Pesticide Biochemistry and Physiology 98 (2010) 68–72

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Development of an enzyme-linked immunosorbent assay for quantitative determination of cyhalofop-butyl

Jun-Dong Wang, Hua-Jun Bao, Hai-Yan Shi, Ming-Hua Wang *

Department of Pesticide Science, College of Plant Protection, Nanjing Agricultural University, Key Laboratory of Monitoring and Management of Crop Diseases and Pest Insects, Ministry of Agriculture, Nanjing 210095, PR China

ARTICLE INFO

Article history:
Received 4 November 2009
Accepted 11 May 2010
Available online 19 May 2010

Keywords:
Cyhalofop-butyl
Aryloxyphenoxypropionate herbicides
Hapten
Polyclonal antibody
ELISA

ABSTRACT

An indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) for cyhalofop-butyl was developed with a polyclonal antibody produced against a hapten (cyhalofop acid) conjugated with bovine serum albumin (BSA). The ELISA of cyhalofop-butyl showed an IC_{50} value of 0.067 ± 0.004 mg/l and the limit of detection (LOD, IC_{10}) of 0.0029 ± 0.0001 mg/l at the optimal conditions. No significant cross-reaction to other structure-related compounds suggested high specificity for cyhalofop-butyl of the method. The average recoveries of cyhalofop-butyl from fortified water and soil were in the range of 83.2–119.7% and 80.1–104.0%, respectively. These data indicate that this method is a convenient analytical technique for monitoring cyhalofop-butyl in water and soil without purification steps.

1. Introduction

Cyhalofop-butyl is a member of the aryloxyphenoxypropionate group of herbicides introduced in the mid 1980s with apparent excellent herbicidal properties and low toxicity. It is a selective post-emergence herbicide, which is registered for control annual perennial grass weeds such as *Echinochloa crusgalli, Leptochloa chinensis* and *Alopecurus aequalis* in rice paddy [1]. As a systemic herbicide to inhibit acetyl CoA carboxylase enzyme [2], cyhalofop-butyl was absorbed from the leaf surface and translocated throughout the plant, moves in both xylem and phloem from the treated foliage to the root system, and accumulated in the meristematic tissue, and thus it inhibits the biosynthesis of fatty acid [3].

Many countries have ruled the maximum residue limits (MRL) for cyhalofop-butyl (for instance, the MRL is 0.01 mg/kg in rice in the Canada and 0.02 mg/kg in all crops in the EU [4]). For quantitative analysis with HPLC [5–7] needing complex sample treatment (such as sample extraction and purification), it is necessary to develop a rapid, sensitive and inexpensive method for determination of cyhalofop-butyl. As is well known, immunoassay would provide a fast, sensitive, and selective method for the detection of pesticides at trace levels [8], and sample purification steps could be reduced to a minimum. Also a number of enzyme-linked immunosorbent assay (ELISA) methods have been reported for the detection of herbicides [9–11], very little information is known about cyhalofop-butyl. In this paper, we developed an ic-ELISA method for cyhalofop-butyl, and the evaluation of the assay's performance in water and soil were described.

2. Materials and methods

2.1. Regents

Quizalofop-p-ethyl, fenoxaprop-p-ethyl, cyhalofop-butyl, (R)-haloxyfop, and metamifop were obtained from the National Pesticide R&D South Center (Jiangsu, China). Bovine serum albumin (BSA), ovalbumin (OVA), 1,3-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), o-phenylenediamine (OPD), goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (GAR-HRP), and Freund's complete and incomplete adjuvants, and polyoxyethylene sorbitan monolaurate (Tween-20) were purchased from Sigma Chemical Co. (Shanghai, China). Phosphate-buffered saline (PBS, 0.01 M, pH 7.4). Carbonate–bicarbonate buffer saline (CBS, 0.05 M, pH 9.6). Phosphate-buffered saline containing 0.05% Tween-20 (PBST). All regents and solvents were analytical grade.

2.2. Apparatus

The 96-Well Polystyrene microplates (Maxisorp) were purchased from Nunc (Roskilde, Denmark). NMR spectrum was obtained via a DRX500 spectrometer (Bruker. Switzerland). Liquid chromatogram-Mass spectra was obtained by a LC-MS^{QDECA} (Finnigan, USA). The characterization of hapten with protein

* Corresponding author. Fax: +86 25 84395479.
E-mail address: wangmha@njau.edu.cn (M.-H. Wang).

0048-3575/$ - see front matter Crown Copyright © 2010 Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.pestbp.2010.05.001

conjugate (UV spectra) was recorded on a Beckman Coulter DU 800 spectrophotometer (Fullerton, CA). The antibody was freeze-dried by AllegraTM 64R Centrifug (Beckman, USA). ELISA plates were washed with a Wellwash Plus (Thermo, USA). ELISA experiments were performed in 96-Well polystyrene microplates and the absorbances were read with the V_{max} reader from TECAN infinite M200 (Thermo, USA) at 490 nm.

2.3. Hapten synthesis

Synthesis of the hapten was carried out as outlined in Fig. 1. Cyhalofop-butyl (3.574 g) in 10 ml of CH_3OH was added 0.6 g of NaOH in water (10 ml). The reaction mixture was stirred at room temperature for 6 h. Then the solution was acidified to about pH 2 with 6 M HCl, white precipitation was filtered. The precipitate recrystallized in ether gave the final product. The white crystal was checked up by NMR and MS: ^{1}H-NMR ($CDCl_3$), δ 1.68 (d, 3H,), 4.78 (q, 1H), 6.87–7.47 (m, 7H). LC-MS, m/z: 324 ($M + Na^+$), 340 ($M + K^+$).

