+H)^+$.

Synthesis of 3-(4-4-tert-butylbenzyloxy)quinazolin-7-ylcarbamoyl)propanoic acid (hapten IV). Compound **5** (0.41 mmol, 125 mg) was dissolved in 2 m*l* chloroform and kept at 0°C. Triethylamine(0.984 mmol, 137.2 μ*l*) and methyl 4-chloro-4-oxobutyrate (0.861 mmol, 106 μ*l*) dissolved in 2 m*l* chloroform were then added dropwise. The mixture was allowed to react at 0°C for 24 h and at ambient temperature for 4 d 20 h to give compound **11** (165.6 mg; yield, 95. 94%). For the hydrolysis of methyl ester at 5~10°C for 6 h, LiOH · H_2O (1.17 mmol, 49.1 mg) dissolved in 1 m*l* distilled water was added to compound **11** (0.39 mmol, 165.6 mg) dissolved in 2 m*l* ethanol. After hydrolysis, the mixture was acidified to pH 4 with 1 N-HCl, added with distilled water, and partitioned with ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulfate and concentrated. The resulting product was purified by flash column chromatography using methylene chloride-ethyl acetate (9 : 1, v/v), methylene chloride-methanol (95 : 5, v/v), methylene chloride-methanol (90 : 10, v/v), methylene chloride-methanol (85 : 15, v/v), methylene chloride-methanol (80 : 20, v/v), methylene chloride-methanol (75 : 25, v/v), and methylene chloride-methanol

(70 : 30, v/v) as eluents. ^{1}H NMR(DMSO-d_6): δ 8.50 (s, 1H, quinazoline ring, C-2), 8.44 (s, 1H, quinazoline ring C-8), 7.95 (d, 1H, *J* = 5.0 Hz, quinazoline ring, C-6), 7.93 (d, 1H, *J* = 5.0 Hz, quinazoline ring, C-5), 7.32 (d, 2H, *J* = 10 Hz, Ar ortho to tert-butyl), 7.23 (d, 2H, *J* = 10 Hz, Ar meta to tert-butyl), 5.05 (s, 2H, O-$\underline{CH_2}$-Ar), 2.43 (t, 2H, *J* = 7.0 Hz, HOOC-$\underline{CH_2}$-CH_2-C(O)-), 2.27 (t, 2H, *J* = 7.0 Hz, HOOC-CH_2-$\underline{CH_2}$-C(O)-), 1.24 (s, 9H, t-butyl).

MS: Low FAB-MS, *m/z* 408.2 $(M+H)^+$.

Synthesis of 2-(quinazolin-4-yloxy)acetic acid (hapten V). Compound **1** (3 mmol, 438.45 mg) was dissolved in 8 m*l* DMF in a 100-m*l* round-bottomed flask placed in an ice-bath and stirred magnetically. Sodium hydride was added to the mixture in small portions until no hydrogen gas evolved. After 20 min at ambient temperature, 3.5 mmol (584.48 mg, 388.1 μ*l*) ethyl bromoacetate was added to the reaction mixture, which was then allowed to react at 80°C for 24 h. After the termination of the reaction, DMF was removed in vacuo. To the remaining residue was added a small amount of distilled water, and the pH was adjusted to 2~3 with 2 N-HCl. The acidified mixture was partitioned with methylene chloride twice, and the methylene chloride extract was dried over anhydrous sodium sulfate and concentrated to dryness. The resulting ethyl ester was hydrolyzed with 1 m*l* of 1 N-NaOH at 65°C for 3 h. The product was flash column- chromatographed sequentially using methylene chloride-methanol (9 : 1, v/v), methylene chloride-methanol (45 : 5, v/v), and methanol as eluents. ^{1}H NMR (300 MHz, CD_3OD): δ 8. 55 (s, 1H quinazoline ring, C-2), 7.851 (d, 1H, *J* = 1.8 Hz, quinazoline ring, C-8), 7.825 (d, 1H, *J* = 1.5 Hz, quinazoline ring, C-5), 7.202 (t, 1H, *J* = 6.9 Hz, quinazoline ring, C-7), 6.54 (t, 1H, *J* = 6.9 Hz, quinazoline ring, C-6), 4.90 (s, 2H, methylene). MS-CI: m/z 205. All the procedures leading to the synthesis of haptens II~V are shown in Fig. 2.

