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Development of an Enzyme-Linked Immunosorbent Assay for the Rapid Detection of Haloxyfop-P-Methyl

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An indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) for the herbicide haloxyfop-P-methyl is reported. Two haptens of haloxyfop-P-methyl with different spacer-arm lengths were synthesized, to which polyclonal antibodies were obtained by immunizing New Zealand rabbits. The most sensitive combination of antibody/coating antigen was selected by homologous and heterologous assays. The results indicated that the spacer-arm length of hapten can affect assay sensitivity. The optimized ic-ELISA showed that the 50% inhibitory concentration (IC_{50}) was 0.0419 mg/L, and the limit of detection (LOD) was 0.0019 mg/L. Recoveries were obtained from the agricultural samples, including water, soil, cucumbers, cabbages, tomatoes, and potatoes; all were in the range of 87.4%−111.8%, well within the requirements of residue detection.

KEYWORDS: Haloxyfop-P-methyl; hapten; polyclonal antibody; ELISA

INTRODUCTION

Haloxyfop-P-methyl is an aryloxyphenoxypropionate herbicide. Due to its excellent herbicidal properties and low mammalian toxicity, it is widely used in many countries to control annual and perennial weeds in many crops, such as soybeans, cucumbers, cabbages, tomatoes, potatoes and peanuts (*1*). Current analyses for haloxyfop-P-methyl herbicide rely on gas chromatography (GC) and high-performance liquid chromatography (HPLC) (*2*, *3*), which are characterized by low limits of detection (LOD) and high precision and sensitivity. However, the instruments are expensive and the procedure is time-consuming (*4*). Immunoassays provide fast, sensitive, cost-effective and selective methods for the detection of herbicide residues, allowing sample purification steps to be reduced to a minimum (*5*, *6*). Several antibody-based tests for aryloxyphenoxypropionate herbicides have been reported. Schwalbe et al. produced antibodies of diclofop-methyl, with LOD of 0.023 mg/L (*7*). Zeng et al. changed the structure of quizalofop-ethyl, and obtained an antibody, giving an assay with an LOD of 0.00192 mg/L, and IC_{50} of 0.03495 mg/L (*8*). Moon et al. obtained three antibodies of metamifop, giving an assay with an LOD of 0.0001 mg/L (*9*).

In this paper, we report an indirect competitive ELISA (ic-ELISA) using polyclonal antibody for the determination of the herbicide haloxyfop-P-methyl. Two haptens with different spacer-arm lengths were synthesized, and the most sensitive combination of antibody/coating antigen was then selected by homologous and heterologous assays, offering a simple, sensitive ELISA for quantification of haloxyfop-P-methyl.

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MATERIALS AND METHODS

Materials. Haloxyfop-P-methyl (94.4%) was supplied by Jiangsu Yangnong Chemical Group Co., Ltd. (Jiangsu, China). *N*,*N*-Dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), dimethyl formamide (DMF), *o*-phenylenediamine (OPD), 4-aminobutyric acid, bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete and incomplete adjuvants, and goat anti-rabbit IgG-horseradish peroxidase were purchased from Sigma Chemical Co. (Shanghai, China). All reagents and solvents were of analytical grade.

Instruments. Nuclear magnetic resonance (NMR) spectrum was obtained on a DRX 500 spectrometer (Bruker, Germany). Mass spectral (MS) data was obtained with a LC-MS^{QDECA} (Finigan, USA). Ultraviolet spectra were recorded on a DU 800 spectrophotometer (Beckman, USA). The 96-well polystyrene microtiter plates were from Roskilde, Denmark. Absorbances were read with a TECAN Infinite M200 Microplate reader (Tecan, Switzerland), and ELISA plates were washed with a Wellwash Plus (Thermo, USA).

Buffer Solutions. Phosphate-buffered saline (PBS, 0.01 M, pH 7.4), carbonate buffer saline (CBS, 0.05 M, pH 9.6), phosphate-buffered saline containing 0.05% Tween-20 (PBST) and substrate solution (37.5 μL of 30% H_2O_2 and 10 mg of OPD per 25 mL of citrate buffer; pH 5.0) were used.

Hapten Synthesis. Synthesis routes for haptens with different spacer-arm lengths Hs and Hj are illustrated in **Figure 1**.

