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Development of a Sensitive Indirect Competitive Enzyme-Linked Immunosorbent Assay (ic-ELISA) Based on the Monoclonal Antibody for the Detection of the Imidaclothiz Residue

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ABSTRACT: An indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) based on monoclonal antibodies (MoAbs) for imidaclothiz was developed. The hapten of imidaclothiz was synthesized and conjugated to bovine serum albumin (BSA) and ovalbumin (OVA) to form the artificial antigens. MoAbs were obtained by immunizing BALB/c mice. Under the optimized conditions (10% methanol, 0.14 M Na^+, and pH 7.4), the half-maximal inhibition concentration (IC_{50}) was 0.0875 ± 0.0034 mg/L and the limit of detection (IC_{20}) was 0.0178 ± 0.0018 mg/L for imidaclothiz. There were no obvious cross-reactivities with most of the structural analogues of neonicotinoid insecticides, except imidacloprid. The recoveries of imidaclothiz in environmental and agricultural samples, including tap water, paddy water, soil, and cabbage, ranged from 80.43 to 113.83%, well within the requirements of residue detection. These results showed that this immunoassay could be used for the determination of imidaclothiz in environmental and agricultural samples.

KEYWORDS: Imidaclothiz, hapten, monoclonal antibodies, ELISA

INTRODUCTION

Imidaclothiz [1-(5-chloro-thiazole methyl)-4,5-dihydro-*N*-nitro-1*H*-imidazole-2-amine] belongs to a new class of neonicotinoid insecticides with a great affinity for insect nicotinergic acetylcholine receptors,[1] which was developed and first commercialized by Nantong Jiangshan Agrochemical and Chemicals Co., Ltd. (China). Because imidaclothiz has greater systemic activity, lower acute mammalian toxicity, and no cumulative long-term toxicity, it has been widely used for controlling whiteflies, thrips, leafhoppers, plant hoppers, and other various harmful pest species in China.[2−5] High-performance liquid chromatography (HPLC) has been used successfully for the detection of imidaclothiz.[6−8] However, current analytical methods are costly, time-consuming, and not suitable for the analysis of the large number of samples.[9,10] Therefore, there is a growing demand for more rapid and economical methods for determining pesticide residues. Enzyme-linked immunosorbent assays (ELISAs) provide fast, sensitive, cost-effective, and selective methods for the detection of pesticide residues.[11] In the past decade, ELISA methods have been developed for the analysis of neonicotinoid insecticides,[12−15] but an ELISA of imidaclothiz has not been reported. In this paper, a sensitive indirect competitive ELISA (ic-ELISA) based on monoclonal antibodies for the detection of imidaclothiz was developed and applied to environmental and agricultural samples.

MATERIALS AND METHODS

Materials. Imidaclothiz (97.82%) was obtained from Nantong Jiangshan Agrochemical and Chemicals Co., Ltd. (Jiangsu, China). Pesticide standards used for cross-reactivity studies were supplied by Jiangsu Qizhou Chemical Group Co., Ltd. (Jiangsu, China). Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvants, *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), *o*-phenylenediamine (OPD), and polyoxyethylene sorbitan monolaurate (Tween-20) were all purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG was purchased from Sino-American Biotechnology Co. (Dalian, China). 3-Mercaptopropionic acid (3-MPA) was from a Johnson Matthey Company. Phosphate-buffered saline (PBS, 0.01 M, pH 7.4), carbonate-buffered saline (CBS, 0.05 M, pH 9.6), phosphate-buffered saline containing 0.05% Tween-20 (PBST), substrate solution (37.5 μL of 30% H_2O_2 and 10 mg of OPD per 25 mL of citrate buffer at pH 5.0) were used. All reagents and solvents were analytical-grade. The BALB/c mice were purchased from the Center of Comparative Medicine of Yangzhou University (Yangzhou, China). All animal studies were performed in accordance with institutional guidelines.