Conjugation of haptens to carrier proteins. Haptens I and II were conjugated to both KLH and BSA using the activated ester method,[7] while haptens III-V were conjugated only to BSA. Hapten I-KLH conjugate and hapten II-KLH conjugate were used as immunogens and all BSA conjugates of haptens I-V were used as coating antigens for homologous and/or heterologous ELISA formats. The prepared conjugates were divided into 2-m*l* cryogenic vials and stored at −80°C until use.

Determination of protein contents and coupling densities. The protein contents of the hapten-protein conjugates were determined by the Bio-Rad protein assay based on the method of Bradford.[8] The free amino groups of each hapten-protein conjugate were determined by a modification of the trinitrobenzenesulfonic acid (TNBSA) method.[9]

Immunization. To produce polyclonal antibodies, six female New Zealand white rabbits weighing 3.0 kg each were used. Each hapten-KLH conjugate (100 μg protein equivalent) dissolved in 0.5 m*l* 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 conjugate and D-F against hapten II-KLH conjugate. Freund's complete adjuvant was used for the first injection and Freund's incomplete adjuvant used for the subsequent boost injections, which were given at 3-week intervals by the same method as above. Bleeding was done 7 days after each boost injection from the jugular vein of the ears to check the titer of each antiserum. The blood samples were placed at room temperature for about 2 h for coagulation and then kept in a refrigerator overnight. The serum was decanted and centrifuged at 800×g. The supernatant was collected, dispensed into cryogenic vials, and stored at −80°C. Boost injections and subsequent bleedings were each performed five times.

Checkerboard titration and indirect ELISA. Checkerboard titrations were performed to examine the titer of each antiserum collected from six rabbits. To obtain the highest sensitivity of the ELISA,[10] the optimum combination between coating antigen concentration and antiserum dilution was chosen using a homologous indirect ELISA. The coating antigen concentration ranged from 0.01 to 1 μg · ml^{-1}, and the antiserum dilution was between 1 : 16000 and 1 : 256000. Microtiter plates were coated with 100 μ*l* /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 five times with 0.1×PBS containing 0.05% Tween 20 (0.1 × PBST) to remove the non-adsorbed antigen. The 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 with 0.1 × PBST. The subsequent procedures were the same as described previously.[11]

Screening of antisera and coating antigens. To decide which antiserum raised against the immunogens (hapten I-KLH and hapten II-KLH) is suitable for the fenazaquin ELISA, competitive inhibition ELISAs were performed by homologous and/or heterologous systems in comparison with the control without the analyte. A competition for antibody binding sites exists between the coating antigen and the analyte fenazaquin at 40 and 25,000 ng · ml^{-1}. The concentration of the coating antigen was 1 μg · ml^{-1}, and the dilution ratios of each antiserum and the secondary antibody were 1 : 16,000 and 1 : 10,000, respectively. The % inhibition was calculated using the following equation: % inhibition = $(A_{control} - A_{fenazaquin})/A_{control} \times 100$. The combination showing the biggest figure was selected for the optimization of the ELISA.

Cross-reactivities. The cross-reactivities of some related compounds to the antibody were tested by a competitive inhibition ELISA (ciELISA), which was run under the optimized conditions. That is, 50 μ*l* of the antiserum and 50 μ*l* of the analyte fenazaquin or 50 μ*l* of the structurally related compounds of various concentrations were added to the plates precoated with hapten III-BSA conjugate (3 μg · ml^{-1}) at 4°C overnight. After mixing and incubating at room temperature for 1 h, the plates were washed as mentioned above. The subsequent procedures were the same as in the indirect ELISA. Standard curves were prepared from the raw data

