JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of an Enzyme-Linked Immunosorbent Assay for Residue Analysis of the Insecticide Emamectin Benzoate in Agricultural Products

MIKA KONDO,[†] HIROSHI YAMASHITA,[†] MIKIKO UCHIGASHIMA,[†] TAKESHI KONO,[†] TOSHIHIDE TAKEMOTO,[§] MASAHIRO FUJITA,[#] MACHIKO SAKA,[#] SEIJI IWASA,[§] SHIGEKAZU ITO,[†] AND SHIRO MIYAKE*,[†]

Research and Development Department, Medical Electronic Systems Division, Horiba, Ltd., 2 Miyanohigashi, Kisshoin, Minami-ku, Kyoto 601-8510, Japan; Department of Materials Science, Toyohashi University of Technology, Tempaku-cho, Toyohashi, Aichi 441-8580, Japan; and The Institute of Environmental Toxicology, 4321 Uchimoriya-machi, Joso-shi, Ibaraki 303-0043, Japan

A direct competitive enzyme-linked immunosorbent assay (dc-ELISA) for the analysis of emamectin residues in agricultural products was developed using a prepared mouse monoclonal antibody. The working range was 0.3−3.0 ng/mL, and the 50% inhibition concentration (IC_{50}) was 1.0 ng/mL. The assay was sufficiently sensitive for analysis of the maximum residue limits in agricultural products in Japan (>0.1 μg/g). Emamectin residues contain the following metabolites: the 4″-epi-amino analogue, the 4″-epi-(*N*-formyl)amino analogue, the 4″-epi-(*N*-formyl-*N*-methyl)amino analogue, and the 8,9-*Z* isomer. The dc-ELISA reacted with these compounds at ratios of 113, 55, 38, and 9.1% of the IC_{50} value of emamectin benzoate. Seven kinds of vegetables were spiked with emamectin benzoate at concentrations of 15−300 ng/g, and the recoveries were 91−117% in the dc-ELISA. The dc-ELISA results agreed reasonably well with results obtained by liquid chromatography−tandem mass spectrometry (LC-MS/MS) using spiked samples and actual (incurred) samples. The results indicate that the dc-ELISA was useful for the analysis of emamectin benzoate residues in agricultural products.

KEYWORDS: Monoclonal antibody; dc-ELISA; kit; residue analysis

INTRODUCTION

Emamectin benzoate (**Figure 1**) is a potent pesticide that was first synthesized from avermectin B1 (*1*). The substitution of the 4″-hydroxy group of avermectin B1 with a methylamino group markedly reduces the insecticidal activity against brine shrimp and two-spotted spider mite and greatly increases the insecticidal activity against Lepidoptera (*2, 3*). Emamectin benzoate contains the B1a analogue (R_1 = *sec*-butyl) and the B1b analogue (R_1 = isopropyl) in a 9:1 ratio, as well as avermectin B1 (*4*). Emamectin benzoate is effective not only against Lepidoptera (*5*) but also against Thysanoptera (*6*) and Coleoptera (*7*), and in 1997 it was registered as a pesticide and an acaricide for vegetables, fruits, and cereals in Japan.

Emamectin benzoate is generally analyzed by means of high-performance liquid chromatography (HPLC). The procedure involves not only extraction of samples with acetone and purification of the extracts with cartridge columns, which are commonly used in pesticide analysis, but also fluorescence inducement (*8, 9*). Therefore, the analysis procedure is more complicated than that used for other pesticides. Liquid chromatography−tandem mass spectrometry (LC-MS/MS) is also used for emamectin benzoate analysis because fluorescence inducement is not required. However, although the LC-MS/MS method is accurate, the instruments are much more expensive than HPLC instruments and require more skill to operate; therefore, the method is not practical for on-site analysis of agricultural products before they are shipped.

The direct competitive enzyme-linked immunosorbent assay (dc-ELISA) is a rapid, simple, and cost-effective alternative method (*10*−*12*). We have already developed dc-ELISA kits for pesticide residue analysis in agricultural products. In Japan, these kits are used for routine on-site checks of agricultural products before shipment. Evaluation data for the imidacloprid kit (*13*−*15*), the chlorfenapyr kit (*16*), the chlorothalonil kit (*17*), the fenitrothion kit (*14*), and the iprodione kit (*18*) have been reported, and these dc-ELISA kits have been shown to be useful for routine checks. Because emamectin benzoate is one of the most widely used insecticides and because it is applied to agricultural products just before harvest, the development of a dc-ELISA for this insecticide is highly desirable. In this paper, we describe the preparation of a monoclonal antibody (MoAb) against emamectin benzoate, a dc-ELISA method using the

* Author to whom correspondence should be addressed (telephone +81 75 325 5834; fax +81 75 325 5103; e-mail shiro.miyake@horiba.com).
[†] Horiba, Ltd.
[§] Toyohashi University of Technology.
[#] The Institute of Environmental Toxicology.