Instruments. Electrospray ionization−mass spectrometry (ESI−MS) was measured using a Mariner 5304 spectrometer (Applied Biosystems, Inc., Foster City, CA). Nuclear magnetic resonance (NMR) spectra were recorded on a DRX500 spectrometer (Bruker, Germany). Ultraviolet−visible (UV−vis) spectra were obtained on a DU-800 spectrophotometer (Beckman Coulter, Inc., Brea, CA). The 96-well polystyrene microplates (Maxisorp) were purchased from Nunc (Roskilde, Denmark). ELISA plates were washed with Wellwash Plus (Thermo Fisher Scientific, Waltham, MA). The absorbances were read with an Infinite M200 microtiter plate reader (Tecan, Switzerland) at 490 nm. The antibody was freeze-dried using an Allegra 64R centrifuge (Beckman Coulter, Inc., Brea, CA).

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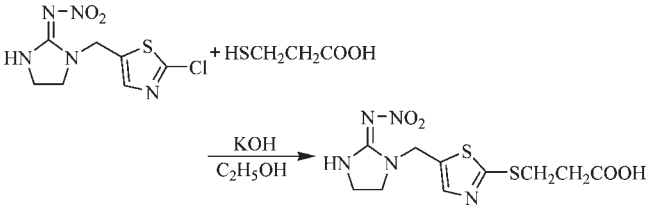


Figure 1. Synthetic route for hapten.

Hapten Synthesis. The hapten synthetic route was illustrated in Figure 1. A mixture of 1.04 g (4.0 mmol) of imidaclothiz, 0.42 g (4.0 mmol) of 3-MPA, and 0.32 g (8.0 mmol) of potassium hydroxide in 20 mL of ethanol was refluxed for 6 h. The reaction mixture was filtered. After the ethanol was concentrated *in vacuo*, the residue was dissolved in water (50 mL) and the solution was adjusted to pH 3 using 2 N HCl and extracted with ethyl acetate (3 × 30 mL). The extract was washed with water (3 × 30 mL), dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residue was recrystallized using methanol to yield a straw yellow solid. The product was characterized by ESI−MS and ^{1}H NMR. ESI−MS *m*/*z*: 330 [M − H$^+$] and 354 [M + Na$^+$]. ^{1}H NMR ($CDCl_3$) δ: 2.94−2.98 (t, 2H, CH_2COO), 3.42−3.47 (t, 2H, SCH_2), 3.61−3.65 (t, 2H, NCH_2), 3.77−3.80 (t, 2H, CH_2NH), 4.69 (s, 2H, CH_2), and 7.67 (s, 1H, ArH).

Preparation of Hapten−Protein Conjugates. Hapten was conjugated with BSA using the carbodiimide method to produce an immunogen[16] and conjugated with OVA by the mixed anhydride method to produce a coating antigen.[17] The conjugates were dialyzed in PBS over 72 h at 4 °C and stored at −20 °C. The formation of conjugates was confirmed by UV−vis spectra, and the hapten densities (the number of hapten molecules per molecule of protein) of the conjugates were estimated directly by the mole absorbance:

$$\text{hapten density} = (\varepsilon_{\text{conjugate}} - \varepsilon_{\text{protein}})/\varepsilon_{\text{hapten}}$$

Immunization and Monoclonal Antibody Preparation. The immunogen emulsion was injected into the peritoneal cavity of 6-week-old BALB/c female mice. The dosage of immunogen for each mouse was 100 μg of immunogen dissolved in physiological saline and emulsified with an equal volume of Freund's complete adjuvant. Four subsequent injections were given at 2 week intervals using immunogen emulsified with incomplete Freund's adjuvant. The week after the fifth immunization, antiserum was obtained from the tail vein of each mouse. The antibody titers and recognition properties for imidaclothiz were tested. The mice that showed strong response reactivity were used for peritoneal cavity injections of 200 μg of immunogen in PBS at 1 week intervals. At 4 days after the booster injection, the cell fusion was performed according to Nowinski et al.[18] Mouse spleen lymphocyt was fused with SP2/0 myeloma cell at a 5:1 ratio. The fused cells were cultured with a hypoxanthine−aminopterin−thymidine (HAT) selection medium for 2 weeks then in hypoxanthine−thymidine (HT) medium for 2 weeks. Culture supernatants were screened for recognizing imidaclothiz, and hybridoma cells in ELISA positive wells were cloned with the limiting dilution method. Stable antibody-producing clones were expanded. Ascites obtained from BALB/c mice were purified using salting out with saturated ammonium sulfate[19] and stored at −20 °C after freeze-drying.