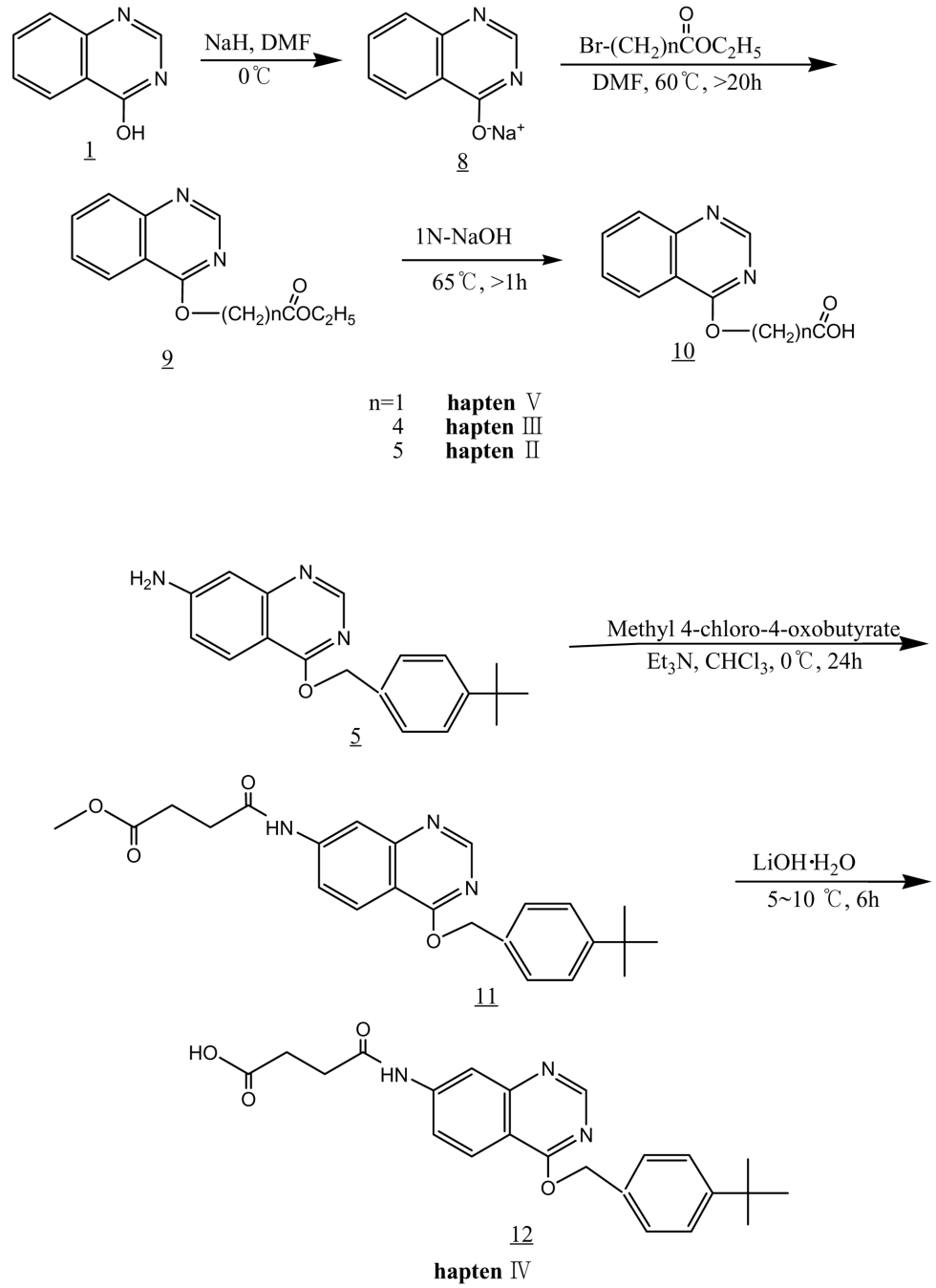


Fig. 2. Procedure for the synthesis of haptens (II~V) for fenazaquin ELISA.

using a four-parameter logistic equation[12] using a commercial software package (Origin, Microcal Software, Inc., MA, USA). Cross-reactivity values were calculated as the ratio of the IC_{50} of the fenazaquin standard to that of the test compounds and expressed as a percentage.