10.1021/jf802788n CCC: $40.75
Published on Web 12/31/2008

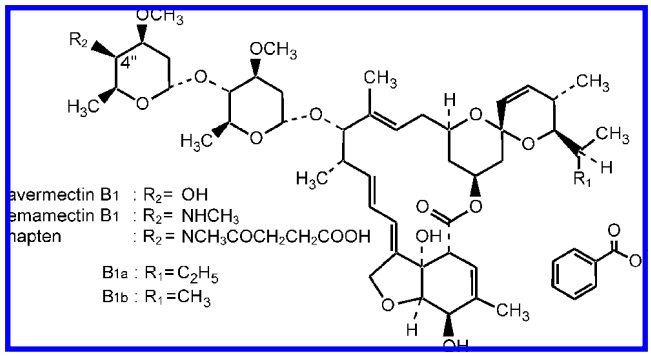

Figure 1. Structure of emamectin benzoate and related compounds.

prepared MoAb, and the performance of the dc-ELISA. We also compared the efficiency of the dc-ELISA with a LC-MS/MS method for the analysis of emamectin benzoate residues in agricultural products.

EXPERIMENTAL METHODS

Reagents and Apparatus. An emamectin benzoate standard was purchased from Hayashi Pure Chemicals Inc. (Osaka, Japan). The 8,9-*Z* isomer was purchased from Kanto Chemicals (Tokyo, Japan). Avermectin B1 and streptomycin sulfate were obtained from Wako Pure Chemical Industries (Osaka, Japan). Validamycin A (purity > 60%) was obtained from Duchefa Biochemie BV (The Netherlands). The other standards were obtained from Hayashi Pure Chemicals. Emamectin used for hapten synthesis was extracted from AFFIRM EC (Syngenta Japan K.K.) and purified by means of silica gel column chromatography. The purity of emamectin was >95% on the basis of ^{1}H NMR. Polyethylene glycol (PEG) 1500, pristane, and keyhole limpet hemocyanin (KLH) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Block Ace was obtained from Dainippon Sumitomo Pharma Co. (Osaka, Japan). Horseradish peroxidase (HRP) was purchased from Toyobo Co. (Osaka, Japan). Rabbit serum albumin (RSA), hypoxanthine thymidine and hypoxanthine aminopterin thymidine (HAT) media, and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Invitrogen Corp. (Carlsbad, CA). HRP-labeled anti-mouse IgG was obtained from ICN Biomedicals Inc. (Aurora, OH). Freund's complete adjuvant and Freund's incomplete adjuvant were obtained from Difco Laboratories (Detroit, MI). The other chemicals used in the examinations were of analytical grade. Microplates with 96 wells for cell culture and microtiter plates with 96 wells for ELISA were obtained from Nalge Nunc International (Roskilde, Denmark). ^{1}H spectra were measured on a Varian Inova-400 (400 MHz) or Mercury-300 (300 MHz) spectrometer. ^{1}H NMR chemical shifts are given in parts per million downfield from tetramethylsilane as an internal standard (ρ 7.71) in $CDCl_3$. ELISA absorbance was measured using an MPR-01 microplate reader from Horiba (Kyoto, Japan).

Hapten Synthesis. For 4″-deoxy-4″-epi-(*N*-3-caboxypropanoyl)-*N*-methylaminoavermectin B1 (hapten) synthesis, emamectin (30 mg, 0.034 mmol), succinic acid (6.0 mg, 0.051 mmol), and 4-(dimethylamino)pyridine (4.0 mg, 0.034 mmol) were dissolved in CH_2Cl_2 (2 mL) under a N_2 atmosphere, and then dicyclohexylcarbodiimide (4.0 mg, 0.034 mmol) was added at 0 °C. The resulting mixture was stirred for 12 h at room temperature. At the end of this period, the solvent was removed under reduced pressure. Purification of the residue by flash column chromatography on silica gel with CH_2Cl_2/CH_3OH (10:1, v/v) as the eluent afforded the adduct (8.0 mg). The purity of this adduct was >90% on the basis of ^{1}H NMR, and it was used as an emamectin hapten without further purification. By the same procedure, 40 mg of additional hapten was prepared.

Hapten−Protein Conjugate Preparation. By means of the activated ester method, the emamectin hapten was conjugated with KLH for use as an immunogen, with RSA for use as a coating antigen in direct-bind ELISA (db-ELISA) and with HRP for use as a tracer in dc-ELISA (*19*). The hapten (20 mg, 0.02 mmol) in 1.5 mL of dry dimethyl sulfoxide was gradually added to a mixture of *N*-hydroxysuccinimide (4.7 mg, 0.04 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (7.8 mg, 0.04 mmol) in 0.5 mL of dry dimethyl sulfoxide. After the mixture had been stirred for 1.5 h at room temperature, 440 μL of the hapten-activated ester solution was added to 1 mL of 0.1 M borate buffer (pH 8.0) supplemented with 150 mM NaCl containing KLH, RSA, or HRP (10 mg), and the mixture was gently stirred for 1.5 h at room temperature and then dialyzed against phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl, pH 7.0) for 4 days at 4 °C, except for the HRP conjugate, which was purified through a gel filtration column rather than by dialysis. The hapten−protein conjugates prepared were stored at −30 °C. The conjugate concentrations were determined by means of a DC Protein Assay Kit from Bio-Rad (Tokyo, Japan).

MoAb Preparation. Seven-week-old female BALB/c mice from Nippon SLC (Shizuoka, Japan) were intraperitoneally immunized with 50 μL of the hapten−KLH conjugate (50 μL, 100 μg/mouse) after it was emulsified with an equal volume of Freund's complete adjuvant. Booster injections (25 μg/mouse) were carried out three times with the emulsion using Freund's incomplete adjuvant at intervals of 2 weeks.