Synthesis of Hapten Hs. Haloxyfop-P-methyl (7.95 g, 0.02 mol) was added to 10.1 mL of 5% LiOH (0.021 mol), and the mixture was stirred at room temperature for 6 h (*10*). Water (20 mL) was added to the mixture and extracted with ethyl acetate. The aqueous phase was adjusted to pH 2.0 with concentrated hydrochloric acid and extracted with ethyl acetate, and the organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain the yellow crude product, which was purified by a silica gel column eluted using 150 mL of ethyl acetate/petroleum (1:9 v/v) and 200 mL of ethyl acetate/petroleum (2:8 v/v). The second fraction was collected and concentrated to obtain product, which

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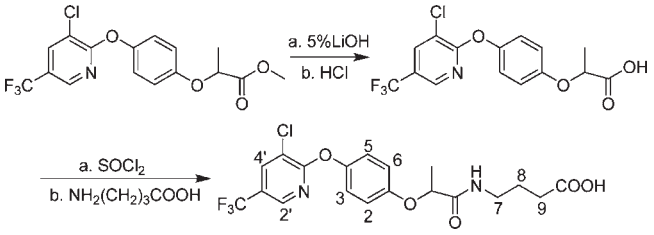


Figure 1. Synthesis of the haptens.

was characterized by ESI-MS and ^{1}H NMR. MS, *m*/*z*: 362 ($M + H^+$), 384 ($M + Na^+$). ^{1}H NMR (500 MHz, DMSO, ppm): δ 1.65 (d, 3H, $-CH_3$), 4.74 (q, 1H, CH), 6.96 (d, 2H, 2,6-H), 7.08 (d, 2H, 3,5-H), 7.97 (s, 1H, 2′-H), 8.25 (s, 1H, 4′-H).

Synthesis of Hapten Hj. 1.085 g (3 mmol) of hapten Hs dissolved in 15 mL of toluene was added to 15 mL of $SOCl_2$, and the mixture was stirred and refluxed at 70−75 °C for 2 h and evaporated to dryness *in vacuo* (50 °C) to obtain yellow acyl chloride, which was dissolved in 20 mL of THF. A mixture of 4-aminobutyric acid (0.31 g, 3 mmol) and NaOH (5 mL, 4 mol/L) was stirred in an ice bath. The acyl chloride obtained in the above was added to the mixture separately in 5 aliquots with an interval of 10 min between additions; 2 mL of NaOH (2 mol/L) was added together during each of the last four additions. The reaction mixture was stirred for 1.5 h in an ice bath (*11*). After acidification to pH 3.0 with concentrated hydrochloric acid, the mixture was extracted with ethyl acetate, and the extract was washed with water, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to afford the crude product, which was purified by a silica gel column eluted using 150 mL of ethyl acetate/petroleum (2:8, v/v) and 200 mL of ethyl acetate/petroleum (3:7, v/v). The second fraction was collected and concentrated to obtain the final product, which was checked by NMR and MS. MS, *m*/*z*: 447 ($M + H^+$), 469 ($M + Na^+$). ^{1}H NMR (500 MHz, DMSO, ppm): δ 1.53 (d, 3H, $-CH_3$), 1.75 (m, 2H, 8-H), 2.24 (t, 2H, 9-H), 3.28 (t, 2H, 7-H), 4.64 (q, 1H, CH), 6.92 (d, 2H, 2,6-H), 7.06 (d, 2H, 3,5-H), 7.96 (s, 1H, 4′-H), 8.20 (s, 1H, 2′-H).

Preparation of Hapten−Protein Conjugates. Haptens Hs and Hj were attached to BSA as an immunogen (hapten−BSA) using the active ester method, and were attached to OVA as coating antigen using a mixed anhydride reaction (*12*). The immunogens and coating antigens were purified by dialysis against PBS at 4 °C for 72 h. UV−vis spectral data was used to confirm the structures of the conjugates. The hapten-to-protein molar ratios were estimated directly by the molar absorptivity at 280 nm (ε_{280}) (*13*):

