Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for Residue Analysis of the Fungicide Azoxystrobin in Agricultural Products

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ABSTRACT: A direct competitive enzyme-linked immunosorbent assay (dc-ELISA) was developed for residue analysis of azoxystrobin in garden crops, for which the maximum residue limits (MRLs) are 0.5−50 mg/kg in Japan. For hapten synthesis, an ethyl carboxyl group was introduced to the 4-position of the 2-cyanophenoxy group in azoxystrobin, and its cyano group was changed to a methyl group. An anti-azoxystrobin monoclonal antibody was prepared from mice immunized with hapten−keyhole limpet hemocyanin conjugate. The dc-ELISA using prepared antibody showed 50−250-fold higher sensitivity compared to the MRLs. The working range of the dc-ELISA was 10−200 ng/mL. The dc-ELISA showed high specificity to azoxystrobin. When methanol extracts from nine kinds of garden crops spiked with azoxystrobin ranging near the MRLs were analyzed, the determined results by the dc-ELISA agreed well with the results of their controls. In addition, azoxystrobin spiked in garden crops homogenates was satisfactorily extracted by methanol solution and easily analyzed. The recovery rate of dc-ELISA was 96−109% and correlated well with the results obtained by HPLC analysis.

KEYWORDS: *monoclonal antibody, immunoassay, dc-ELISA, strobilurin, azoxystrobin*

■ INTRODUCTION

Strobilurin fungicide azoxystrobin, methyl (2E)-2-(2-{[6-(2-cyanophenoxy)pyrimidin-4-yl]oxy}phenyl)-3-methoxyacrylate, was synthetically modified from a natural fungicidal substance, strobilurin A, produced by wood-rotting fungi. The modification improved the fungicidal activity against major plant pathogenic fungi (Ascomycete, Basidiomycete, Deuteromycete, and Oomycete), the systemic property, and the photochemical stability, which are necessary for a fungicide.[1] Azoxystrobin has also low toxicity: its LD_{50} value is >5000 mg/kg in rat.[2] On the basis of enough evidence of its efficacy and safety, azoxystrobin has been widely applied to control pathogenic fungi for many kinds of garden crops.

The maximum residue limits (MRLs) of azoxystrobin for garden crops have been set to 0.5−50 mg/kg in Japan.[3] Reported residue levels of garden crops were in the range of $1/5$ to $1/20$ of their MRLs as summarized in Table 1.[4−8] Azoxystrobin is a major residual pesticide, the detection of which in garden crops was actually reported in Japan between 1995 and 2009.[9] Monitoring is therefore important to prevent contaminated crops from being distributed in the market. The development of a rapid, simple, and low-cost monitoring method has been required for azoxystrobin residue analysis in garden crops.

Azoxystrobin is generally analyzed by a gas chromatograph equipped with a nitrogen−phosphorus detector[7] or by a high-performance liquid chromatograph (HPLC) equipped with an

Table 1. Residue Levels of Azoxystrobin Applied to Garden Crops

agricultural product	application	residue level (mg/kg) after application		MRL (mg/kg)	ref
		immediately	days after		
tomato	greenhouse	0.19	0.15 (7 days)	1	4
strawberry	field	0.55	0.15 (15 days)	3	5
grape	field	1.13	1.01 (15 days)	10	6
grape	field	0.50	NT[a]	10	7
grapefruit	postharvest	0.11	NT	1	8

[a]NT is not tested.

ultraviolet absorption detector.[10] Although such instrumental analyses are sufficiently sensitive and accurate, they are not practical for the on-site monitoring of garden crops because they are complicated, time-consuming, and expensive. As an alternative monitoring method that can be widely used for the on-site analysis, immunoassays have been developed for many
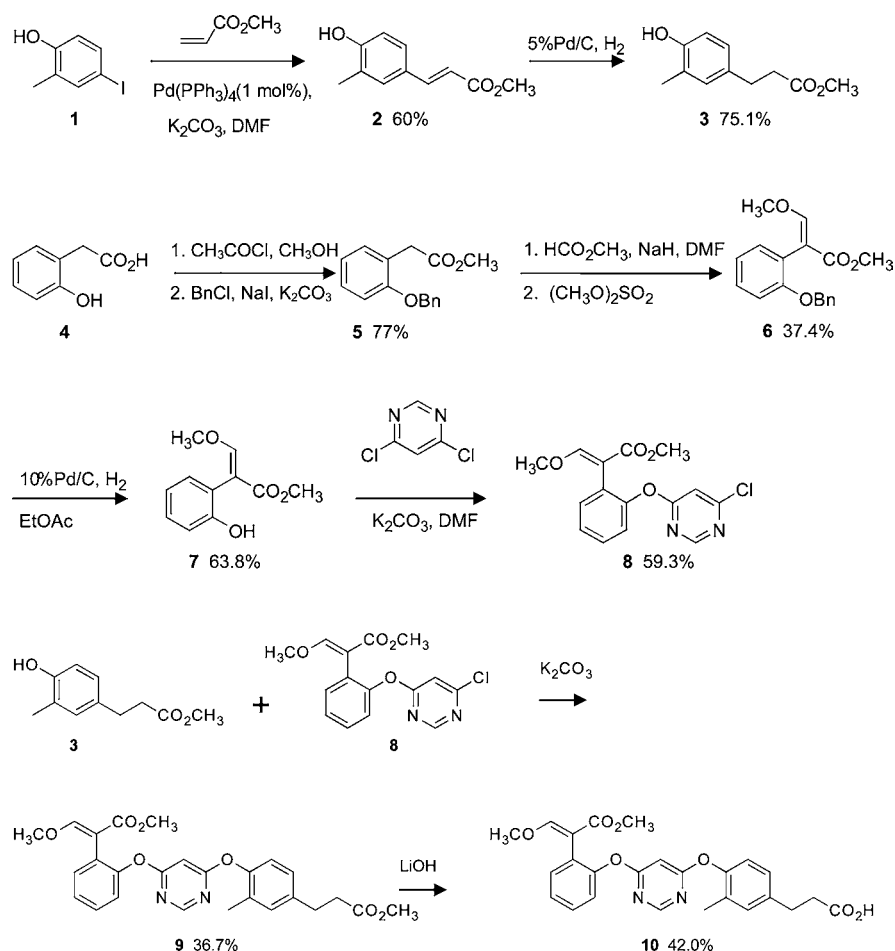
Figure 1. Scheme of new hapten synthesis.