Three days after the last injection, spleen cells from the mouse were fused with P3-X63-AG8.653 myeloma cells (*20*) according to the conventional method (*21*). Briefly, the spleen cells (5×10^8 cells) were fused with the myeloma cells (5.5×10^7 cells) using PEG 1500. The fused cells were suspended at 2.5×10^6 cells/mL in HAT medium, transferred to the wells of a 96-well microplate, and incubated at 37 °C under 5% CO_2. Ten days after the incubation, we screened the culture media in which the hybridomas formed a colony by determining the reactivity with the hapten−RSA conjugate in a db-ELISA and by determining the reactivity with emamectin benzoate in an indirect competitive ELISA (ic-ELISA) as described below. Hybridomas grown in the positive well were cloned by means of the limiting dilution technique, and the representative cell clone was used for preparation of the MoAb.

For MoAb preparation, BALB/c mice were pretreated by intraperitoneal injection of each mouse with 0.5 mL of pristane, and 1 week after the pretreatment, the mice were inoculated with 2×10^7 cells of a viable cell clone. Seven to 10 days after the inoculation, the ascite fluids produced were collected from the mice, and MoAb in the fluid was purified with a protein G column from GE Healthcare Bio-Sciences KK (Tokyo, Japan). The concentration of MoAb was determined with an extinction coefficient of 1.4 for 1 mg/mL of IgG.

db-ELISA and ic-ELISA for MoAb Screening. A db-ELISA and an ic-ELISA were used to screen for a hybridoma producing anti-emamectin benzoate MoAb, as described previously (*22*). In brief, each well of 96-well microtiter plates was coated with 100 μL of hapten−RSA conjugate (4 μg/mL) in PBS, and the plates were incubated overnight at 4 °C. After washing, the wells were blocked by the addition of 300 μL of 25% Block Ace in PBS. For the db-ELISA, 50 μL of cultured fluids of the hybridoma was added to the wells, and the plates were incubated for 1 h at 25 °C. After the plates had been washed three times, 100 μL of HRP-labeled rabbit anti-mouse IgG antibody (1 μg/mL) in PBS modified with 10% Block Ace was added to each well. The plates were incubated for 1 h at 25 °C and then washed three times with PBS. HRP substrate solution (100 μL; 2 mg/mL of 3,3′,5,5′-tetramethylbenzidine and 0.006% H_2O_2 in 0.1 M sodium acetate buffer, pH 5.5) was added to each well, and the plates were incubated for 10 min at 25 °C. Color development by the HRP reaction was stopped by the addition of 100 μL of 0.5 M sulfuric acid, and the absorbance at 450 nm was measured using a microplate reader.

For the ic-ELISA, emamectin benzoate was dissolved to 0.1−100 ng/mL in PBS modified with 10% Block Ace, and the cultured fluid of the hybridoma was also diluted 2-fold with PBS modified with 10% Block Ace; the concentration of the diluted cultured fluid shows 50% of maximum absorbance in the db-ELISA described above. The emamectin benzoate solution (50 μL) was added to the blocked wells prepared in the db-ELISA, and an equal volume of the diluted cultured fluid was immediately added to the wells. The plate was incubated for 1 h at 25 °C, and the following procedures were performed in addition to the db-ELISA.

dc-ELISA. A dc-ELISA for residue analysis of emamectin benzoate was constructed as described previously (*23*). Rabbit anti-mouse IgG

antibody (100 μL, 10 μg/mL) in PBS was added to each well of a 96-well microtiter plate, and the plate was incubated overnight at 4 °C. After the wells had been washed with PBS, they were blocked by the addition of 300 μL of 25% Block Ace in PBS and subsequent incubation for 1 h at 25 °C. The representative purified MoAb at 3.0−300 ng/mL in PBS (100 μL) modified with 10% Block Ace was added to each well, and the MoAb was bound to the coated rabbit antibody by incubation for 1 h at 25 °C. Emamectin benzoate was dissolved to 0.1−100 ng/mL in PBS modified with 10% Block Ace and 1−40% methanol. A hapten−HRP conjugate was adjusted to the concentration in PBS modified with 10% Block Ace, on which the absorbance at 450 nm without emamectin benzoate was developed to 1.0 in this dc-ELISA. The emamectin benzoate or sample solution was mixed with an equal volume of the hapten−HRP conjugate solution, and the mixture was added to the MoAb-coated wells. The plate was incubated for 1 h at 25 °C and then washed three times with PBS. Color development and absorbance measurement were performed as described above for the db-ELISA.

Treatment of Test Samples for Determination of Emamectin Benzoate. To investigate the recovery of emamectin by means of the dc-ELISA, we collected vegetable samples containing no emamectin benzoate from a market; the pesticide-use history of the vegetables was traceable. To compare the results of dc-ELISA and LC-MS/MS, we also collected vegetable samples from a market; the pesticides were not traceable, but the absence of emamectin benzoate and its metabolites in these samples was confirmed by LC-MS/MS. Vegetable samples containing emamectin benzoate residues (as well as negative control samples) for comparison of the dc-ELISA and LC-MS/MS results were kindly provided by Syngenta Japan. The vegetables were harvested from a Japanese agricultural field to which an emulsion formulation containing 1.0% emamectin benzoate as an active ingredient had been applied, and the negative controls were obtained from a field that had not been treated with the emulsion formulation.