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

Production of Polyclonal Antibodies. Two male New Zealand white rabbits weighing approximately 2 kg were immunized with the immunogen to raise polyclonal antibodies according to the method described previously (*14*). The first injection was of immunogen (2 mg) diluted in physiological saline and emulsified with an equal volume of Freund's complete adjuvant. This was injected intradermally at multiple sites on each rabbit's back. A further 3 mg of immunogen emulsified with Freund's incomplete adjuvant was injected as a booster shot after 3 weeks. Booster shots were given four times at two-weekly intervals. One week after the last injection, blood samples were obtained from each rabbit's heart. The antiserum was prepared by allowing the blood to clot overnight at 4 °C, and then isolated by centrifugation. Antibody was separated by salting out with caprylic acid−ammonium sulfate, and stored at −20 °C after freeze-drying (*15*).

Screening of Antibody. The sera titers were determined by ic-ELISA (*16*). The 96-well microtiter plates were coated during overnight incubation at 4 °C with hapten−OVA (100 μL per well) in CBS. The plates were washed three times with PBST and were blocked by incubation with 1% OVA in PBS (200 μL per well) for 0.5 h. After another washing step, 100 μL per well of antiserum previously diluted with PBS was added. After incubation for 1 h at 37 °C, the plates were washed. Subsequently, 100 μL per well of a diluted (1:3000) goat anti-rabbit IgG-horseradish peroxidase was added and incubated for 1 h at 37 °C, and after another washing step, 100 μL per well of substrate solution was added. After 15 min, the reaction was stopped by adding 50 μL of H_2SO_4 solution (2 mol/L) to each well. The absorbance was measured at 490 nm.

Table 1. Homologous and Heterologous ci-ELISA for Haloxyfop-P-methyl

antibody	coating antigen	IC_{50} (mg/L)
Hs-Ab	Hs−OVA	0.05761
	Hj−OVA	1.65959
Hj-Ab	Hs−OVA	0.07468
	Hj−OVA	2.68324

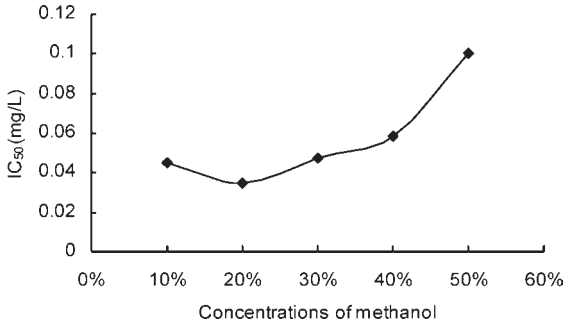


Figure 2. Effects on the IC_{50} of haloxyfop-P-methyl with different concentrations of methanol.

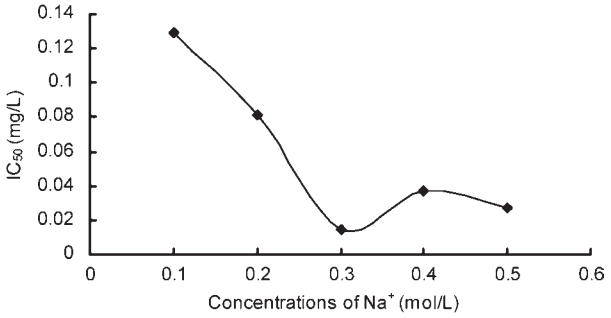


Figure 3. Effects on the IC_{50} of haloxyfop-P-methyl with different concentrations of Na^+.

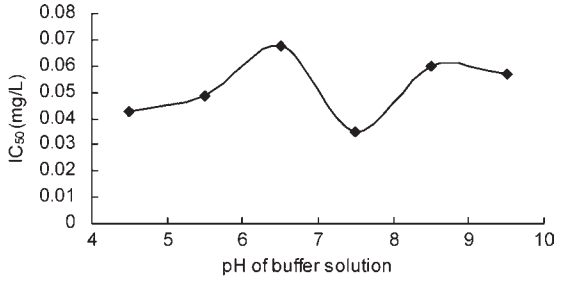


Figure 4. Effects on the IC_{50} of haloxyfop-P-methyl with different pH values.

