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Development of a sensitive ELISA for the analysis of the organophosphorous insecticide fenthion in fruit samples

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Abstract

Two fenthion haptens, 4-(4-(dimethoxyphosphorothioyloxy)-2-methylphenylamino)-4-oxobutanoic acid (H1) and 6-(methoxy(4-(methylthio)phenoxy)phosphorothioylamino)hexanoic acid (H2), were synthesized. H1 was conjugated with bovine serum albumin (BSA) and H2 with ovalbumin (OVA) by the active ester method. Then H2-OVA conjugate was used as coating antigen, while H2-BSA conjugate was used to produce polyclonal antibodies. After optimization, an effective competitive indirect enzyme-linked immunosorbent assay (ELISA) for determination of fenthion was established with the new combination of antibody/antigen, I_{50} of which was 0.01 ng/ml, and there was only cross reactivity (CR) with fenitrothion (4.5%), and CRs with other tested pesticides were all below 0.1%. The recoveries obtained by standard fenthion addition to the different fruit samples such as grape, peach, pear and tomato were all from 79.8% to 106.0%. Therefore, the optimized ELISA may become a new convenient and satisfied analytical tool for monitoring fenthion residues in agricultural samples.

Keywords: Organophosphorus pesticide; Fenthion; Hapten; Antibody; ELISA

1. Introduction

Organophosphorous insecticides (OPs) are a group of highly toxic compounds which are extensively used as agricultural and domestic pesticides (Costa, 1988). OP toxicants generally elicit their effects by inhibition of acetylcholinesterase, which leads to the accumulation of the neurotransmitter acetylcholine (Ach) in synapses, over stimulates the post synaptic cholinergic receptors with consequent signs of neurotoxicity (Ecobichon, 1996; Gallo & Lawryk, 1991). So non-target organisms, such as human, fish, bee and so on, are also threatened by the insecticides.

Fenthion, *O*,*O*-dimethyl *O*-[3-methyl-4-(methyl-thio)-phenyl]phosphorothioate is one of OP insecticides. It is also moderately toxic to mammals (Brun, Garcés-García, Puchades, & Maquieira, 2004). Due to its extensive application to crops and cattle, the occurrence of fenthion residues in food and the environment has been widely reported (Tsatsakis et al., 2003). Thus, many countries classified fenthion as a restricted use pesticide and gave a strict standard of maximum residue limited (MRLs) for the chemical in farm produce, and China government is not an exception.

Several methods have been described for the determination of fenthion at trace levels, including high performance liquid chromatography (Cabras, Plumitallo, & Spanedda, 1991), flow injection (Hernandez, Carabias, Becerro, & Jiménez, 1988), colorimetric and bioassay techniques (Devi, Mohandas, & Visalakshy, 1986), cholinesterase-based biosensors (Lee, Kim, Cho, & Lee, 2002), and gas

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chromatography (Arrebola, Martínez Vidal, Gonzaález-Rodríguez, Garrido-Frenich, & Sánchez Morito, 2003). However, more specific, sensitive, rapid and economical analytical methods for the detection of fenthion residues are needed. In particular, an immunoassay method would be a useful analytical tool (Brun et al., 2004). Immunoassays for fenthion have been reported (Brun et al., 2004; Cho, Kim, Hammock, Lee, & Lee, 2003; Kim et al., 2003a, Kim, Cho, Lee, & Lee, 2003b) recently. In our previous work, five haptens with different spacer-arm attachment sites on the structure of the organophosphorus insecticide fenthion were synthesized and 15 combinations of immunizing/coating hapten were selected for studies of assay sensitivity and specificity for fenthion. Finally, we found the most sensitive combination which was different from other people'. With this novel combination of immunizing/coating hapten, we developed a sensitive ELISA for detection of fenthion residues in several fruit samples.

2. Materials and methods

2.1. Chemicals and instruments

Chemical reagents for hapten synthesis and pesticide standards used for cross-reactivity studies were supplied by Jiangsu Pesticide Research Institute (Nanjing, China). Analytical grade solvents were from Sinopharm Group Chemical Reagent Co., Ltd (Shanghai, China). Tween 20, *N*-hydroxysuccinimide (NHS), *N*,*N*′-dicyclohexylcarbodiimide (DCC), bovine serum albumin (BSA), ovalbumin (OVA), and complete and incomplete Freund's adjuvant were purchased from Sigma (St. Louis, USA). Tetramethylbenzidine (TMB) and peroxidase-labeled goat anti-rabbit immunoglobulins (GAR-HRP) were obtained from Huamei Biotechnology Co. (Luoyang, China). All other reagents used were analytical grade. Thin-layer chromatography (TLC) was performed on 0.25 mm, precoated silica gel 60 F254 on aluminum sheets (Merck, Darmstat, Germany). Column chromatographic purifications were carried out with silica gel (60–230 mesh), from Qingdao Haiyang Chemical Co., Ltd (Qingdao, China).

