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Analytical Methods

Development of a McAb-based immunoassay for parathion and influence of the competitor structure

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ABSTRACT

A specific monoclonal antibody (McAb) for parathion was produced. Based on this McAb, a battery of competitors as coating antigens were used to develop homologous and heterologous indirect competitive enzyme-linked immunosorbent assays (ELISAs) for parathion. The relationship between the heterology degree of the competitor and the sensitivity of the corresponding immunoassay was investigated. Results showed that, when the specific McAb was used in the ELISA experiment, competitors should have a certain degree of homology with the immunizing hapten for immunoassays, and the best performance occurred when the competitor hapten was highest heterologous to the target analyte. With the most suitable competitor, a sensitive and selective ELISA was developed. The IC_{50} value of the ELISA was 2.94 ng/ml with a detection limit (IC_{20}) of 0.70 ng/ml. The average recoveries of parathion in spiked water, soil, cucumber and rice were 88.09%, 93.15%, 91.37% and 83.42%, respectively.

1. Introduction

Organophosphate pesticides are considered as the substances of high environmental impact due to their toxicity and persistence (Roex, Keijzers, & Gestel, 2003). They can be found in many compartments of the ecosystem and in the organisms. Parathion (O,O-diethyl O-4-nitrophenyl phosphorothioate), belongs to the organophosphorus pesticides, can deactivate the enzyme acetylcholinesterase, disrupt nerve function, and lead to paralysis and death (Buratti, Volpe, Meneguz, Vittozzi, & Testai, 2003; Fenske, Lu, Barr, & Needham, 2002). Due to its high toxicity and persistence, parathion is fully prohibited to be used in agriculture in many countries over the world. Nowadays, the residue of parathion is still one of the important items in the detection of pesticide residues in the international trade. The conventional methods for detection of parathion involving gas chromatography (GC) (Bai, Zhou, & Wang, 2006; Basheer, Alnedhary, Rao, & Lee, 2007) and high-performance liquid chromatography (HPLC) (Rotich, Zhang, & Li, 2003) are sensitive and reliable. However, these applications are relatively slow and usable only in laboratory scale. For high sample throughput and on-site monitoring of pesticide residues, it is important to develop and explore technologies that more rapid, economical and without need of complicated derivatization steps.

Enzyme-linked immunosorbent assay (ELISA) is proven to be simple, cost-effective, and do not require sophisticated instrumentation (Abad et al., 1999; Nunes, Toscano, & Barceló, 1998). Further more, the immunoassay is demonstrated as a powerful tool involved in high sample throughput and on-site screening in pesticide monitoring programs (Ferguson, Kelsey, Fan, & Bushway, 1993; Gabaldón, Maquieira, & Puchades, 1999). All of these features make the immunoassay a very promising analytical tool. Many ELISA methods have been established for parathion. A radioimmunoassay was developed by Ercegovich et al. (1981). The resulting assay did detect parathion, but the limit of detection (LOD) was very high. Ibrahim, Morsy, and Hewedi (1994) produced a monoclonal antibody (McAb) to parathion and ELISA was developed. But the immunoassay showed significant cross-reactivity with parathion-methyl (50%). Garrett, Appleford, Wyatt, Lee, and Morgan (1997) produced a recombinant anti-parathion antibody (ScFv). LOD for the McAb-based and ScFv-based ELISAs were 16 and 20 ng/ml, respectively. Recently, Zeng et al. (2007) developed a McAb-based ELISA for parathion with the sensitivity (IC_{50}) and the detection limit (IC_{10}) of 360 and 26 ng/ml, respectively.

In our previous work (Liu et al., 2007), we developed a more sensitive polyclonal antibody-based immunoassay for parathion (LOD was 0.31 ng/ml). Because McAb offered a more definite specificity than polyclonal antibodies and an unlimited production, in this work, a specific McAb was produced. Furthermore, as an increase of the detectability by introducing a certain degree of heterology in the chemical structure of the competitor was often reported (Galve, Baeza, Camps, & Marco, 2002; Holthues, Fukumura, Sound, & Baumann, 2005; Kim, Cho, Lee, & Lee, 2003; Kim, Kim, Lee, & Lee, 2007), the influence of the competitor structure on McAb-based immunoassay sensitivity and specificity was

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also investigated. Additionally, theoretical computer models were used to objectively assess the degree of heterology existing between the analyte and the haptens. Finally, a heterologous indirect ELISA (the immunizing hapten and the coating hapten differ in their molecular structures) based on this McAb was developed, and applied to detect parathion residue in water, soil, cucumber and rice samples.

2. Experimental

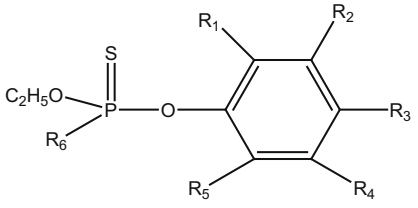
2.1. Immunoreagents, chemicals and instruments

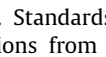
Table 1 showed the structures of parathion and the haptens (hapten **1**–**14**) used in this study. The preparation of the immunizing antigen (hapten **1**-BSA) and the coating antigen (hapten **1**–**14**-OVA) had been described in a previous paper (Liu et al., 2007). Parathion and other pesticide standards were obtained from National Standards Company (China).