ic-ELISA. The ELISA was carried out on 96-well polystyrene microplates. All incubations were carried out at 37 °C. Briefly, microplate wells were coated with the coating antigen (100 μL/well in CBS at pH 9.6) overnight at 4 °C. The plate was washed 4 times with PBST and blocked by adding 1% OVA/PBS (200 μL/well). After incubation for 0.5 h, the plate was washed, antibody diluted in PBS was added (100 μL/well), and the plate incubated for 1 h. The plate was washed and further incubated with the goat anti-mouse IgG−HRP (1:10 000 in PBST, 100 μL/well) for 1 h. After another washing, substrate solution was added (50 μL/well). The reaction was stopped with sulfuric acid (2 mol/L, 50 μL/well) after 15 min of incubation. The absorbance was measured at 490 nm.

Table 1. Effect of the Organic Solvent, Ionic Strength, and pH Value on the ELISA Sensitivity

methanol (v/v, %)	IC_{50} (mg/L)	ionic strength (M)	IC_{50} (mg/L)	pH	IC_{50} (mg/L)
0	0.12	0.1	0.15	5.5	0.13
10	0.09	0.14	0.13	6.5	0.12
20	0.16	0.2	0.14	7.4	0.10
30	0.19	0.3	0.21	8.5	0.16
40	0.23	0.4	0.20	9.5	0.17

The ic-ELISA was performed by blocking each well with the appropriate concentration analyte (50 μL/well), followed by the addition of the diluted antibody (50 μL/well). The plate was shaken for 1 min and then processed as indicated above.

Optimization of the ELISA. The experimental parameters, including organic solvent, ionic strength, and buffer pH, were studied sequentially to improve the sensitivity of the immunoassay. Evaluation of the immunoassay was based on IC_{50} and the coefficient of determination (R^2) of the linear equation.[20] The effects of methanol, a commonly organic solvent used in ELISA procedures to improve analyte solubility,[21] were studied using PBS containing 0, 10, 20, 30, and 40% methanol to dilute the imidaclothiz standard. PBS buffers of different ionic strength (0.14−0.5 M Na$^+$) were tested to determine the effects of ionic strength. The effects of pH values were evaluated using different PBS solutions ranging from pH 5.5 to 9.5.

A standard curve for imidaclothiz was obtained under the optimum condition by plotting percent binding (% B/B_0) versus the logarithm of the concentration of imidaclothiz (log C). % B/B_0 was calculated using

$$\%\ B/B_0 = [(A_x - A_{\min})/(A_{\max} - A_{\min})] \times 100$$

where A_x is the absorbance of the sample, $A_{\max}$ is the absorbance in the absence of analyte, and $A_{\min}$ is the absorbance of the background.

Cross-reactivity (CR). CR between antibodies and compounds that are structurally similar to the target compound is an inherent problem with ELISA.[21] The CR between antibodies with some analogues of imidaclothiz was tested using ic-ELISA. The CR was calculated in accordance with the following formula:

$$\text{CR}\ \% = (\text{IC}_{50}\ \text{of imidaclothiz}/\text{IC}_{50}\ \text{of analogue}) \times 100$$

Recovery. The spiked recoveries were used to confirm the accuracy of ic-ELISA. Tap water and paddy water samples were spiked with imidaclothiz at 0.5, 0.1, and 0.05 mg/L. The samples were diluted twice with PBS containing 20% methanol and analyzed by ELISA to estimate the recoveries. A total of 10 g of soil and cabbages (the leaves of cabbages were chopped into fine pieces) were spiked with imidaclothiz at 0.5, 0.1, and 0.05 mg/kg and overnight. They were mixed with 30 mL of dichloromethane and ultrasonic extraction for 10 min and then centrifuged for 10 min at 4000 rpm. The supernatants (15 mL) were filtered through anhydrous sodium sulfate and evaporated *in vacuo*. The remainder was dissolved with PBS containing 10% methanol and determinated by ic-ELISA. Each analysis was performed in triplicate.

RESULTS AND DISCUSSION

Identification of Conjugations. UV−vis spectra showed qualitative differences between the hapten, carrier protein, and conjugates, especially at 280 nm. The results showed that the carrier protein and hapten had been coupled successfully.