Each sample was homogenized, and an aliquot (5 g) was transferred to a 50 mL plastic tube. One milliliter of a methanol solution containing emamectin benzoate (75−1500 ng) was added, and the tube was vigorously shaken. After the tube had been allowed to stand for 30 min, 24 mL of methanol was added to the tube, which was shaken for 30 min (120 times/min). For the Syngenta samples, 25 mL of methanol was added, and the tube was shaken. The extracts were filtered through filter paper, and 1 mL of the filtrate was diluted to 8.5 mL with distilled water. The diluted solutions were mixed with the hapten−HRP conjugate solutions (described above) for use as samples for the dc-ELISA.

Conventional Method for Emamectin Benzoate Analysis. For cleanup and analysis of the emamectin benzoate samples, we used a conventional method for emamectin benzoate analysis, with some modifications (*24*). The samples were extracted with methanol, cleaned up on a GL-Pak PLS-2 cartridge (GL science) and a Sep-Pak NH_2 cartridge (Waters), and analyzed by LC-MS/MS (ESI, Quattro Premier, Waters).

RESULTS AND DISCUSSION

Preparation of the Anti-Emamectin MoAb. Hapten design is important to produce a highly reactive antibody against a low molecular weight compound, and a distant position from a unique epitope of the compound is generally chosen as an attachment site for the carrier protein (*25, 26*). For emamectin benzoate, a secondary amino group on the opposite side of the molecule from the unique lactone ring was chosen as the attachment site. The emamectin hapten was synthesized by reaction of emamectin with succinic acid in the presence of an equimolar amount of a dehydrating reagent, and the carboxypropionyl group was introduced as a spacer arm as shown in **Figure 1**. Selective introduction of the spacer arm was confirmed from the ^{1}H NMR spectrum, in which the *N*-methyl group signal of the amide on the synthesized hapten was shifted downfield (around 3.2 ppm) relative to the signal (2.55 ppm) on the unmodified emamectin.

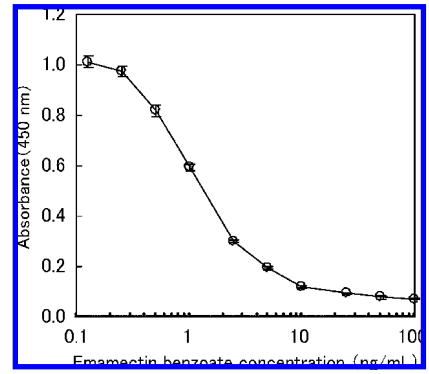

Figure 2. Standard curve for emamectin benzoate concentration in the dc-ELISA. The data points are means ± SD (n = 3).

The hapten was conjugated with KLH, and BALB/c mice were immunized with the conjugate. The mice, in which the antibody titer against emamectin was raised, were used for spleen cell preparation for cell fusion. Ten days after cell fusion, wells in which a hybridoma was grown were screened in a db-ELISA, and 4 of 1824 wells were selected as positive wells. Three of the four selected wells showed IC_{50} values of <10 ng/mL for emamectin benzoate in an ic-ELISA. The cells in each positive well were cloned by the limiting dilution technique. MoAb EMT2-17H7-2 secreted from the representative cell clone showed the highest reactivity with emamectin benzoate (IC_{50} = 2.6 ng/mL in the ic-ELISA), and EMT2-17H7-2 was used as the representative MoAb for the construction of a dc-ELISA.

dc-ELISA Construction. dc-ELISA performance generally depends on the combination of the first antibody and the hapten−HRP conjugate. The dc-ELISA for emamectin benzoate was optimized under the following conditions: the first antibody, EMT2-17H7-2, was coated at 30 ng/mL on each well, and the corresponding hapten−HRP was used at a final concentration of 100 ng/mL in the competitive reaction mixture. The standard curve in the dc-ELISA is shown in **Figure 2**. The working concentration range was chosen such that the lowest concentration was the 20% inhibition concentration (IC_{20}) and the highest concentration was the 80% inhibition concentration (IC_{80}); the range was 0.3−3.0 ng/mL. The IC_{50} value was 1.0 ng/mL under these conditions.

The standard curve in the working range was almost linear (slope = −0.27, r^2 = 0.999) and agreed well with a line drawn between the points for 0.3 and 3.0 ng/mL. We believe that a two-point linear standard should be used for test kit development because the use of fewer standard points can increase the numbers of samples required for routine analysis of agricultural products.

Methanol Influence in the dc-ELISA. Emamectin benzoate is highly lipophilic (the K_{ow} log P value is 5.0), although the salt form dissolves easily in water (*5*); emamectin benzoate must be extracted with a water-miscible solvent from agricultural products for residue analysis. Methanol is generally used as extraction solvent for application to dc-ELISA because it has less effect on antigen−antibody interactions and HRP activity than other organic solvents (*13, 17, 27−30*). Therefore, we examined the methanol tolerance of the dc-ELISA to determine whether methanol could be used for extraction of emamectin benzoate. The absorbance decreased with increasing methanol concentration, and the decrease was gradual at methanol concentrations up to 15% (**Figure 3**). At 40% methanol, the absorbance was half that in 1% methanol. In contrast, the working range was not changed by the changes in methanol

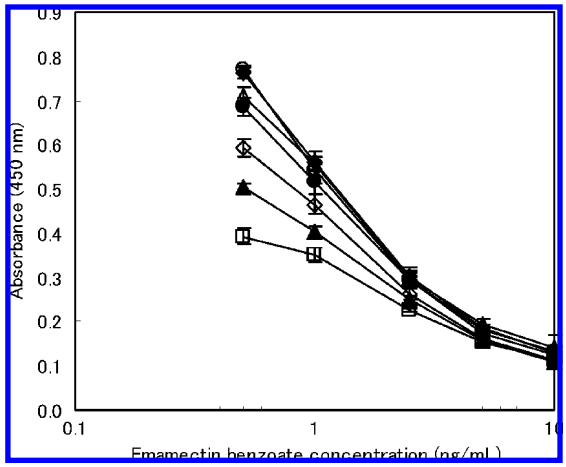

Figure 3. Effect of methanol concentration on competitive reaction in the dc-ELISA: (○) 1% methanol; (◆) 5%; (△) 10%; (●) 15%; (◇) 20%; (▲) 30%; (□) 40%. The methanol concentrations are expressed as percentages before mixing with the hapten−HRP solution. The data points are means ± SD ($n = 4$).