Ovalbumin (OVA, MW45000), bovine serum albumin (BSA, MW67000), peroxidase-labelled goat anti-mouse immunoglobulins, culture media RPMI-1640, hypoxanthine–aminopterin–thymidine (HAT) and hypoxanthine–thymidine (HT) medium supplements, pristane, complete and incomplete Freund's adjuvants, dimethyl sulphone (DMSO), polyethylene glycol (PEG) 3350 were purchased from Sigma–Aldrich (Spain). SP2/0 mouse plasmacytoma line was from Shanghai Institute of Cell Biology (China). *O*-phenylenediamine (OPD) and Tween 20 were purchased from Shanghai Chemical Reagents Company (China). Phosphate-buffered saline (PBS, 10 mM, pH 7.4), carbonate-buffered saline (CBS, 50 mM, pH 9.5) and phosphate–citrate buffer (pH 5.6) were self-prepared. All other chemicals and organic solvents were of analytical grade or better.

The ELISA was carried out in 96-well polystyrene microplates (COSTAR, High Binding Plates, US). Plates were washed with a DEM plate washer (Beijing Tuopu Analytical Instruments Co. Ltd., China) and absorbencies were read with a 550 plate reader (Bio-Rad, America).

Table 1
Chemical structures of parathion and its haptens.



Compound	R_1	R_2	R_3	R_4	R_5	R_6
Parathion	H	H	NO_2	H	H	C_2H_5O
Hapten **1**	H	H	NO_2	H	H	$NH(CH_2)_4COOH$
Hapten **2**	H	H	NO_2	H	H	$NH(CH_2)_3COOH$
Hapten **3**	H	H	NO_2	H	H	
Hapten **4**	F	H	NO_2	H	H	$NH(CH_2)_3COOH$
Hapten **5**	NO_2	H	H	H	H	$NH(CH_2)_3COOH$
Hapten **6**	H	NO_2	H	H	H	$NH(CH_2)_3COOH$
Hapten **7**	NO_2	H	F	H	H	$NH(CH_2)_3COOH$
Hapten **8**	NO_2	H	H	F	H	$NH(CH_2)_3COOH$
Hapten **9**	H	H	NH_2	H	H	C_2H_5O
Hapten **10**	H	H	C_2H_5	H	H	$NH(CH_2)_3COOH$
Hapten **11**	H	H	$C(CH_3)_3$	H	H	$NH(CH_2)_3COOH$
Hapten **12**	Cl	H	Cl	H	H	$NH(CH_2)_3COOH$
Hapten **13**	Cl	H	F	H	H	$NH(CH_2)_3COOH$
Hapten **14**	OCH_3	H	H	H	OCH_3	$NH(CH_2)_3COOH$

2.2. McAb production and characterisation

2.2.1. Immunization

BALB/c female mice (8–10 weeks old) were immunized with hapten **1**-BSA conjugate. First dose consisting of 100 μg of the conjugate was intraperitoneally injected as an emulsion of sterilized physiological saline and complete Freund's adjuvant. Booster injections were given 2, 4 and 6 weeks after the initial dose, with the same amount of immunogen emulsified in incomplete Freund's adjuvant. One week after the last injection, mice were tail-bled and titres of antisera were determined by indirect ELISA. After a resting period of at least 3 weeks from the last injection in adjuvant, mice selected to be spleen donors for hybridoma production received a final soluble intraperitoneal injection of 100 μg of conjugate in physiological saline (without adjuvant), 3 days prior to cell fusion.

2.2.2. Cell fusion

SP2/0 murine myeloma cells (screened by 8-Azaguanine) were cultured in RPMI-1640 media supplemented with 2 mM glutamine 4.5 g/l glucose, 15 mM HEPES and 15% foetal bovine serum. Cell fusion procedures were carried out essentially as described by Nowinshi, Lostrom, Tam, Stone, and Burnette (1979). Mouse spleen lymphocytes were fused with myeloma cells at 5:1 ratio using PEG 3350 as the fusing agent. The fused cells were distributed in 96-well culture plates (approximately 4×10^5 cells/100 μl of RPMI-1640/well), and 100 μl of HAT selection medium (RPMI-1640 medium supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) was added to each well 2 or 3 days after plating. Half of the medium of the wells was replaced by fresh HAT medium on the 6 day post-fusion and by HT medium (HAT medium without aminopterin) on the 10 day post-fusion.

2.2.3. Hybridoma selection and cloning

Twelve to fourteen days after cell fusion, culture supernatants were screened with homologous coating conjugate for the presence of antibodies that recognised parathion. Selected hybridomas were subcloned by limiting dilution. Stable antibody-producing clones were expanded and stored in liquid nitrogen.

2.2.4. McAb characterisation

Selected antibody-producing clones were cultured in 150-ml flasks, and the supernatant was collected. Class and subclass determination was performed using Pierce ImmunoPure Monoclonal Antibody Isotyping Kit (HRP/ABTS). With the supernatant of cell culture medium with proper dilution and the coating antigen hapten **1**-OVA, established competitive indirect ELISA for parathion.

2.3. Competitive indirect ELISA

For competition assays, the antibody and coating antigen concentrations were optimised with checkerboard titration. All incubations were carried out at 37 °C. Standards were prepared in 10% methanol–PBS by serial dilutions from a stock solution in methanol, using borosilicate glass tubes. The ELISA was run as described as a preceding paper (Liu et al., 2007). Briefly, Microtiter plates were coated with the optimised concentrations of antigens in CBS (100 μl/well) by incubation for 2 h. The plates were washed and then blocked by incubation with 2% of degreasant milk in PBS (300 μl/well) for 30 min. Serial dilutions (50 μl/well) of the analyte in methanol–PBS were added, followed by adding 50 μl/well of a previously determined McAb concentration. After incubation for 1 h, 100 μl per well of diluted (1/10000) peroxidase-labelled goat