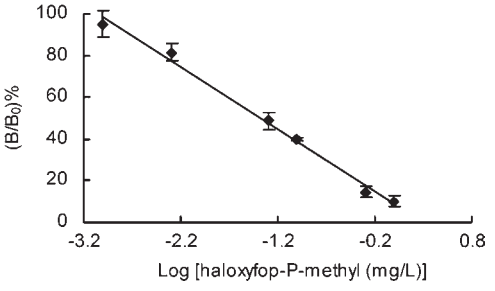
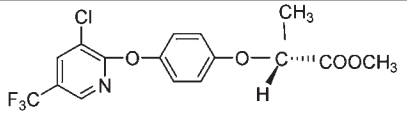
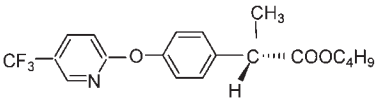
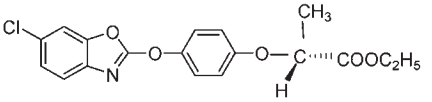
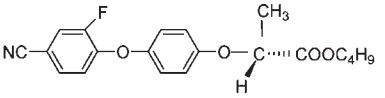
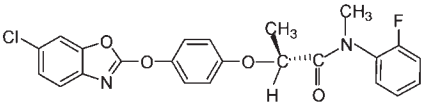
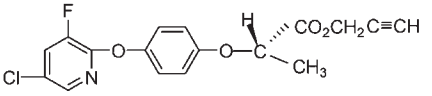


Figure 5. Standard curve of the optimized ELISA for haloxyfop-P-methyl.

Homologous and Heterologous Assays. The effect of the spacer-arm length between the immunogen and the coating antigen on the ELISA sensitivity was evaluated by homologous and heterologous assays.

Table 2. Cross-Reactivity of Analogues of Haloxyfop-P-methyl

Chemicals	Structure	IC_{50} (mg/L)	CR (%)
Haloxyfop-P-methyl		0.042	100.0
Fluazifop-P-butyl		12.65	0.33
Fenoxaprop-p-ethyl		96.64	0.04
Cyhalofop-butyl		254.70	0.02
Metamifop		36.33	0.12
Clodinafop-propargyl		10.66	0.39

Competition curves were obtained. The IC_{50} value was calculated by ic-ELISA (*17*). The steps of the ic-ELISA are the same as described above, except the "100 μL per well of antiserum" was changed to "50 μL per well of the haloxyfop-P-methyl and 50 μL per well of the antiserum."

Assay Optimization. To improve the immunoassay features, using the best coating antigen/antiserum combination, the experimental parameters, including methanol concentration, ionic strength, and pH, were studied sequentially as previously described (*18*). A standard curve for haloxyfop-P-methyl was obtained under the optimum conditions by plotting percent binding (% (B/B_0)) versus the logarithm of the concentration of haloxyfop-P-methyl (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-Reactions. The cross-reactivity of the antibody with some analogues of haloxyfop-P-methyl was determined by ic-ELISA. The cross-reactivity (CR %) of the ELISA procedure was calculated in accordance with the following formula:

$$CR\ (\%) = (IC_{50}\ \text{of haloxyfop-P-methyl})/(IC_{50}\ \text{of analogues}) \times 100$$

Analysis of Spiked Samples. Water was spiked with haloxyfop-P-methyl standards at 0.01, 0.05, 0.1, 0.5 mg/L and analyzed by ELISA to estimate the recoveries. Soil, cucumbers, beans, tomatoes, cabbages and potatoes samples (20 g) were spiked with haloxyfop-P-methyl standards at 0.05, 0.5, 1 mg/kg. Each was shaken with 30 mL of acetonitrile. After ultrasonic extraction for 10 min, the suspensions were filtered, and the filtrate was added to 5 mL of saturated sodium chloride solution and vigorously shaken. After standing 10 min, the supernatant (15 mL) was evaporated *in vacuo*. The residue was dissolved in 10 mL of PBS containing 20% methanol, and analyzed by ELISA. Each analysis was done in triplicate.

RESULTS AND DISCUSSION

Identification of Artificial Antigens and Coupling Ratio. The coupling ratio of the hapten and carrier proteins was estimated using the UV method. The UV−vis spectra of hapten−BSA, hapten−OVA, hapten, BSA, and OVA were obviously different at 280 nm, indicating these conjugates were coupled successfully. The coupling ratios of hapten to protein were 33:1, 37:1, 2.5:1 and 8:1 for Hs−BSA, Hj−BSA, Hs−OVA, and Hj−OVA, respectively.