Table 1. Cross-Reactivities of the MoAb EMT2-17H7-2 in the dc-ELISA

compound	cross-reactivity[a] (%)
emamectin ($R_2 = NHCH_3$)	100
metabolite 1 ($R_2 = NH_2$)	113 ± 20
metabolite 2 ($R_2 = NHCHO$)	55 ± 8.9
metabolite 3 ($R_2 = N(CH_3)CHO$)	38 ± 5.1
8,9-*Z* isomer	9.1 ± 0.9
avermectin B1 ($R_2 = OH$)	78 ± 11
milbemectin A4	<0.1
spinosyn A	<0.1
validamycin A	<0.1
streptomycin sulfate	<0.1

[a] Cross-reactivity (%) = IC_{50} of emamectin/IC_{50} of test compound × 100. The data are means ± SD ($n = 3$).

concentration. These results indicate that the methanol concentration should be kept below 15% for dc-ELISA. We therefore decided to use 10% methanol to prepare samples for the dc-ELISA.

Specificity of the dc-ELISA. For emamectin residue analysis, it is important to determine three kinds of major metabolites and a stereoisomer simultaneously because the compounds often coexist with emamectin in agricultural products, as described previously (*31*). We expected that emamectin and related compounds would show high reactivity and that other pesticides that might be present in samples would show no reactivity or less reactivity than emamectin. As shown in **Table 1**, metabolite 1 ($R_2 = NH_2$) showed the highest reactivity (113%) among the compounds. Metabolite 2 ($R_2 = NHCHO$) and metabolite 3 [$R_2 = N(CH_3)CHO$] showed relatively high reactivity (55 and 38%, respectively). All of the metabolites were substituted at the R_2 position of emamectin as well as the synthesized hapten. We believe that modification of the R_2 position did not produce so much difference structures as the epitope of EMT2-17H7-2. In contrast, the 8,9-*Z* stereoisomer showed relatively low reactivity (9.1%), which indicates that the stereostructure of the lactone ring strongly influenced the affinity for EMT2-17H7-2. Avermectin B1 showed 78% cross-reactivity. This relatively high cross-reactivity, like the high cross-reactivities of metabolites 1−3, was due to the fact that avermectin B1 differs from emamectin only at R_2. The other pesticides (milbemectin A4, spinosyn A, validamycin A, and streptomycin sulfate), which (like emamectin) are antibiotics and have different structures, did not show any cross-reactivity. These results showed that EMT2-17H7-2 was specific for emamectin and related compounds and that the dc-ELISA constructed using the MoAb can be used to determine emamectin residues, although the reactivities of metabolites 2 and 3 and the stereoisomer were not quite as high as the reactivity of emamectin.

Table 2. Recovery of Emamectin Benzoate from Spiked Vegetable Samples by the dc-ELISA

vegetable	concentration (ng/g)	recovery (%)	SD (%)
Chinese cabbage	150	94	4.6
	50	91	1.8
	15	114	7.8
spinach	150	99	2.0
	50	111	5.2
	15	110	2.4
lettuce	150	99	0.2
	50	111	1.0
	15	113	8.0
Welsh onion	150	109	2.4
	50	114	4.4
	15	110	6.4
tomato[a]	300	104	2.9
	100	100	4.2
	30	109	3.6
cucumber[a]	300	103	6.0
	100	115	3.9
	30	117	9.8
cabbage[a]	300	100	3.6
	100	105	7.5
	30	109	1.3

[a] Sample solutions were diluted 2-fold with 10% methanol before measurement. The data are means ± SD ($n = 3$).

Application of the dc-ELISA to Agricultural Products. Methanol extracts from agricultural products are known to often suppress reaction between a hapten−HRP conjugate and an antibody in a dc-ELISA and to decrease absorbance of the dc-ELISA (*32*−*34*). In the dc-ELISA constructed using EMT2-17H7-2, this suppression, the so-called matrix effect, should be checked for each agricultural product. Because emamectin benzoate is frequently used as an insecticide for many agricultural products and because it is used immediately before the harvest in Japan, we examined the matrix effect for seven kinds of commonly grown vegetables. The criterion for ruling out the matrix effect was that the negative control did not show a determined concentration of >0.3 ng/mL (IC_{20} value). Homogenized Chinese cabbage, spinach, lettuce, and Welsh onion were spiked with emamectin benzoate at 0, 15, 50, and 150 ng/g, and the concentrations were confirmed by the dc-ELISA after the standard sample treatment. Tomato, cucumber, and cabbage were spiked at 0, 30, 100, and 300 ng/g and were analyzed after the sample treatment and after 2-fold further dilution with 10% methanol because the matrix effect was found with the standard sample treatment (data not shown). High recoveries (91−117%) were observed for all of the samples (**Table 2**). Some of the vegetables showed estimated recoveries of >100%, even though they contained no emamectin benzoate other than what was added. The overestimation occurred mainly at relatively low concentrations of emamectin, and we therefore speculated that the overestimation might be due to minor matrix effects. Their minor overestimations were common, but extreme under- or overestimation (<70 or >120%) was not observed under these conditions. Therefore, we believe that dc-ELISA can be used for emamectin residue analysis for these agricultural products.