agrochemical determinations.[11−18] They are not only simple, rapid, and cost-effective methods compared to instrumental analyses but also practically sensitive and accurate. An immunoassay was developed also for the determination of azoxystrobin.[19] The immunoassay was highly sensitive and showed the working range of 0−400 pg/mL. Such high sensitivity is useful to determine azoxystrobin concentration in the environment. However, the sensitivity was too high for azoxystrobin residue analysis near the MRLs in garden crops, for which >1000-fold dilution was necessary.

We have previously reported that the direct competitive enzyme-linked immunosorbent assay (dc-ELISA) developed for the insecticide emamectin benzoate is applicable to the residue analysis of garden crops with only 50-fold dilution as a sample pretreatment.[20] The dilution rate was appropriated for azoxystrobin analysis to neglect the matrix influence from garden crops. Our purpose in this paper is the development of a 50-fold or more sensitive dc-ELISA for azoxystrobin than the MRLs. This paper describes the preparation of a new hapten and a monoclonal antibody (MoAb) as key materials for the dc-ELISA and the constitution and performance of the dc-ELISA.

MATERIALS AND METHODS

Materials. Strobilurin fungicide azoxystrobin standard was purchased from Hayashi Pure Chemicals (Osaka, Japan). Two kinds of azoxystrobin metabolites (Z-isomer (98.0%) and hydrolysate (100%)) were kindly provided by Syngenta Japan (Tokyo, Japan). For hapten synthesis, dimethylformamide (DMF) dehydrated was purchased from Tokyo Chemical Industry Co., Ltd. Methanol

(MeOH) dehydrated was purchased from Sigma-Aldrich Co. Tetrahydrofuran (THF) dehydrated was purchased from Kanto Chemical Co., Inc. All other starting materials used were commercially available. For immunization, keyhole limpet hemocyanin (KLH) was purchased from Wako Pure Chemical. Freund's complete adjuvant and incomplete adjuvant were from Difco Laboratories (Detroit, MI). Seven-week-old female BALB/c mice were from Nippon SLC (Shizuoka, Japan). For cell fusion and cell culture, polyethylene glycol (PEG) 1500 reagent, RPMI 1640 medium, hypoxanthine thymidine (HT), and hypoxanthine aminopterin thymidine (HAT) reagents were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). Culture flasks and 96-well microplates were purchased from Thermo Fisher Scientific (Rockford, IL). For ELISA constitution, horseradish peroxidase (HRP) was purchased from Toyobo (Osaka, Japan). Bovine serum albumin (BSA) was from Sigma-Aldrich. Gel filtration column (Sephadex G-25, Super-Fine) and Protein G column were from GE Healthcare U.K. (Buckinghamshire, U.K.). HRP-labeled goat anti-mouse IgG antibody and 96-well microtiter plates were purchased from Thermo Fisher Scientific. All other chemicals and reagents were of analytical grade purchased from Wako Pure Chemical or Nacalai Tesque (Kyoto, Japan).

Hapten Synthesis. General Considerations. A new hapten synthesis is summarized in Figure 1. All reactions were performed under an atmosphere of argon unless otherwise noted. All reactions were monitored by thin layer chromatography (TLC); glass plates were precoated with silica gel Merck KGaA 60 F254, layer thickness = 0.2 mm. The products were visualized by irradiation with UV light or using I₂. Flash column chromatography was performed using silica gel (Merck, article no. 7734). ¹H NMR (400 or 300 MHz) and ¹³C NMR (100 or 75 MHz) spectra were recorded on a Varian Inova-400 or a

Mercury-300 spectrometer. Chemical shifts are reported as δ values (ppm) relative to CDCl$_3$ (7.26 ppm). IR spectra were recorded by using a JASCO FT/IR-230 spectrometer and are reported in reciprocal centimeters (cm^{-1}). Elemental analyses were measured on a Yanaco CHN CORDER MT-6.

α,β-Unsaturated Ester 2. To a mixture of 4-iodo-2-methylphenol **1** (2.34 g, 10.0 mmol), K$_2$CO$_3$ (6.91 g, 50.0 mmol), and Pd(PPh$_3$)$_4$ (11.6 mg, 0.01 mmol) in degassed DMF (10.0 mL) was added methyl acrylate (2.70 mL, 30 mmol). The reaction mixture was heated to 80 °C under argon atmosphere for 19 h. After the reaction mixture cooled to room temperature, the product was extracted with ether and dried over Na$_2$SO$_4$ anhydrous. The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 3:1→1:2 v/v) to afford Heck−Mizoroki reaction product **2** in 60% yield (1.16 g) as a yellow solid: R_f = 0.21 (hexane/EtOAc = 3:1 v/v); IR (neat) v 3233, 3032, 2952, 2851, 2367, 1683, 1636, 1598, 1508 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 2.26 (s, 3H), 3.79 (s, 3H), 5.15 (s, 1H), 6.30 (d, J = 16.2 Hz, 1H), 6.78 (d, J = 8.5 Hz, 1H), 7.28 (dt, J = 10.7, 1.9 Hz, 2H), 7.62 (d, J = 15.7 Hz, 1H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 15.9, 51.9, 114.9, 115.5, 124.7, 127.1, 127.8, 131.2, 145.3, 156.4, 168.4. Anal. Calcd for C$_{11}$H$_{12}$O$_3$: C, 68.74%; H, 6.29%. Found: C, 68.49%; H, 6.41%.