Analysis of fruit samples. Because fenazaquin has been mainly used in fruits, apple and pear were analyzed by the ELISA as the representative fruit samples. For the recovery from fortified samples, sliced apple and pear samples (5 g each) were fortified with the fenazaquin stock solution to 1.5, 3, 4.5, and 6 ppm; and to 0.3, 0.6, 0.9, and 1.2 ppm, respectively. Each fortified sample was extracted with 50 m*l* acetone by sonication for 20 min and filtered by suction using 2 spoonfuls of celite 545, and the residue was washed with 20 m*l* acetone. The combined filtrate was concentrated to dryness, redissolved in 1×PBS to 50 m*l* (10-fold dilution), and after sonication, was successively passed through a cotton filter and a syringe filter (CAMEO 25AS; acetate; pore size, 0.45; Osmonics). Five milliliters of the filtrate were diluted with 20 m*l* of 1×PBS (50-fold dilution) for apple and with 5 m*l* of 1×PBS (20-fold dilution) for pear, and were subjected to ELISA.

Table 1. Protein contents and coupling densities of hapten-protein conjugates

Hapten-protein conjugate	Use	Protein contents (mg · ml^{-1})	Coupling densities[a] (%)
H-I-BSA	coating antigen	3.22	6.95
H-I-KLH	immunogen	2.01	6.50
H-II-BSA	coating antigen	3.22	19.19
H-II-KLH	immunogen	3.40	5.26
H-III-BSA	coating antigen	3.32	19.23
H-IV-BSA	coating antigen	3.58	8.91
H-V-BSA	coating antigen	2.83	5.06

[a]Determined by trinitrobenzenesulfonic acid method 100–[(Absorbance of hapten–protein conjugate)/Absorbance of protein×100] %

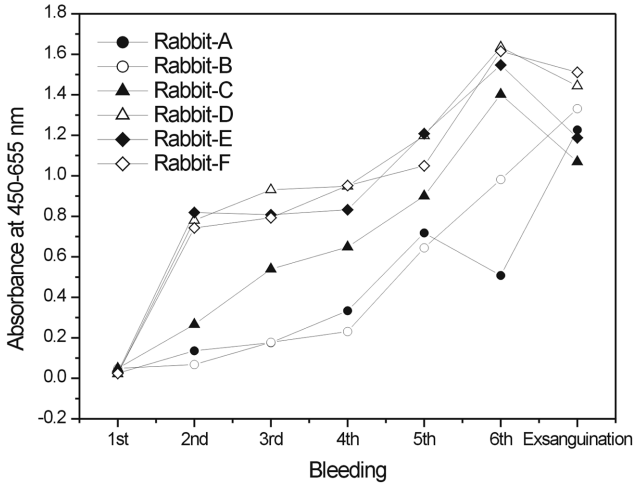


Fig. 3. Titers of the antisera produced by six rabbits. Rabbits A-C were immunized against hapten I-KLH, and rabbits D-F against hapten II-KLH. Boost injections were given every three weeks, and bleeding was performed 1 week after each boost to check the titers of the antisera by homologous indirect ELISA. Each antiserum was diluted 16,000-fold. Each well was coated with a plate-coating antigen at 1 μg · ml^{-1}.

Results and Discussion

Synthesis of haptens. Attempt to synthesize the haptens with spacer arms on the quinazoline ring in the fenazaquin structure through the nitration of fenazaquin was not successful. As an alternative, haptens I and IV were synthesized, because the haptens devoid of one methylene group in the 4-tert-butylphenethyl moiety would not affect the immunization and/or the subsequent antigen-antibody interactions. The haptens II, III, and V each were designed to contain a carboxylic group with different carbon lengths, instead of the 4-tert-butylphenethyl moiety in the fenazaquin structure, for use as immunogens and coating antigens. Schlaeppi *et al.*[13] reported that monoclonal antibodies generated with a simple hapten corresponding only to the chloroethoxy sulfonamide moiety of the sulfonylurea herbicide triasulfuron with an additional succinic acid spacer showed much higher affinity for triasulfuron than those obtained with a hapten consisting of the complete molecule with an aminoalkyl spacer attached to the triazine ring. Furthermore, Mercader *et al.*[14] showed that a hapten consisting of the two original rings of azinphos-methyl attached to a spacer arm was suitable for protein conjugation and monoclonal antibody production.