The determination range in the agricultural products tested under the standard sample treatment conditions was calculated to be 15−50 ng/g (50 times the working range of 0.3−3.0 ng/mL) in the dc-ELISA, and we suggest that the 2-fold further dilution was effective for eliminating the matrix effect. The

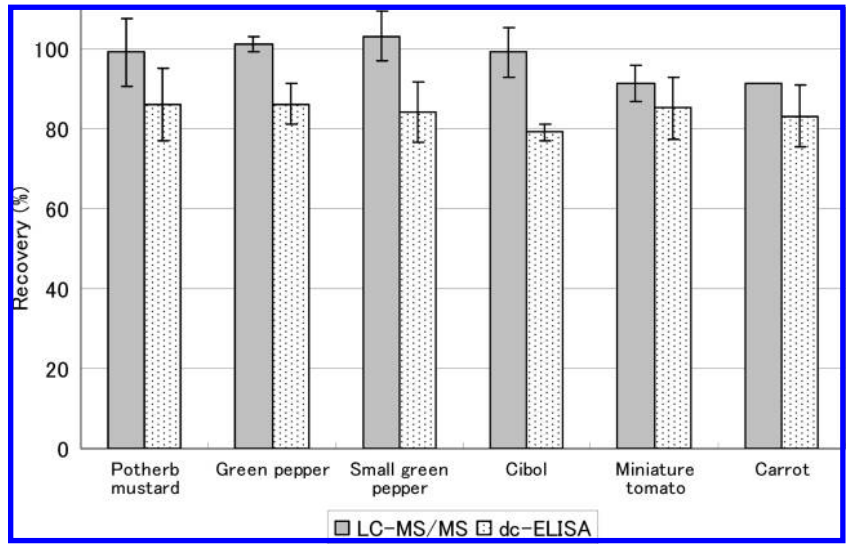

Figure 4. Recoveries of emamectin benzoate from spiked samples by the dc-ELISA and the conventional method (LC-MS/MS). Each sample was spiked with emamectin at 0.1 mg/kg. The data are means ± SD ($n = 3$).

sensitivity of the method was good for analysis of emamectin benzoate residues in agricultural products because the maximum residue levels (MRLs)—the total residues of the parent compound, its three metabolites, and the 8,9-*Z* isomer—were set at 0.1−0.5 μg/g, except for cottonseed and oilseeds, in Japan (*35*).

Comparison with the Conventional Method for Spiked Samples. To confirm the applicability of the dc-ELISA to agricultural product samples, we compared results of the dc-ELISA to the LC-MS/MS method. **Figure 4** showed the recoveries of emamectin benzoate from spiked samples for the dc-ELISA and for the LC-MS/MS method. The mean recoveries of emamectin benzoate from samples were 79−91% (dc-ELISA) and 85−103% (LC-MS/MS), respectively.

The recoveries for the dc-ELISA were a little lower than those for LC-MS/MS. The difference may have been due to differences in the sample treatment, especially with regard to the solvent for sample extraction, and in the standard preparations for the dc-ELISA and LC-MS/MS. Recoveries for the dc-ELISA were also a little lower than those obtained in the investigation of the matrix effect. This difference may have been due to differences between the samples and the different examination dates, and we intend to validate the results in future studies. Although minor differences were observed, we consider the dc-ELISA to be as effect as LC-MS/MS because the recovery rates for both methods were in the 70−120% range.

Comparison with the Conventional Method for Incurred Samples. Residue levels in actual (incurred) samples were determined by the dc-ELISA, and the results were compared to those determined by the conventional method using LC-MS/MS. Although the LC-MS/MS data showed that the samples did not contain the metabolites or the 8,9-*Z* isomer (except for green pepper), the residue levels obtained by the dc-ELISA were approximately twice those obtained by the conventional method (**Table 3**). It was difficult to obtain accurate data with the dc-ELISA, because the residue levels of the incurred samples were close to the IC_{20} value for the dc-ELISA, and at these levels the matrix effect occurred more frequently, as described above. However, the recovery data for the incurred samples (0.02 and 0.03 mg/kg) and the respective control samples (<0.02 mg/kg) (data not shown) were clearly different. Thus, the dc-ELISA seems to be applicable for emamectin benzoate residue analysis in actual samples, but accurate data for residue levels close to the IC_{20} value should be confirmed by means of LC-MS/MS or some other conventional method.

Table 3. Residue Levels in Potherb Mustard and Green Pepper Determined by dc-ELISA and LC-MS/MS

sample	residue level[a] (mg/kg)	
	dc-ELISA	LC-MS/MS[b]
potherb mustard A	0.02	0.010
potherb mustard B	0.02	0.008
green pepper	0.03	0.016[c]

[a] The data are means ($n = 2$). Both of the dc-ELISA results were the same level. Difference of the LC-MS/MS results was 0.001 mg/kg. [b] The emamectin benzoate metabolites and the 8,9-*Z* isomer were not present in potherb mustards A and B (<0.005 mg/kg, LOQ), and metabolite 3 was present at 0.006 mg/kg in green pepper (<0.002 mg/kg, LOQ). [c] The emamectin benzoate concentration excluding metabolite 3.