Saturated Ester 3. α,β-Unsaturated ester **2** (1.16 g, 6.02 mmol) was dissolved in MeOH (10.0 mL) and simply hydrogenated by using 5 wt % Pd/C (640.0 mg, 0.30 mmol) and H$_2$ (balloon). The reaction was monitored by TLC, and after 20 h, the reaction mixture was filtered off under vacuum. The solvent was evaporated under reduced pressure, and the residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 1:1 v/v) to give the saturated ester **3** in 75.1% yield (877.4 mg) as a white solid: R_f = 0.66 (hexane/EtOAc = 1:1 v/v); IR (neat) v 3418, 3016, 2944, 2914, 2850, 2722, 1718, 1612, 1519, 1443, 1374, 1251, 1187 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 2.22 (s, 3H), 2.59 (t, J = 8.2 Hz, 2H), 2.85 (t, J = 8.0 Hz, 2H), 3.67 (s, 3H), 4.67 (s, 1H), 6.69 (d, J = 8.0 Hz, 1H), 6.90 (dd, J = 8.0, 1.9 Hz, 1H), 6.95 (s, 1H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 15.9, 30.2, 36.2, 51.8, 115.0, 124.1, 126.7, 131.0, 132.3, 152.6, 174.2. Anal. Calcd for C$_{11}$H$_{14}$O$_3$: C, 68.02%; H, 7.27%. Found: C, 67.70%; H, 7.44%.

Methyl 2-(2-Hydroxyphenyl)acetate 5. Acetyl chloride (neat, 5.68 g, 72.3 mmol) was carefully added to dry MeOH (25.0 mL), followed by the addition of 2-hydroxyphenylacetic acid **4** (5.00 g, 32.9 mmol), and the resulting mixture was stirred for 3 h at room temperature. The solvent was removed under reduced pressure, and the residue was treated with saturated NaHCO$_3$ (6.0 mL). The methyl ester product was extracted with ether and dried over Na$_2$SO$_4$ anhydrous, and the solvent was evaporated under reduced pressure. To a mixture of the crude methyl ester, K$_2$CO$_3$ (1.36 g, 98.6 mmol), and NaI (4.93 g, 32.9 mmol) in DMF (60.0 mL) was added a solution of benzyl chloride (4.17 g, 32.9 mmol) in DMF (15.0 mL) at 0 °C. The resulting mixture was stirred for 24 h at room temperature followed by the filtration of K$_2$CO$_3$ and NaI. After the addition of water (80 mL), the product was extracted with ether and dried over Na$_2$SO$_4$ anhydrous, and the solvent was evaporated under reduced pressure. The crude benzyl ether product was purified by column chromatography on silica gel (hexane/EtOAc = 6:1 v/v) to furnish **5** in 77% yield (6.49 g, for two steps) as a white solid: R_f = 0.42 (hexane/EtOAc = 6:1 v/v); IR (neat) v 2945, 1732, 1598, 1459, 1333, 1246, 1214 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 3.63 (s, 3H), 5.09 (s, 2H), 6.94 (t, J = 8.2 Hz, 2H), 7.19−7.43 (m, 7H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 36.2, 51.9, 70.0, 111.9, 120.9, 123.6, 127.2, 127.9, 128.6, 128.7, 131.1, 137.2, 156.7, 172.4. Anal. Calcd for C$_{16}$H$_{16}$O$_3$: C, 74.98%; H, 6.29%. Found: C, 75.00%; H, 6.37%.

Benzyl Ether 6. A solution of ester **5** (800.0 mg, 3.12 mmol) was added to a suspension of 60 wt % NaH (washed with dry hexane, 249.7 mg, 6.24 mmol) in dry DMF (7.0 mL) at 0 °C. The mixture was stirred for 45 min followed by the addition of methyl formate (0.58 mL, 9.36 mmol) via syringe at 0 °C. After an additional 1 h of stirring at room temperature, dimethyl sulfate (0.6 mL, 6.24 mmol) was added at 0 °C. After 20 min, the reaction was quenched with saturated NH$_4$Cl solution (5.0 mL), and the organic product was extracted with ether and dried over Na$_2$SO$_4$ anhydrous. The solvent was removed

under reduced pressure, and the residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 4:1 v/v) to give **6** in 37.4% yield (347.9 mg) as a white solid: R_f = 0.55 (hexane/EtOAc = 2:1 v/v); IR (neat) v 2943, 1708, 1636, 1495, 1445, 1377, 1254, 1119 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 3.65 (s, 3H), 3.80 (s, 3H), 5.08 (s, 2H), 6.92−7.01 (m, 2H), 7.20−7.42 (m, 7H), 7.51 (s, 1H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 51.6, 61.8, 70.1, 108.9, 112.6, 120.7, 122.4, 126.9, 127.7, 128.5, 129.2, 132.0, 137.6, 156.4, 159.5, 168.5. Anal. Calcd for C$_{18}$H$_{18}$O$_4$($^1/_{10}$H$_2$O): C, 72.03%; H, 6.11%. Found: C, 72.01%; H, 6.08%.

Phenol Derivative 7. A suspension of 10 wt % Pd/C (60.0 mg, Pd: 0.03 mmol) and **6** (347.9 mg, 1.17 mmol) in MeOH (9.0 mL) was stirred under H$_2$ atmosphere (balloon) at room temperature for 25 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 2:1 v/v) to afford the desired compound **7** in 63.8% yield (155.0 mg, 0.74 mmol) as a white solid: R_f = 0.19 (hexane/EtOAc = 2:1 v/v); IR (neat) v 3385, 2947, 1689, 1498, 1446, 1265, 1134 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 3.77 (s, 3H), 3.89 (s, 3H), 6.19 (s, 1H), 6.93 (dt, J = 7.5, 1.4 Hz, 1H), 6.98 (dd, J = 8.2, 1.4 Hz, 1H), 7.16 (dd, J = 7.7, 1.6 Hz, 1H), 7.22 (dd, J = 7.7, 1.6 Hz, 1H), 7.63 (s, 1H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 52.3, 62.5, 107.9, 117.5, 119.5, 120.4, 129.7, 132.0, 153.7, 161.1, 169.1. Anal. Calcd for C$_{11}$H$_{12}$O$_4$ ($^1/_{10}$H$_2$O): C, 62.91%; H, 5.86%. Found: C, 63.09%; H, 6.00%.