Determination of protein contents and coupling densities. Table 1 shows protein contents and coupling densities of the hapten-protein conjugates. Although the overall coupling densities were very low, however, hapten II-

Table 2. Screening of antisera obtained from test rabbits by homologous and heterologous ciELISAs for the inhibition by the analyte fenazaquin

Coating antigen	Concn of fenazaquin (ng · ml^{-1})	Immunogen					
		H-I-KLH			H-II-KLH		
		rabbit[b]					
		A	B	C	D	E	F
H-I-BSA[a]	25000	23.1[c]	2.1	14.3	27.7	1.1	37.0
	40	-7.6	2.3	-1.0	0.5	-0.5	-3.9
H-II-BSA	25000	-8.3	-75.0	89.1	NI[d]	-16.7	1.5
	40	0	-50.0	-10.9	NI	33.3	1.0
H-III-BSA	25000	138.5	37.0	98.4	51.8	42.0	-0.7
	40	-15.4	25.9	4.9	-4.3	-3.5	-0.8
H-IV-BSA	25000	25.7	17.4	54.3	-2.0	-2.2	-3.7
	40	4.1	0.5	-8.8	-4.7	8.7	0.0
H-V-BSA	25000	NI	NI	138.5	NI	NI	NI
	40	NI	NI	7.7	NI	NI	NI

[a]No titer was observed using BSA alone as a coating antigen.
[b]antiserum (1:16,000 dilution)
[c]%inhibition = [(A–B)/A]×100, where A and B are absorbances of negative control and fenazaquin, respectively.
[d]No inhibition.

Table 3. Effect of various factors on the sensitivity of the ELISA

Factor	A_{max} (A)	Slope (B)	IC_{50} (ng · ml^{-1}) (C)	A_{min} (D)	A/D
			Blocking agent		
No blocking	1.14	0.74	2164.1	0.59	1.93
1% gelatin	0.97	0.60	798.2	0.48	2.02
3% skim milk	0.14	0.97	186.1	0.04	3.5
3% ovalbumin	0.29	1.02	196.9	0.15	1.93
			Detergent		
Tween 20(%)					
0	0.13	0.99	228.01	0.02	6.5
0.05	0.07	0.91	250.86	0.03	2.33
			Concn of the assay buffer (ionic strength)		
0.5×PBS	0.54	0.76	153.2	0.18	3
1×	0.21	0.55	239.31	0.03	7
1.5×	0.11	0.92	523.74	0.02	5.5
			pH		
4.5	0.08	0.40	891.48	0.02	4
5.5	0.13	0.89	486.68	0.03	4.33
6.5	0.22	0.52	254.06	0.04	5.5
7.5	0.21	0.55	239.31	0.03	7
8.5	0.31	0.72	741.31	0.04	7.75
9.5	0.33	0.80	419.19	0.05	6.6
			Solvent		
Acetone (%)					
0	0.11	0.74	96.97	0.02	5.5
5	0.07	0.60	367.38	0.03	2.33
10	0.05	0.55	778.07	0.02	2.5
Methanol (%)					
0	0.11	0.74	96.97	0.02	5.5
5	0.14	0.76	349.40	0.03	4.67
10	0.16	0.91	698.91	0.03	5.33
15	0.17	0.72	757.25	0.02	8.5
20	0.19	0.84	1027.5	0.05	3.8

BSA conjugate and hapten III-BSA conjugates showed relatively high coupling densities (19.19 and 19.23%, respectively).

Titration of antisera. Titers of the antisera produced by six rabbits were examined (Fig. 3). Most titers of antisera increased until the 6th bleeding, then decreased slightly at exsanguination. Each antiserum was diluted 16,000-fold, and the concentration of the coating antigen was 1 μg · ml^{-1}.

Screening and selection of antisera. To select the desired antiserum for the ELISA, each antiserum produced by six rabbits was screened using the homologous and heterologous formats for inhibition at two concentration levels (40 and 25,000 ng · ml^{-1}) of the analyte fenazaquin dissolved in the assay buffer. The inhibition ratio was calculated based on the difference in absorbance between the assay buffer containing the analyte fenazaquin and that without the analyte. The antisera produced by rabbits A and C immunized against H-I-KLH exhibited 138.5 and 98.4% inhibitions, respectively, using 25,000 ng · ml^{-1} of H-III-BSA as the coating antigen (Table 2). The antiserum of rabbit C was used for the subsequent optimization and characterization of the ELISA, because the antiserum of rabbit A showed higher IC_{50} values in the subsequent ELISAs.