The dc-ELISA constructed with the anti-emamectin benzoate MoAb EMT2-17H7-2 showed good characteristics for analysis of emamectin residues in agricultural products. Because the dc-ELISA is simple, rapid, and cost-effective and because it offers high throughput relative to the conventional method, it should be suitable for the analysis of agricultural products, especially for routine on-site analysis by farmers and cooperatives. When the residue concentrations determined by the dc-ELISA exceed the MRLs, the concentrations should be confirmed by LC-MS/MS analysis. We expect that the dc-ELISA will be used for emamectin benzoate analysis as an alternative tool to the conventional method.

ACKNOWLEDGMENT

We express our appreciation to Syngenta Japan for the vegetables containing emamectin benzoate residues and to Atsushi Kadowaki, Hiroyuki Takahashi, and Narihiro Oku for their support of this work.

LITERATURE CITED

(1) Lasota, J. A.; Dybas, R. A. Avermectins, a novel class of compounds: implications for use in arthropod pest control. *Annu. Rev. Entomol.* **1991**, *36*, 91–117.

(2) Mrozik, H. Advances in research and development of avermectins. In *Natural and Derived Pest Management Agents*; Hedin, P., Hollingworth, R., Menn, J., Eds.; American Chemical Society: Washington, DC, 1994; Vol. 551, pp 54−73.