Monocoupling Pyrimidine Derivative 8. To a suspension of phenol derivative **7** (49.9 mg, 0.24 mmol) and K$_2$CO$_3$ (49.8 mg, 0.36 mmol) in DMF (4.0 mL) was added a solid amount of 4,6-dichloropyrimidine (107.1 mg, 0.72 mmol), and the resulting mixture was stirred for 3 days at room temperature. The product was extracted with ether and dried over Na$_2$SO$_4$ anhydrous, and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 2:1 v/v) to give **8** in 59.3% yield (45.6 mg) as a yellow oil: R_f = 0.37 (hexane/EtOAc = 2:1 v/v); IR (neat) v 2947, 1708, 1635, 1554, 1491, 1443, 1258, 1200, 1131, 1056 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 3.60 (s, 3H), 3.73 (s, 3H), 6.78 (d, J = 0.8 Hz, 1H), 7.17 (dq, J = 8.0, 0.5 Hz, 1H), 7.44−7.28 (m, 3H), 7.45 (s, 1H), 8.58 (d, J = 0.9 Hz, 1H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 51.7, 62.0, 107.0, 107.3, 122.0, 126.0, 126.2, 129.4, 132.9, 150.0, 158.6, 160.7, 161.9, 167.3, 170.4. Anal. Calcd for C$_{15}$H$_{13}$ClN$_2$O$_4$ ($^1/_3$EtOAc)($^1/_3$H$_2$O): C, 55.09%; H, 4.62%; N, 7.87%; Found: C, 54.82%; H, 4.25%; N, 7.83%.

Azoxystrobin Hapten Ester 9. To a suspension of **3** (23.3 mg, 0.12 mmol) and K$_2$CO$_3$ (24.9 mg, 0.18 mmol) in DMF (0.3 mL) was added a solution of **8** (37.6 mg, 0.12 mmol) in DMF (0.3 mL), and the resulting mixture was stirred for 4 days at room temperature. The product was extracted with ether and dried over Na$_2$SO$_4$ anhydrous, and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc = 2:1 v/v) to furnish **9** in 36.7% yield (21.0 mg) as a yellow solid: R_f = 0.21 (hexane/EtOAc = 2:1 v/v); IR (neat) v 3056, 2985, 1711, 1636, 1577, 1496, 1447, 1387, 1263, 1202, 1148 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 2.13 (s, 3H), 2.64 (t, J = 7.4 Hz, 2H), 2.94 (t, J = 8.5 Hz, 2H), 3.59 (s, 3H), 3.69 (s, 3H), 3.74 (s, 3H), 6.19 (d, J = 0.8 Hz, 1H), 6.96 (d, J = 8.2 Hz, 1H), 7.04−7.44 (m, 6H), 7.46 (s, 1H), 8.40 (d, J = 0.8 Hz, 1H); ^{13}C NMR (100 MHz, CDCl$_3$) δ 16.4, 30.4, 35.7, 51.6, 51.8, 62.0, 91.4, 107.4, 121.8, 122.2, 125.8, 126.0, 127.2, 129.2, 130.6, 131.6, 132.7, 138.4, 149.4, 150.4, 158.5, 160.7, 167.7, 171.5, 171.6, 173.4. Anal. Calcd for C$_{26}$H$_{26}$N$_2$O$_7$($^1/_2$EtOAc): C, 64.36%; H, 5.79%; N, 5.36%. Found: C, 64.72%; H, 5.96%; N, 5.46%.

Azoxystrobin Hapten 10. To a solution of LiOH (1.0 mg, 0.04 mmol) in THF/H$_2$O (1:1 v/v, 0.6 mL) was added a solid amount of **9** (19.1 mg, 0.04 mmol), and the resulting mixture was stirred for 2 h followed by the addition of saturated NH$_4$Cl solution (1 drop). The product was extracted with CHCl$_3$ and dried over Na$_2$SO$_4$ anhydrous, and the solvent was removed under reduced pressure to afford the pure carboxylic acid derivative **10** in 42.0% yield (8.0 mg) as a yellow solid. This product was used for the preparation of the antibody. **10**: R_f = 0.33 (EtOAc); IR (neat) v 2925, 1709, 1573, 1450, 1388, 1254, 1200, 1141, 992 cm^{-1}; ^{1}H NMR (300 MHz, CDCl$_3$) δ 2.11 (s, 3H),

2.65 (t, J = 7.2 Hz, 2H), 2.92 (t, J = 7.1 Hz, 2H), 3.58 (s, 3H), 3.74 (s, 3H), 6.19 (s, 1H), 6.94 (d, J = 8.3 Hz, 1H), 7.09 (t, J = 8.5 Hz, 2H), 7.20 (d, J = 8.5 Hz, 1H), 7.27–7.43 (m, 4H), 7.46 (s, 1H), 8.39 (s, 1H); ^{13}C NMR (75 MHz, CDCl$_3$) δ 16.3, 29.9, 30.6, 51.6, 62.0, 77.4, 91.5, 107.6, 121.9, 122.3, 125.8, 126.1, 127.2, 129.2, 130.6, 131.6, 132.8, 138.6, 149.6, 150.6, 158.5, 160.7, 167.7, 171.6, 171.7.