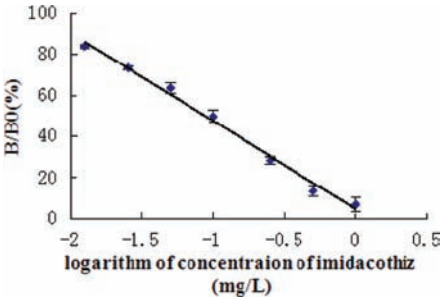
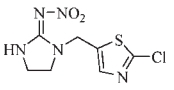
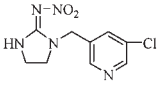
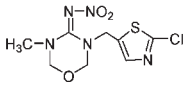
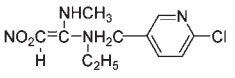
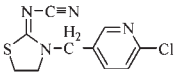
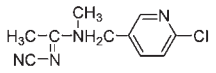
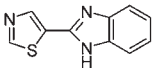


Figure 2. Standard curve of the ic-ELISA for imidaclothiz.

Table 2. CR of the Analogues of Imidaclothiz

Compound	Structure	IC_{50} (mg/L)	CR (%)
Imidaclothiz		0.0875	100
Imidacloprid		0.0954	91.7
Thiamethoxam		>10	<0.05
Nitenpyram		>10	<0.05
Thiacloprid		>10	<0.05
Acetamiprid		5.62	<0.05
Triabendazole		>10	<0.05

The molar ratios were estimated as 15:1 and 8:1 for immunogen and coating antigen, respectively.

Optimization of the ELISA. As shown in Table 1, the organic solvent could influence the sensitivity (IC_{50}) for the ELISA and the IC_{50} was the lowest at 10% methanol. The IC_{50} increased gradually as the buffer salt concentration increased, and 0.14 M of salt concentration was selected because the IC_{50} was lowest and the $R^2 = 0.99$. The pH had an insignificant effect on the sensitivity of the assay, with the IC_{50} values ranging from 0.10 to 0.17 mg/L. pH 7.4 was selected for the competition step of the immunoassays, with an IC_{50} value of 0.10 mg/L. Optimum parameters of the ELISA procedure were 10% methanol, pH 7.4, and ionic strength of 0.14 M.

CR. The inhibition standard curve of imidaclothiz was obtained under the optimum conditions (Figure 2). The curve was plotted with % B/B_0 as the lateral coordinates (y) and the logarithm of concentration of imidaclothiz (mg/L) as the longitudinal coordinates (x): $y = -42.998x + 4.475$, with $R^2 = 0.9917$. The linear range was in the range of 0.0178−0.745 mg/L. The IC_{50} and limit of detection (IC_{20}) were 0.0875 ± 0.0034 and 0.0178 ± 0.0018 mg/L, respectively.

Table 3. Recovery of Imidaclothiz in Spiked Samples

sample	spiked concentration (mg/L and mg/kg)	mean recovery ± SD (%)	CV (%)
tap water	0.5	96.72 ± 8.02	8.32
	0.1	93.01 ± 2.54	2.71
	0.05	106.81 ± 3.93	3.75
river water	0.5	93.05 ± 2.51	2.73
	0.1	106.25 ± 5.27	4.92
	0.05	99.78 ± 7.81	7.83
soil	0.5	92.42 ± 2.04	2.21
	0.1	113.83 ± 8.15	7.16
	0.05	80.43 ± 0.86	1.08
cabbage	0.5	81.40 ± 2.16	2.66
	0.1	92.03 ± 4.92	5.34
	0.05	94.69 ± 8.13	8.92

The CRs for the analogues of imidaclothiz were tested (Table 2). The result showed that the antibody did not recognize other related analytes, except imidacloprid, which showed significant CR (91.7%). The structure of imidacloprid with that of imidaclothiz has a similar imidazole ring and $=N-NO_2$. Therefore, it could be assumed that the imidazole ring and $=N-NO_2$ play an important role in the immunoreaction and the ELISA system could also be used for the determination of imidacloprid.

Analysis of Spiked Samples. Table 3 shows the recoveries and the coefficient of variations (CVs) of the tested samples. Acceptable recoveries of 80.43−113.83% and CVs of 1.08−8.92% were obtained. The results showed that the ELISA could accurately measure the imidaclothiz residue in environment and agricultural samples. This immunoassay could play an important role for environmental monitoring of imidaclothiz.

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