2.4. Preparation of hapten–protein conjugates

The hapten was conjugated to BSA by active ester method [12]. In brief, the hapten (90.8 mg) was dissolved in *N*,*N*-dimethylformamide (DMF, 1 ml), and then a solution of NHS (104.8 mg) and DCC (92.8 mg) in DMF (2 ml) was added. The mixture was stirred for 12 h at room temperature. Supernatant liquor was added to BSA (40 mg) in 4 ml PBS with stirring. The mixture was stirred at 4 °C overnight. The solution was dialyzed in PBS at 4 °C for 72 h and stored at −20 °C. Characterization of the conjugate was identified by UV spectrophotometer. The conjugate was applied as an immunogen (hapten–BSA).

The hapten was coupled to OVA via a mixed anhydride reaction [13]. The hapten (75.6 mg) and tri-n-butylamine (60 μl) were dissolved in DMF (1 ml), isobutylchloroformate (30 μl) was added, and the formation of the mixed anhydride was allowed to proceed for 1 h at room temperature. The solution was added dropwise to OVA (60 mg) in 5 ml of PBS with thorough stirring. The mixture was stirred for 2 h and then dialyzed in PBS at 4 °C for 72 h and stored at −20 °C. Characterization was finished using UV spectrophotometer [12]. The hapten was covalently attached to OVA as coating antigen (hapten–OVA). The UV spectra of hapten–BSA, hapten–OVA, hapten, BSA, and OVA have obviously different at 280 nm, indicating these conjugates were coupled successfully.

2.5. Immunization procedure and antisera preparation

Polyclonal antibody against cyhalofop-butyl were prepared following the method of Shan GM [14]. Hapten–BSA was used as immunogen to immunize two male New Zealand white rabbits (about 2 kg). The antigen (2 mg hapten–BSA) dissolved in PBS (3 ml) was emulsified with Freund's complete adjuvant (1:1 v/v) and injected intradermally at multiple sites on the back of each rabbit. After the first injection, the rabbits were boosted four times at 2-week intervals with the immunogen (1.5 mg/kg) in Freund's incomplete adjuvant (1:1 v/v). Each rabbit was bled from the ear vein 7 days after each boosting injection. After blood was obtained by heart, the serum was separated by the method of salting out (with caprylic acid–ammonium sulfate) [15] and freeze-dried.

2.6. Indirect competitive ELISA development

A checkerboard titration assay was carried out with different amounts of the coating antigen (hapten-OVA) in 0.05 M CBS and the antisera in PBS [16]. After the screening of antisera and coating antigens, an indirect competitive ELISA was developed as follows: a microplate was coated with coating antigen (4 mg/l, 100 μl/well) by incubation at 37 °C for 2 h. The plate was washed three times with PBST and blocked with 1% OVA in PBS (200 μl/well) by incubation at 37 °C for 0.5 h. After another washing step with PBST, 50 μl/well various concentrations of analyte in PBS containing 10% methanol and 50 μl/well of the antibody were added and shake up for 1 min, the plates incubated for at 37 °C 1 h. The plate was washed again and further incubated with goat anti-rabbit IgG-HRP (1:3000 in PBS, 100 μl/well) at 37 °C for 1 h. Then a further washing, 100 μl/well of OPD solution (0.4 mg/ml in 0.05 M citrate–phosphate with 0.03% H_2O_2, pH 5.0) was added. The reaction was stopped with 2 M of sulfuric acid solution (50 μl/well) after an incubation of 15 min at 37 °C. The absorbance at 490 nm was read immediately. A linear dose–response standard curve was prepared by plotting logarithm of analyte concentration versus percent binding ($\%(B/B_0) = (A - A_{min})/(A_{max} - A_{min}) \times 100$, where A is the absorbance at a given concentration of the analyte, A_{max} is the absorbance at zero dose of the analyte, and A_{min} is the background absorbance).

2.7. Effects of solvents, salt concentrations, and buffer pH

The effects of the variables were examined by running standard curves in containing various percentages of methanol, salt concentrations, and pH values. The effects of the variables on A_{max} and midpoint inhibition concentration (IC_{50}) were evaluated. To evaluate solvent effects, methanol was diluted in PBS of 0, 10, 20, 30 and 40% (v/v). To determine effects of salt concentrations, cyhalofop-butyl was diluted in PBS and NaCl was added to give its concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 M. To determine effects of pH, cyhalofop-butyl was diluted in PBS with pH values of 4.5, 5.5, 6.5, 7.5, and 8.5.

2.8. Cross-reactivity

The optimized assays were applied to cross-reactivity studies by using the standard solution of the cyhalofop-butyl and other related compounds. Cross-reactivity (CR) values were calculated as follows: CR% = (IC_{50} of cyhalofop-butyl/IC_{50} of related compound) × 100.

2.9. Recovery

The optimized ELISA was used for cyhalofop-butyl determination in three water samples from different sources, river water (Yangtze river, Nanjing, China) and paddy irrigation water (experimental paddy field, Nanjing Agricultural University), and commercial bottled water. Water samples showed a pH between 5.8 and 6.6. While river water and commercial bottled water were used without any preparation, paddy irrigation water was filtered. In

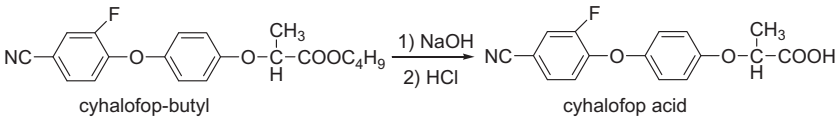

Fig. 1. Synthetic routes for the preparation of the hapten.