Hapten−Protein Conjugate Preparation. Azoxystrobin hapten was conjugated with proteins (KLH, BSA, and HRP) by the activated ester method as previously described.[20] The hapten (20 μmol) was dissolved in 1 mL of dried dimethyl sulfoxide. N-Hydroxysuccinimide (40 μmol) and 1-ethyl-3-(3-dimethylamiopropyl) carbodiimide hydrochloride (40 μmol) were added in the solution. The solution was stirred at room temperature for 1.5 h to esterify the carboxylic acid and the succinimide. An amount of 206 μL of the stirred solution was added to KLH (20 mg) dissolved in 1 mL of borate-buffered saline (100 mmol/L sodium borate, 150 mmol/L NaCl, pH 8.0). A second portion of the stirred solution (277 μL) was added to BSA (10 mg) solution. A third portion of the stirred solution (200 μL) was added to HRP (10 mg) solution. Each of the mixtures was gently stirred at room temperature for 1.5 h to combine the activated carboxyl group of the hapten with the amino group of L-lysine residues in the proteins. The combined hapten−KLH and the hapten−BSA conjugates were dialyzed against 10 mmol/L phosphate-buffered saline (PBS; 10 mmol/L sodium phosphate, 150 mmol/L NaCl, pH 7.2) at 4 °C for 4 days to remove the uncombined chemicals. On the other hand, the hapten−HRP conjugate was purified through the gel filtration column. The prepared hapten−KLH conjugate was used to immunize mice, the hapten−BSA conjugate was used to constitute a direct-bind ELISA (db-ELISA) and an indirect competitive ELISA (ic-ELISA), and the hapten−HRP conjugate was used to constitute a dc-ELISA.

MoAb Preparation. MoAbs were prepared by a slight modification of the previously described procedure.[21] BALB/c mice were intraperitoneally immunized with 100 μL of the hapten−KLH conjugate (100 μg per mouse) after it had been emulsified with an equal volume of Freund's complete adjuvant. Booster immunization (25 μg per a mouse) was performed four times using the emulsion with Freund's incomplete adjuvant at intervals of 2 weeks. On the third day after the last immunization, spleen cells from the mice (5 × 10^8 cells) were fused with P3U1 myeloma cells (5.5 × 10^7 cells) by using PEG 1500 reagent. The fused cells were suspended at 2.5 × 10^6 cells/mL (spleen cells) in RPMI 1640 medium modified with 10% FBS and with HT reagent; 100 μL of each was transferred to the wells of a 96-well microplate. The microplate was incubated at 37 °C for 24 h in 5% CO$_2$, and then 40 μL of RPMI 1640 modified with 10% FBS and with HAT reagent was added to each well. After it had been confirmed that the growing hybridoma had formed a colony, each of the secreted antibodies in the cultured fluids was screened on the basis of reactivity with the hapten−BSA conjugate by the db-ELISA. Fluids in the positive wells were secondarily screened on the basis of reactivity with azoxystrobin by the ic-ELISA. Each hybridoma grown in the positive wells was cloned by limiting dilution technique (two times), and the representative cell clone was used for preparation of the MoAb.

For MoAb preparation, BALB/c mice were pretreated by intraperitoneal injection with 0.5 mL of pristane, and 1 week after the pretreatment, the mice were inoculated with 2 × 10^7 viable cells. Seven to 10 days after the inoculation, ascitic fluids produced were collected from the mice, and the MoAb in the fluid was purified on a protein G column. The MoAb concentration was determined from the extinction coefficient (1.4 for 1.0 mg/mL of IgG).

db-ELISA and ic-ELISA. A db-ELISA and an ic-ELISA were constituted by a slight modification of the previously described procedure.[20] For both of the ELISA constitutions, 100 μL of hapten−BSA conjugate (1.0 μg/mL) dissolved in PBS was initially added to each well of a 96-well microtiter plate. The conjugate was physically bound to the well by incubation at 4 °C overnight. After three washings with PBS, the well surface was blocked with BSA by addition of 300 μL of 0.4% BSA dissolved in PBS. The blocked well was washed again with PBS.

For a db-ELISA, each 50 μL of cultured fluids of the hybridoma or the diluted fluids with PBS modified with 0.2% BSA (PBS−BSA) was added to the blocked well. The microtiter plate was incubated at 25 °C for 1 h. After the plate was washed three times with PBS, 100 μL of HRP-labeled goat anti-mouse IgG antibody diluted to 8000-fold with PBS−BSA was added to each well. The plate was incubated at 25 °C for 1 h and then washed three times with PBS. One hundred microliters of HRP substrate solution (2 mg/mL of 3,3′,5,5′-tetramethylbenzidine and 0.006% H$_2$O$_2$ dissolved in 0.1 mol/L sodium acetate buffer, pH 5.5) was added to each well, and the plate was incubated at 25 °C for 10 min to develop color reaction. The reaction was stopped by the addition of 100 μL of 0.5 mol/L sulfuric acid. The absorbance was measured at a wavelength of 450 nm in the microplate reader (MPR-01, HORIBA, Kyoto, Japan).

For an ic-ELISA, azoxystrobin was dissolved and serially diluted with MeOH. The diluents were further diluted to 10-fold with PBS−BSA: the prepared concentrations were 12.5−5000 ng/mL. The cultured fluid was diluted with PBS−BSA to 2-fold concentration to which 50% of maximum absorbance was developed by the above db-ELISA. Each 50 μL of the azoxystrobin solutions was added to the above blocked wells, and then 50 μL of the diluted cultured fluid was immediately mixed in the wells. The plate was incubated at 25 °C for 1 h. The subsequent steps were taken as described in the above db-ELISA.

dc-ELISA. A dc-ELISA was constituted by a slight modification of the previously described procedure.[20] A rabbit anti-mouse IgG antibody (100 μL, 10 μg/mL) dissolved in PBS was added to each well of a 96-well microtiter plate. The plate was incubated at 4 °C overnight. After the antibody-coated plate was washed three times with PBS, the wells were blocked with BSA by the addition of 300 μL of 1% BSA dissolved in PBS. The plate was incubated at 25 °C for 1 h. One hundred microliters of purified MoAb (100 ng/mL) dissolved in PBS−BSA was added to each well, and the MoAb was bound to the coated antibody based on antigen−antibody reaction by incubation at 25 °C for 1 h.