Screening Antisera. An indirect noncompetitive ELISA was used to characterize the reactivity of the antiserum to homologous coating antigens. Titers of the two antibodies (Hs-Ab and Hj-Ab) were 1.28×10^5 ($OD_{490} = 1.0$).

Homologous and Heterologous Assays. To establish a sensitive ELISA, all combinations between coating antigens and antisera were screened by ic-ELISA. The results are shown in **Table 1**.

Table 1 show that a shorter aliphatic spacer-arm of the hapten enhanced assay sensitivity, the combination of Hs-Ab and Hs−OVA exhibited higher sensitivity.

Assay Optimization. The effects of the organic solvent, ionic strength and pH of solution were evaluated. As seen in **Figure 2**, **Figure 3**, and **Figure 4**, the optimum organic solvent was found to be 20% methanol. The ionic strength of the assay phosphate buffer was found to be 0.3 mol/L of Na^+, and pH 7.5 was found to be optimal, where the IC_{50} value was the lowest.

Standard Curve. A competitive standard curve for haloxyfop-P-methyl (**Figure 5**) was obtained using the optimized conditions. This curve was plotted with % (B/B_0) on the y-axis and log C on the x-axis; $y = -29.765x + 9.0044$ (with $R^2 = 0.9927$); the assay showed that the IC_{50} was 0.0419 mg/L, and the LOD (IC_{10}) was 0.0019 mg/L. The working range was at 0.0019−0.9260 mg/L. The antibody showed high sensitivity and specificity to haloxyfop-P-methyl.

Cross-Reactions. The specificity of the antibody was evaluated with CR % to analogues of haloxyfop-P-methyl. As seen in **Table 2**, the results show that CRs with structurally related compounds were all lower than 0.5%. It was suggested that the antibody had high specificity to haloxyfop-P-methyl. The cross-reactions of fluazifop-P-butyl and clodinafop-propargyl were significantly greater than those of other herbicides. So we believe that the heterocyclic component of haloxyfop-P-methyl was the antigenic determinant, and their heterocyclic parts were apparently similar to that of haloxyfop-P-methyl.

Analysis of Spiked Samples. The recoveries of haloxyfop-P-methyl from agricultural samples are presented in **Table 3**. The recoveries of water were 88.2%−93.4%, and coefficients of variation were 2.62%−4.42%. The recoveries from soil were in

Table 3. Spiked Recovery of Haloxyfop-P-methyl in Fortified Samples

sample	spiked concn (mg/L, mg/kg)	mean recovery ± SD (%)	CV (%)
tap water	0.01	93.43 ± 2.45	2.62
	0.05	91.50 ± 3.72	4.07
	0.1	88.53 ± 3.91	4.42
	0.5	88.23 ± 2.84	3.21
soil	0.05	111.80 ± 4.14	3.70
	0.5	102.43 ± 1.09	1.06
	1	93.33 ± 4.47	4.80
cucumber	0.05	94.72 ± 1.02	1.08
	0.5	87.43 ± 3.11	3.56
	1	95.09 ± 3.11	3.27
soybean	0.05	90.84 ± 11.41	12.56
	0.5	93.32 ± 0.67	0.72
	1	87.86 ± 3.60	4.10
tomato	0.05	88.63 ± 1.20	1.35
	0.5	89.47 ± 2.78	3.12
	1	92.34 ± 6.01	6.59
cabbage	0.05	93.77 ± 11.10	11.84
	0.5	89.96 ± 4.32	4.81
	1	97.88 ± 7.20	7.20
potato	0.05	95.53 ± 0.10	0.11
	0.5	106.58 ± 2.93	2.75
	1	103.85 ± 4.85	4.67

the range 93.3%−111.8%, with a coefficient of variation between 1.06% and 4.80%. The recoveries of six other kinds of samples were in the range 87.4%−106.6%, with a coefficient of variation between 0.11% and 12.56%. The result shows that this method has good correlation with instrumental analysis methods. These data are well within the requirements of residue analysis, and the method is suitable for quantitative detection of haloxyfop-P-methyl at trace levels.

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