(3) Fisher, M. H.; Recent progress in avermectin research. In *Pest Control with Enhanced Environmental Safety*; Duke, S. O., Menn, J. J., Plimmer, J. R., Eds.; American Chemical Society: Washington, DC, 1993; Vol. 524, pp 169−182.
(4) *The Pesticide Manual*, 12th ed.; TomlinC. D. S., Ed.; British Crop Protection Council: Surrey, U.K., 2000; pp 341−142.
(5) Jansson, R. K.; Peterson, R. F.; Mookerjee, P. K.; Halliday, W. R.; Argentine, J. A.; Dybas, R. A. Efficacy of solid formulation of emamectin benzoate at controlling lepidopterous pests. *Fla. Entomol*. **1996**, *79*, 434–449.
(6) Ishaaya, I.; Kontsedalov, S.; Horowitz, A. R. Emamectin, a novel insecticide for controlling field crop pests. *Pest Manag. Sci*. **2002**, 1091–1095.
(7) Grosman, D. M.; Upton, W. W. Efficacy of systemic insecticides for protection of lablolly pine against southern pine engraver beetles (Coleoptera: Curculionidae: Scolytine) and wood borers (Coleoptera: Carambycidae). *J. Econ. Entomol*. **2006**, *99*, 94–101.
(8) Prabhu, S. V.; Wehner, T. A.; Egan, R. S.; Tway, P. C. Determination of 4″-deoxy-4″-(epimethylamino)avermectin B1 benzoate (MK-0244) and its delta 8,9-isomer in celery and lettuce by HPLC with fluorescence detection. *J. Agric. Food Chem*. **1991**, *39*, 2226–2230.
(9) Yoshii, K.; Kaihara, A.; Thumura, Y.; Ishii, S.; Tonogai, Y. Simultaneous determination of residues of emamectin metabolites, and milbemectin, ivermectin, and abamectin in crops by liquid chromatography with fluorescence detection. *J. AOAC Int*. **2001**, *84*, 910–917.
(10) Jung, F.; Gee, S. J.; Harrison, R. O.; Goodrow, M. H.; Karu, A. E.; Braun, A. L.; Li, Q. X.; Hammock, B. D. Use of Immunochemical Techniques for the Analysis of Pesticides. *Pestic. Sci*. **1989**, *26*, 303–317.
(11) Rittenburg, J. H.; Grothaus, G. D.; Fitzpatrick, D. A.; Lankow, R. K. Rapid on-site immunoassay systems, agricultural and environmental applications. In *Immunoassays for Trace Chemical Analysis*; Vanderlaan, M., Stanker, L. H., Watkins, B. E., Roberts, D. W., Eds.; American Chemical Society: Washington, DC, 1991; Vol. 451, pp 28−39.
(12) Sherry, J. P. Environmental chemistry: the immunoassay option. *Crit. Rev. Anal. Chem*. **1992**, *23*, 217–300.
(13) Watanabe, E.; Eun, H.; Baba, K.; Arao, T.; Ishii, Y.; Endo, S.; Ueji, M. Evaluation and validation of a commercially available enzyme-linked immunosorbent assay for the neonicotinoid insecticide imidacloprid in agricultural samples. *J. Agric. Food Chem*. **2004**, *52*, 2756–2762.
(14) Amano, S.; Yano, H. Matrix effect of cucumber on fenitrothion and imidacloprid residue analysis by a commercially available ELISA kit. *J. Pestic. Sci* **2006**, *31*, 425–230.
(15) Watanabe, E.; Eun, H.; Baba, K.; Arao, T.; Ishii, Y.; Endo, S.; Ueji, M. Rapid and simple screening analysis for residual imidacloprid in agricultural products with commercially available ELISA. *Anal. Chim. Acta* **2004**, *521*, 45–51.
(16) Watanabe, E.; Baba, K.; Eun, H.; Arao, T.; Ishii, Y.; Ueji, M.; Endo, S. Evaluation of a commercial immunoassay for the detection of chlorfenapyr in agricultural samples by comparison with gas chromatography and mass spectrometric detection. *J. Chromatogr., A* **2005**, *1074*, 145–153.
(17) Watanabe, E.; Miyake, S.; Ito, S.; Baba, K.; Eun, H.; Ishizaka, M.; Endo, S. Reliable enzyme immunoassay detection for chlorothalonil: fundamental evaluation for residue analysis and validation with gas chromatography. *J. Chromatogr., A* **2006**, *1129*, 273–282.
(18) Watanabe, E.; Miyake, S. Immunoassay for iprodione: key estimation for residue analysis and method validation with chromatographic technique. *Anal. Chim. Acta* **2007**, *583*, 370–376.
(19) Karu, A. E.; Goodrow, M. H.; Schmidt, D. J.; Hammock, B. D.; Bigelow, M. W. Synthesis of haptens and derivation of monoclonal antibodies for immunoassay of the phenylurea herbicide diuron. *J. Agric. Food Chem*. **1994**, *42*, 301–309.
(20) Kearney, J. F.; Radbruch, A.; Liesegang, B.; Rajewsky, K. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody hybrid cell lines. *J. Immunol*. **1979**, *123*, 1548–1550.
(21) Miyake, S.; Beppu, R.; Yamaguchi, Y.; Kaneko, H.; Ohkawa, H. Polyclonal and monoclonal antibodies specific to the chrysanthemic acid moiety of pyrethroid insecticides. *Pestic. Sci*. **1998**, *54*, 189–194.
(22) Miyake, S.; Morimune, K.; Yamaguchi, Y.; Ohde, K.; Kawata, M.; Takewaki, S.; Yuasa, Y. Hapten design and antibody preparation on immunoassay toward the insecticide oxamyl. *J. Pestic. Sci*. **2000**, *25*, 10–17.
(23) Miyake, S.; Ishii, Y.; Yamaguchi, Y.; Ohde, K.; Motoki, M.; Kawata, M.; Ito, S.; Yuasa, Y.; Ohkawa, H. Simple determination of herbicides in rice paddy water by immunoassay. In *Environmental Fate and Effects of Pesticides*; Coats, J. R., Yamamoto, H., Eds.; American Chemical Society: Washington, DC, 2003; Vol. 853, pp 124−138.
(24) The Notified Analytical Methods of Pesticide Residues, Ministry of Health, Labour and Welfare in Japan, 2005 (in Japanese).
(25) Harrison, R. O.; Goodrow, M. H.; Gee, S. J.; Hammock, B. D. Hapten synthesis for pesticide immunoassay development. In *Immunoassays for Trace Chemical Analysis*; Vanderlaan, M., Stanker, L. H., Watkins, B. E., Roberts D. W., Eds.; American Chemical Society: Washington, DC, 1991; Vol. 451, pp 14−27.
(26) Goodrow, M. H.; Sanborn, J. R.; Stoutamire, D. W.; Gee, S. J.; Hammock, B. D. Strategies for immunoassay hapten design. In *Immunoanalysis of Agrochemicales*; Nelson, J. O., Karu, A. E., Wong, R. B., Eds.; American Chemical Society: Washington, DC, 1995; Vol. 566, pp 119−139.
(27) Mercader, J. V.; Montoya, A. Development of monoclonal ELISAs for azinphos-methyl. 2. Assay optimization and water sample analysis. *J. Agric. Food Chem*. **1999**, *47*, 1285–1293.
(28) Abad, A.; Montoya, A. Development for 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.
(29) Chin, T. E.; Wong, R. B.; Pont, J. L.; Karu, A. E. Haptens and monoclonal antibodies for immunoassay of imidazolinone herbicides. *J. Agric. Food Chem*. **2002**, *50*, 3380–3389.
(30) Cho, Y. A.; Kim, Y. J.; Hammock, B. D. Development of a Microtiter plate ELISA and a dipstick ELISA for the determination of the organophosphorus insecticide fenthion. *J. Agric. Food Chem*. **2003**, *51*, 7854–7860.
(31) *Food Sanit. Res.* **2000**, *50*, 125−128.
(32) Skerritt, J. H.; Rani, B. E. A., Detection and removal of sample matrix effects in agricultural immunoassays. In *Immunoassay for Residue Analysis*; Beier, R. C., Stanker, L. H., Eds.; American Chemical Society: Washington, DC, 1996; pp 29−43.
(33) Skerritt, J. H.; Hill, A. S.; Sashidhar Rao, R. B.; Beasley, H. L.; Rani, B. E. A.; Udaya Kumari, C. G.; Vijayashankar, Y. N.; Venugopal, N. B. R. K.; Karanth, N. G. K. Sample matrix interference in immunoassays for organochlorine residues in plant-derived foods and some strategies for their removal. *Food Agric. Immunol*. **2003**, *15*, 17–34.
(34) Wang, S.; Zhang, J.; Yang, Z.; Wang, J.; Zhang, Y. Development of two enzyme-linked immunosorbent assays for detection of endosulfan residues in agricultural products. *J. Agric. Food Chem*. **2005**, *53*, 7377–7384.
(35) Announcement 370. Ministry of Health, Labour and Welfare, Japan, 1969; Dec 28 (updated Feb 5, 2007).

Received for review September 9, 2008. Revised manuscript received November 24, 2008. Accepted November 24, 2008.

JF802788N