Azoxystrobin was dissolved and serially diluted with MeOH. The diluents were further diluted to 10-fold with PBS−BSA: the prepared concentrations were 0.1−100 ng/mL. A hapten−HRP conjugate was diluted to the adequate concentration (1.5 ng/mL) with PBS−BSA, so that the maximum absorbance showed around 1.3 at 450 nm by this dc-ELISA. Each of the azoxystrobin solutions or sample solutions prepared from garden crops was mixed with an equal volume of the hapten−HRP conjugate solution, and the mixture was added to the above well. The plate was incubated at 25 °C for 1 h. After the plate was washed three times with PBS, color development steps were taken as described in the above db-ELISA.

Treatment of Garden Crop Samples. Garden crop samples were treated on the basis of the previously described procedure.[20] Cucumber, lettuce, tomato, leek, grape, melon, peach, strawberry, and watermelon were collected from a vegetable shop selling pesticide-free garden crops. Each sample was homogenized, and the homogenized sample (5 g) was put in a 50 mL plastic tube. Twenty-five milliliters of MeOH was added to each tube, and the tube was hermetically sealed with a screw cap. It was vigorously shaken for 30 min to extract azoxystrobin. The extract was filtered through a filter paper, and 1 mL of the filtrate was diluted with 7.5 mL of distilled water. The diluted sample was used for the dc-ELISA: the MeOH concentration was equivalent to 10%. Further dilutions of the diluted sample were carried out with water/MeOH (9:1, v/v; 10% MeOH).

For determination of azoxystrobin spiked in garden crop extract, 100 μL of azoxystrobin dissolved in MeOH was added to 5.9 mL of the above MeOH extract: the concentrations were equivalent to 0.5−10 mg/kg in garden crops. Separately, 100 μL of azoxystrobin was added to the above homogenized crop samples (5 g) for recovery examination: the concentrations were equivalent to 0.6−9.2 mg/kg.

HPLC Analysis. HPLC analysis was carried out on the basis of the standard procedure in Japan.[10] Each homogenized sample (20 g) was mixed with 100 mL of MeOH. The mixture was immediately shaken for 30 min. The extract was filtered through, and the residue was refiltered after mixing with 50 mL of MeOH. The combined filtrate
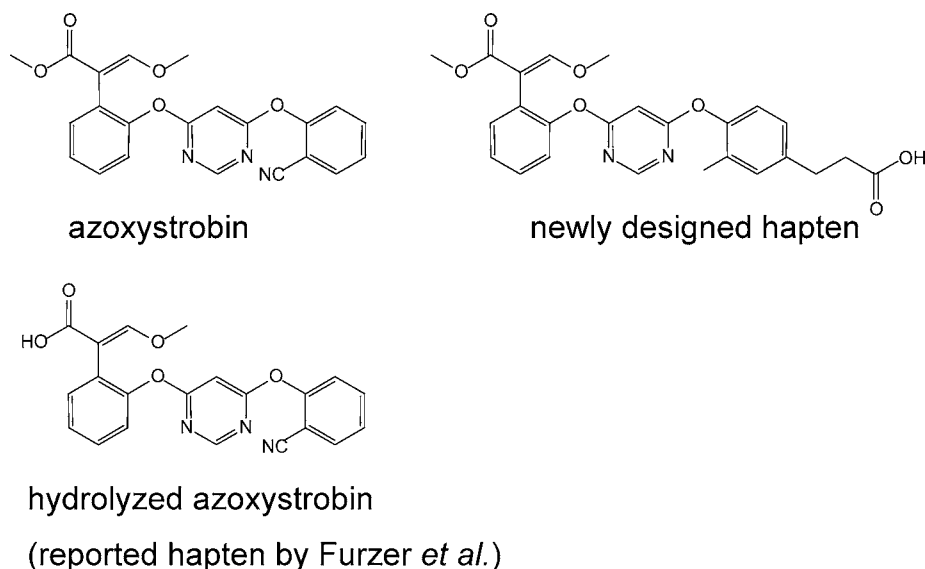
Figure 2. Structures of azoxystrobin and its haptens.

was filled to 200 mL with MeOH. The aliquot (100 mL) was concentrated to approximately 10 mL by evaporation, and then 10 mL of water and NaCl (1.0 g) were added. The solution was applied to macroporous diatomaceous material column and allowed to stand for 10 min. The column was eluted with 100 mL of acetyl acetate. The eluate was dried by evaporation and redissolved with 1 mL of acetone. The solution was mixed with 19 mL of hexane. The solution was applied to a Florisil column (910 mg). The column was washed with 10 mL of a mixture of hexane and acetone (90:10, as volume) and then eluted with 20 mL of a mixture of hexane and acetone (70:30, as volume). The eluate was dried by evaporation and redissolved with 5 mL of mixture of hexane and acetone (90:10, as volume). The dissolved solution was applied to a silica gel column (690 mg). The column was washed with 5 mL of a mixture of hexane and acetyl acetate (70:30, as volume) and then eluted with 20 mL of a mixture of hexane and acetyl acetate (50:50, as volume). The eluate was dried by evaporation and then redissolved with a mixture of acetonitrile and water (50:50, as volume).

The solution was applied to the Jasco 900 series Gulliver HPLC system (Jasco Corp., Tokyo, Japan). The column was a CAPCELL PAK C18 UG80 (250 mm × 4.6 mm, 5 μm particle size). The mobile phase was a mixture of acetonitrile and water (60:40, as volume). The flow rate was 1.0 mL/min. The column oven temperature was 40 °C. The ultraviolet absorption at 240 nm was used for azoxystrobin detection.

■ RESULTS AND DISCUSSION

Preparation of an Anti-azoxystrobin MoAb. Furzer et al. reported that a hydrolyzed azoxystrobin as shown in Figure 2 was a useful hapten for the preparation of a highly reactive antibody to azoxystrobin. The ic-ELISA constituted with the hapten and the prepared antibody showed 0–400 pg/mL of measurement range.[19] The sensitivity is too high to determine azoxystrobin concentration near the MRLs in Japan, 0.5–50 mg/kg for garden crops. The reported reactivity is 1000–100 000 times higher than the expected level. As a result, the measurement sample needs to be diluted by many fold for ic-ELISA application, and such high dilution makes the ic-ELISA inconvenient and inaccurate. Preparation of less reactive antibody has been, therefore, required to constitute an adequately sensitive immunoassay for azoxystrobin residue analysis in garden crops.