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a Bruker ARX pectrometer (300 MHz, Rheinstetten, Germany). Chemical shift values are given in parts per million (ppm) downfield from internal standard deuterium chloroform. Coupling constants are expressed in Hz and the abbreviations s, d, t, m and ar, represent singlet, doublet, triplet, multiplet and aromatic, respectively. UV–Vis spectra were recorded on a Bechman 640 spectrophotometer. Polystyrene 96-well microtiter plates were from Costar (Corning, Massachusetts, USA). A microplate washer from Prolong New Technology Co. (Beijing, China) was used to wash ELISA plates. Absorbance (A) was measured using a microtiter plate reader (Thermo Electron Co., United States); this device was controlled by a personal computer containing the standard software package EasySoftware.

2.2. Synthesis of haptens

2.2.1. Synthesis of immunizing hapten (H1)

To a solution of compound **1** (2.1 g, 7 mmol) in ethyl ether (40 mL) were added 9:1 acetic acid:HCl (21 mL) and zinc dust (4.1 g, 60 mmol). The reaction mixture was stirred for 30 min at room temperature, and then refluxed for 30 min. The mixture was decanted from the reaction flask and the zinc was washed with ether. The combined organic phase was then washed with water and dried over K_2CO_3. The solvent was evaporated and the residue was subjected to column chromatography (dichloromethane, R_f: 0.55) to give the product as a yellow-red syrup (1.8 g, 79%).

^{1}H NMR ($CDCl_3$) δ: 2.15 (s, 3H), 3.82 (s, 3H, CH_3-OP), 3.87 (s, 3H, CH_3-OP), 6.61 (d, $J = 8.5$ Hz, 1H, Ar), 6.84 (s, 1H, Ar), 6.86 (d, $J = 8.5$ Hz, 1H, Ar); ^{13}C NMR ($CDCl_3$) δ: 123.0, 119.4, 115.7, 77.9, 77.5, 77.1, 55.5, 18.0; MS (EI) m/z (%): 247 (M^+, 80), 138 (100).

To a solution of compound **2** (1.7 g, 6 mmol) in dichloromethane (90 mL) was added succinic anhydride (0.7 g, 6 mmol). After the mixture was stirred at room temperature for 18 h, the solution was concentrated. The recrystallization of the residue from ethyl ether gave H1, 4-(4-(dimethoxyphosphorothioyloxy)-2-methylphenylamino)-4-oxobutanoic acid, as a brown solid (1.9 g, 90%).

^{1}H NMR ($CDCl_3$) δ: 2.23 (s, 3H), 2.70 (t, $J = 3.1$ Hz, 2H), 2.80 (t, $J = 3.1$ Hz, 2H), 3.84 (s, 3H, CH_3-OP), 3.88 (s, 3H, CH_3-OP), 7.02 (d, $J = 9.2$ Hz, 1H, Ar), 7.38 (s, 1H, Ar), 7.68 (d, $J = 9.2$ Hz, 1H, Ar), 8.25 (s, 1H, NH); ^{13}C NMR ($CDCl_3$) δ: 178.1, 171.2, 148.2, 132.9, 125.6, 123.1, 119.2, 119.1, 55.6, 31.7, 29.9, 18.3; MS (ESI) m/z (%): 346 ($M-H^+$, 44).

2.2.2. Synthesis of coating hapten (H2)

Compound **6** (H2), 6-(methoxy(4-(methylthio)phenoxy)phosphorothioylamino)hexanoic acid, was prepared as Kim et al.described (2003a). The product was a colorless liquid and its ^{1}H NMR was same as the reference (Kim et al., 2003a). MS (ESI) m/z (%): 376 ($M-H^+$, 21), 753 ($2M-H^+$, 100).

2.3. Preparation of hapten–protein conjugates

For immunization purposes, H1 was covalently attached through its carboxylic acid moiety to the lysine groups of BSA by the active ester method (Langone & van Vunakis, 1982). Additionally, H2 was coupled to OVA to obtain coating antigens with the same method as the above. Then, the immunogen (H1-BSA) and the coating antigen (H2-OVA) were purified by dialysis (Zhang et al., 2006) against phosphate buffer (PB: 0.02 mol /L, pH 6.8). The conjugates were stored at −20 °C until use. Finally, UV–Vis spectral data supported the structures of the final conjugates. The hapten density (the number of the hapten molecules per molecule of protein) of conjugates was estimated directly by mole absorbance ε.