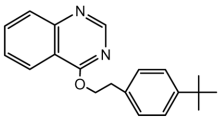
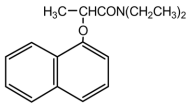
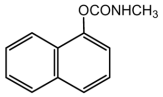
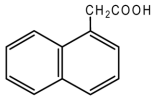
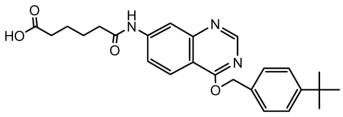
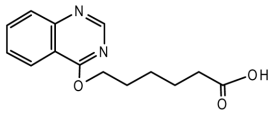
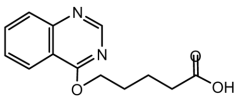
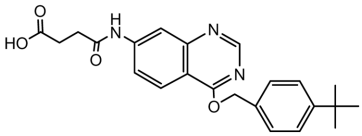
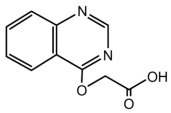
Table 4. Optimized ELISA conditions and some results obtained therefrom

Immunogen	Hapten I-KLH conjugate
Coating antigen	Hapten III-BSA (3 μg · ml^{-1})
Blocking agent	3 skim milk at 37°C
Antiserum(dilution)	Rabbit C (1:8,000)
Assay buffer(pH 7.5)	1×BS
Average IC_{50}	96.97 ng · ml^{-1}
Detection range	14.9-631 ng · ml^{-1}
Lowest limit of detection (LOD)	8 ng · ml^{-1}

Optimization. On the basis of the results obtained through the screening of the antisera (Table 2), rabbit C antiserum and hapten III-BSA conjugate were selected as the antiserum and the coating antigen, respectively, for the subsequent ELISA. To obtain the most sensitive and desirable ELISA, various factors were evaluated (Table 3).

Effect of blocking agents. To prevent nonspecific sorption of an antibody onto the less-coated plate, a few blocking agents were evaluated. Because 3% skim milk exhibited the lowest IC_{50} value (186.1 ng · ml^{-1}) among the blockers tested, with

Table 5. Cross-reactivity of some structurally related compounds to the rabbit C antiserum in the ELISA

Compound	Chemical structure	IC_{50} ($ng \cdot ml^{-1}$)	CR (%)
Fenazaquin		176.67	100
Napropamide		1298.9	13.60
Carbaryl		2974.1	5.94
NAA(1-naphthyl acetic acid)		2786.9	6.34
Hapten		9.60	1840.31
Hapten		687.43	25.70
Hapten		338.51	52.19
Hapten		0.11	160609.09
Hapten		12.77	1383.48

*% Cross-reactivity (CR) = (IC_{50} of fenazaquin/IC_{50} of test compound)×100

the highest A/D ratio of 3.5 (Table 3), it was used as the blocker for the ELISA.

Effect of detergent. Although Tween 20 is known to reduce nonspecific interactions and enhance sensitivity,[15] its presence (0.05%, v/v) did not enhance the sensitivity of the fenazaquin ELISA, but instead showed a negative effect. This negative effect of Tween 20 on ELISA was also reported for other chemicals.[16~22]

Concentration of the assay buffer. Among the concentration levels tested, 0.5×PBS gave the lowest IC_{50} value (153.2 $ng \cdot ml^{-1}$).

Effect of pH. Of the pH values evaluated in the range of pH 4.5-9.5, the lowest IC_{50} value was obtained at pH 7.5 of the assay buffer.

Effect of solvents. To clarify the effect of organic solvents used in the preparation of the ELISA, acetone and methanol were tested at various concentrations. The two solvents affected the sensitivity of the ELISA, as evidenced by an increase in IC_{50} values in the presence of the solvents.

Some results obtained under the optimized conditions. Table 4 summarizes the optimized ELISA conditions and some important results obtained therefrom. Hapten-KLH conjugate, which has a spacer arm attached to the carbon-7 position on the quinazoline ring and maintains the 4-tert-butylbenzyloxy moiety, functioned better as the immunogen for ELISA than hapten-II-KLH conjugate with a spacer arm attached to the carbon-4 position of the quinazoline ring in

Table 6. Recovery of the analyte fenazaquin from fortified fruit samples by the ELISA