A hapten shown in Figure 2 was newly designed in this study. Carboxylic acid and methylene linker (C = 2) were introduced to the 4-position of the 2-cyanophenoxy group in azoxystrobin. The introduced position was changed to the opposite end from the reported hapten to adjust the reactivity and specificity of prepared antibodies to azoxystrobin. Furthermore, a methyl group was introduced instead of the cyano group to suppress reactivity. Polyclonal antibody (PoAb) was prepared from mice immunized with the hapten−KLH conjugate, and its reactivity was examined by ic-ELISA. As shown in Figure 3, 50% of
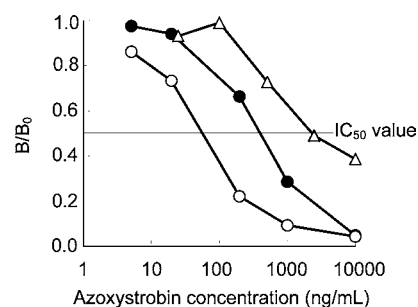

Figure 3. Reactivity of prepared antibodies with azoxystrobin in ic-ELISA: (○) MoAb 11A; (●) MoAb 2B; (△) PoAb. Each data point is the mean of duplicates.

inhibition concentration (IC_{50}) value of the PoAb was 2400 ng/mL. The level of reactivity is too low to determine azoxystrobin residues in garden crops near the MRLs.

MoAbs have been expected to reach to dozens-fold higher reactivity compared to the PoAb from the same immunized mouse as previously described.[20,22−25] An adequately reactive MoAb should be, therefore, prepared by using the immunized mice. Two MoAbs, that is, MoAb 11A and MoAb 2B, were actually prepared from the mice. MoAb 11A, of which subclass was IgG_1 and light chain was kapper, was 40-fold reactive than the PoAb on the basis of the IC_{50} values by ic-ELISA as shown in Figure 3: the IC_{50} value was 58 ng/mL. The IC_{50} value signifies that MoAb 11A is applicable to constitute dc-ELISA for the determination of azoxystrobin residues near the MRLs.

dc-ELISA Constitution Based on MoAb 11A. A dc-ELISA is usually adopted for the development of commercially available immunoassay test kits for agrochemicals[26−32] because the dc-ELISA needs fewer assay steps than the ic-ELISA. The dc-ELISA for azoxystrobin was therefore adopted: the coating quantity of MoAb 11A was 10 ng per well. The working concentration range of the constituted dc-ELISA was between 10 ng/mL (IC_{20} value) and 200 ng/mL (IC_{80} value) as the azoxystrobin concentration as shown in Figure 4. The
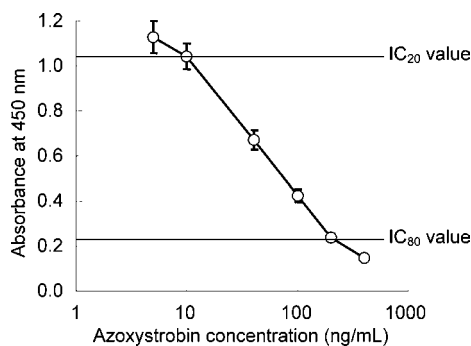

Figure 4. Reactivity of MoAb 11A with azoxystrobin in dc-ELISA. Each data point is the mean of four replicates; error bars indicate standard deviations.

maximum absorbance was 1.3 at 450 nm, and the IC_{50} value was 38 ng/mL. The standard deviation (SD) also showed high repeatability (5.5−7.0% as coefficient of variation, CV), although the SD data were preliminary because there were only four replicates. Such high repeatability is considered to come from tight binding between MoAb 11A and the hapten−HRP conjugate and to be achieved by the choice of high-affinity MoAb against the hapten−BSA conjugate on the first MoAb screening. The constituted dc-ELISA is expected to be adequately reactive to determine the residual azoxystrobin near the MRLs.

The reactivity of MoAb 11A in the dc-ELISA was examined with azoxystrobin itself and a stereoisomer (Z-isomer) and a metabolite of it. As described in Table 2, the Z-isomer gave

Table 2. Cross-Reactivity of MoAb 11A with Azoxystrobin Isomer and Metabolite in dc-ELISA

compound	structure	cross-reactivity (%)
azoxystrobin		100
Z-isomer		30
hydrolyzed azoxystrobin		<0.1

cross-reactivity of 30%. This shows that MoAb 11A recognizes differences of stereoisomers to some extent. On the other hand, hydrolyzed azoxystrobin showed no cross-reactivity. Such a

drastic change of reactivity may be caused by the ionization of the carboxylic acid residue, as well as by the change of shape and polarity. The binding site of MoAb 11A is considered to be a nonpolar pocket from the methyl ester structure of the hapten, whereas the carboxylic acid residue of hydrolyzed azoxystrobin can be ionized due to physiological condition during the examination. The ionization of hydrolyzed azoxystrobin is supposed to have a strong influence on the binding ability with MoAb 11A. Actually, we previously experienced a similar influence on reactivity of a prepared MoAb against chrysanthemic acid, which is an acid moiety of pyrethroid insecticides.[24] The prepared MoAb showed weak reactivity with the chrysanthemic acid at physiological pH, whereas the reactivity increased by pH shift to the lower side in the solution.

The results of the cross-reactivity examination suggest that MoAb 11A can recognize slight differences of characteristic structures in azoxystrobin and its related compounds. This ability may be derived from the fact that the linker direction of the hapten is positioned to the opposite end from the methoxyacrylate residue discussed above.

As a result, it is found that the dc-ELISA with the hapten and MoAb 11A has adequate characteristics, for example, reactivity and specificity, for azoxystrobin residue analysis near the MRLs.

Determination of Azoxystrobin Spiked in Garden Crop Extracts. Azoxystrobin spiked in garden crop extracts was determined and compared to the control results (10% MeOH with azoxystrobin), to confirm the influence of the sample matrix coextracted from the garden crops. The typical pretreatment condition for immunoassays is as described in previous papers:[20,27−32] addition of 5 parts of MeOH to 1 part of sample homogenates, extraction by vigorous shaking for 30 min, and dilution of the extracts with purified water to 8.5-fold (10% MeOH adequate).

Azoxystrobin was added to nine kinds of garden crop extracts: cucumber, lettuce, tomato, leek, grape, melon, peach, strawberry, and watermelon. The concentrations were equivalent to 0.5, 1.0, 5.0, and 10 mg/kg in the garden crops. The extracts were applied for determination of azoxystrobin by the dc-ELISA. As shown in Figure 5, results from the extracts highly agreed with the control results for cucumber, tomato, leek, and strawberry. The other crops also showed good agreement (data not shown). These results demonstrate that the crop matrices treated under the above condition show no influence in the dc-ELISA and that the dc-ELISA is applicable to the analysis of the residual azoxystrobin in MeOH extracts.

Recovery Examination of Azoxystrobin Spiked in Garden Crop Homogenates. Recovery examination was carried out to confirm the extraction efficiency using cucumber, tomato, leek, and strawberry. Azoxystrobin was spiked at 0.6, 2.0, and 9.2 mg/kg in the crop homogenates, respectively. After standing for 30 min, the homogenates were extracted with MeOH under the same conditions as in the previous section. As described in Table 3, all of the recovery results were close to the spiking concentration. The obtained recovery rates were 96−109%. Such high recovery rates may be achieved because the dc-ELISA can determine azoxystrobin with only extraction and dilution steps and without purification. The repeatability was also good, although the data were preliminary due to only three replicates: the CV was <6.6%. The results suggest that the dc-ELISA has a large potential to quantitatively determine residual azoxystrobin in the crops, even though aged azoxystrobin, which may be contained in real samples, was
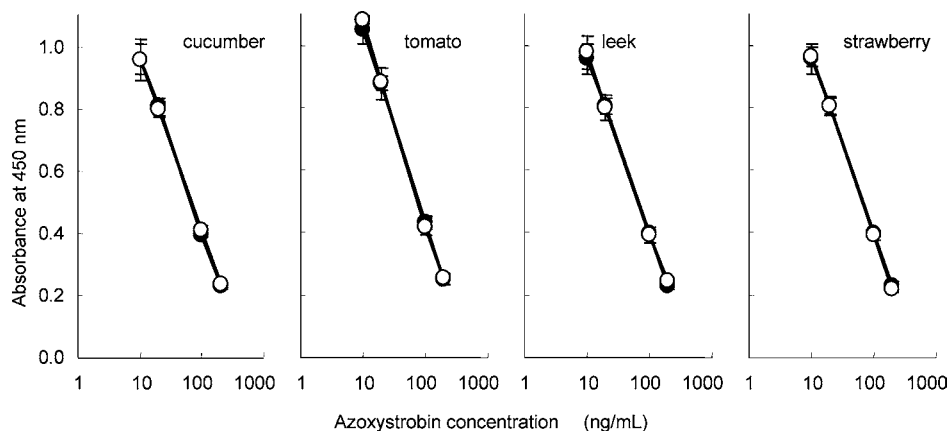
Figure 5. Comparison of standard curve for 10% methanol (○) and the corresponding curve for garden crop extracts (●) in dc-ELISA. Each data point is the mean of duplicates.

Table 3. Recovery of Azoxystrobin Spiked in Garden Crop Homogenates

concentration[b] (mg/kg)	recovery[a] (%)			
	cucumber	tomato	leek	strawberry
0.6	103 ± 1.8	102 ± 2.2	100 ± 2.1	96 ± 2.1
2.0	103 ± 3.0	99 ± 2.2	101 ± 2.1	109 ± 2.7
9.2	104 ± 1.6	101 ± 1.4	103 ± 1.8	100 ± 6.6

[a]Values are the mean of three replicates ± SD. [b]The concentrations are expressed as value in garden crops.

not determined in this examination. Comparison of HPLC and the dc-ELISA using real samples is desirable as a future work. The recovery rates of >100% might be derived from uncertainty in sample treatment process and/or minor matrix influence. These results suggest that it would not be a problem to use the dc-ELISA for azoxystrobin residue analysis because 70−120% recoveries are permitted in the United States and Japan.[33,34]

The recovery results were also confirmed by HPLC analysis instead of dc-ELISA, using cucumber, tomato, and leek. Azoxystrobin was spiked to the crop homogenates and extracted under the same conditions as described above. The recovery results were 75−89% for all of the examined samples (data not shown). The results correlated well with those obtained by dc-ELISA; however, the recovery rate was approximately 20% lower than the dc-ELISA results. As azoxystrobin was stable through all steps in the examination, the difference might be caused by loss on sample purification for the HPLC analysis.

In conclusion, for cucumber, tomato, and strawberry, for which the MRLs are 1 mg/kg, the dc-ELISA could determine azoxystrobin near the MRL without any additional pretreatments. On the other hand, for leek, for which the MRL is 7.5 mg/kg, the dc-ELISA could determine azoxystrobin near the MRL by 5-fold additional dilution with 10% MeOH. As most of the MRLs are set at concentrations >1 mg/kg, for example, 5−30 mg/kg for leaf vegetables, the developed dc-ELISA using MoAb 11A will be applicable for the residual azoxystrobin analysis in garden crops.

The dc-ELISA is simple, rapid, cost-effective, and high throughput, being comparable to conventional instrumental analysis. The performance of the dc-ELISA must be suitable for routine on-site analyses. It is expected that the dc-ELISA will be widely used for azoxystrobin analysis as an alternative method, with additional evaluations in several laboratories.

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Notes
Safety: This study was approved by the Biomanagement Committee at HORIBA, Ltd., and carried out according to the guidelines of the committee.

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