Sample	Fortified ($ng \cdot ml^{-1}$)	Theoretical concn in the ELISA ($ng \cdot ml^{-1}$)[b]	Detected concn ($ng \cdot ml^{-1}$)	Mean recovery (%, n=4)	Coefficient of variation (%)	Remark, MRL[a] (in Korea) $\mu g \cdot ml^{-1}$
Apple	0	0	0	0	1	0.1
	1500	30	28.86	96.21	12	
	3000	60	55.91	93.18	6	
	4500	90	94.29	104.77	5	
	6000	120	115.20	96.00	4	
Pear	0	0	0	0	1	0.3
	300	15	15.47	103.14	3	
	600	30	33.59	111.95	2	
	900	45	46.71	103.79	3	
	1200	60	47.64	79.40	7	

[a]Maximum residue limits

[b]Fortified concentrations were 50- and 20-fold diluted in apple and pear, respectively.

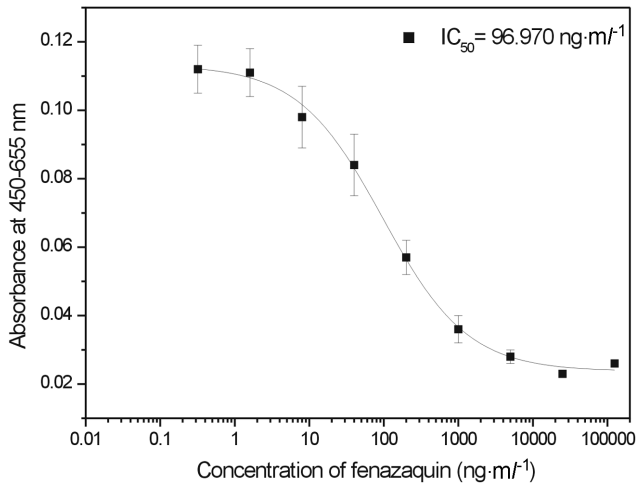


Fig. 4. Competitive standard curve of the fenazaquin ELISA drawn under the optimized conditions. Error bars represent standard deviations calculated from replicate calibration curves, which were obtained using the same set of standards (n=4).

place of the 4-tert-butylbenzyloxy moiety as observed in the inhibition test (Table 2). An average IC_{50} value of 96.97 $ng \cdot ml^{-1}$ was obtained, which is not low enough. To enhance the sensitivity, various trials are currently being conducted.

Cross-reactivities (CR). Specificity of the antiserum C produced against hapten-I-KLH conjugate was evaluated with some structurally related compounds using the optimized assay format (Table 5). The highest cross-reactivity with hapten IV indicates that the antiserum C recognizes very well the quinazoline ring, 4-tert-butylphenyl, and an adequate length of a spacer arm (currently under investigation). The cross-reactivity with hapten V is 53.8-fold higher than that with hapten II, and 26.5-fold higher than that with hapten III. This fact suggests that the length of the spacer arm affects the recognition of the quinazoline ring by the antibody. Cross-reactivity of hapten IV is 87.3-fold higher than that of hapten I. This result also indicates that the length of the spacer arm affects the recognition of the quinazoline ring and the 4-tert-butylphenyl moiety by the antiserum. The big difference in cross-reactivity between the chemicals containing 1-naphthyl group and those containing quinazoline ring proves that the antibody has a higher affinity for the latter than for the former.

Recovery of fenazaquin from fortified fruit samples. In the case of the apple sample, the mean recovery was in the range of 93.18-104.77% (n = 4) at 30 to 120 ppb. Likewise, in the case of pear, the recovery range was 79.40-111.95% (n = 4) at 15 to 60 ppb (Table 6), within the acceptable range in ELISA. Considering that the maximum residue limits set in Korea for apple and pear are 0.1 and 0.3 ppm, respectively, the ranges tested for recovery could be applied to monitoring fenazaquin residues in agricultural samples.

Conclusion

In Korea, fenazaquin has been in wide use to control two-spotted spider mite, European red mite, and citrus red mite in various fruits, and to determine its residues in agricultural produce, HPLC is usually used. The ELISA developed in this investigation could be a substitute for the HPLC method, which is time-consuming, laborious, and requires sophisticated technique. Even though the sensitivity of the developed ELISA is not high enough, it is nevertheless convenient for the purpose of monitoring residues in various agricultural and environmental samples. More sensitive ELISA formats are presently under investigation.

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