Article

Immunoassay Development for the Class-Specific Assay for Types I and II Pyrethroid Insecticides in Water Samples

Qi Zhang, Wen Zhang, Xiuping Wang and Peiwu Li *

Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan 430062, China; E-Mails: zhangqi521x@yahoo.cn (Q.Z.); zhangwen@oilcrops.cn (W.Z.); xiupinwang@sina.com (X.P.W.)

* Author to whom correspondence should be addressed: E-Mail: peiwuli@oilcrops.cn.

Received: 28 October 2009; in revised form: 10 December 2009 / Accepted: 29 December 2009 / Published: 4 January 2010

Abstract: Five generic haptens of pyrethroid insecticides, which were classified as three types, were designed and synthesized: the first (hapten 1) is for type I pyrethroids without a cyano group, the second (hapten 2 and XQ) for type II pyrethroids with a cyano group, and the third (hapten 4 and 5) for both types of pyrethroids with loss of the ester group. The hapten structures were confirmed by MS and $^1$H-NMR. Hapten 1 and 2 were conjugated with BSA respectively and haptens 1-5 were conjugated with OVA. Four polyclonal antisera were raised against BSA conjugates including a mixture conjugate, and twenty antibody/coating conjugate combinations were selected for studies of assay sensitivity and specificity for pyrethroids. The study revealed the best combination, which showed equal high sensitivities ($I_{50}$ is around 0.02 µg mL$^{-1}$) to both types of pyrethroids. The immunity results suggest that, with a mixture conjugates, a polyclonal antibody against a group of insecticides can be prepared for multi-residue assays.

Keywords: hapten; class-specific antibody; sensitivity; immunoassay; pyrethroid insecticides

Abbreviations: THF, tetrahydrofuran; DMF, dimethyl formamide; NHS, N-hydroxysuccinimide; DCC, N,N-dicyclohexylcarbodiimide; BSA, bovine serum albumin; OVA, ovalbumin; CR, cross-reactivity; ELISA, enzyme-linked immunosorbent assay; TMB, Tetramethylbenzidine; GAM-HRP, peroxidase-labeled goat anti-mouse immunoglobulins; $I_{50}$, concentration giving 50% inhibition of maximum response; LOD,
Introduction

Pyrethroid insecticides have been being used widely from agricultural uses [1-4] to home pest control [5,6] and are effective against a broad range of pests. The synthetic pyrethroids and natural pyrethrins can be divided into two groups of compounds on the basis of their chemical structure and mechanism of action at insect target sites (Figure 1): The type I compounds are simple cyclic alcohol esters of 2,2-dimethyl-3-(2-methyl-1-propenyl)-cyclopropanecarboxylic acid, and the type II compounds are esters of an aryl cyanohydrin [7]. Synthetic pyrethroid residues have been seen in agricultural products [8,9] and food [10]. Lower and lower amounts are being allowed worldwide by regulatory agencies [11]. Many pyrethroids act as a neurotoxin, they are also highly toxic to aquatic life, particularly fish [12,13], so it is very important to develop a rapid, sensitive, specific methods to monitor pyrethroid residues in food products.

Figure 1. Structures of some synthetic pyrethroids.
relatively intensive, time-consuming, expensive, and not particularly suitable for large numbers of samples.

Immunoassays have been considered as a valuable supplement to existing, and rapidly developing, chromatographic techniques, because they have attractive features including high sensitivity and selectivity, rapid detection, and the possibility of analysis of difficult matrices without extensive pre-treatment [21].

When an immunoassay for a single analyte was developed in the past years, people all expected CRs as lower as possible [22–27]. With the increase in requests for detection of chemical residues, there has been a gradual focus on total and multi-residues immunoassays. So, with higher CRs, some immunoassays for a type of compounds are also expected and some class specific antibodies against organophosphorus [28–30], type I or type II pyrethoid insecticides have been reported [7,31,32]. Here we aimed to develop a combination of coating conjugates and antibodies with equal high sensitivity to both type I and type II pyrethoids.

**Scheme 1.** Synthetic schemes for the preparation of generic haptens 1, 2, 4, and 5 and the structure of hapten XQ.
Results and Discussion

Hapten design and conjugate verification

Obtaining haptens with proper chemical structures is the key to development of novel antibodies and immunoassays. According to the characteristics of the two types of synthetic pyrethroid pesticides [32], here we designed three types of haptens (Scheme 1): the first (hapten 1) is for type I pyrethroids without a cyano group, the second (hapten 2 and XQ) for type II pyrethroids with a cyano group, and the third (hapten 4 and 5) for both types of pyrethroids with loss of the ester group. Most of these haptens were synthesized simply with just one or two steps and without any rigorous condition.

Through scanning the UV-Vis spectrum, hapten/protein ratios were calculated by measuring the absorbance of the hapten, the protein, and the hapten-protein conjugate at the same wavelength, and the ratios of BSA-hapten 1, BSA-hapten 2, OVA-hapten 1, 2, 4, 5, XQ were 14, 13, 9, 6, 8 and 4.

Here, hapten 1 and hapten 2 were used as immunizing haptens. To attempt to produce specific antibodies against both types of pyrethroid insecticides, a mixture of conjugates of hapten 1 and hapten 2 was also regarded as an immunogen. All of the above OVA conjugates, (OVA-hapten 1 (CAg-1), OVA-hapten 2 (CAg-2), OVA-hapten 4 (CAg-4), OVA-hapten 5 (CAg-5), OVA-hapten XQ (CAg-XQ)) were used as coating antigen. According to references, some generic immunizing haptens of each type of pyrethroids have been described [7,31–33]. Comparing with the reported, both hapten 1 and hapten 2 retain the ester group but lose a cyclopropane group.

Effect of the haptens on affinity of the antisera for the coating antigens

The most important precondition is that a sufficient titer value for combination of antibody and antigen exist [34]. To investigate homo- and heterologous affinity, twenty antibody/coating conjugate combinations were tested by a noncompetitive ELISA protocol. Some titer values, which were found to be difference among the combinations are shown in Table 1.

| Immunogen          | Antiserum | Antibody dilution | Coating antigen (1 μg mL⁻¹) |
|--------------------|-----------|-------------------|-----------------------------|
|                    |           |                   | cAg-1 | cAg-2 | cAg-4 | cAg-5 | cAg-XQ |
| BSA-hapten 1       | pAb-1      | 1: 8000           | 1.65  | 0.398 | 0.629 | 0.412 | 0.257  |
| BSA-hapten 2       | pAb-2      | 1: 8000           | 0.573 | 1.484 | 0.384 | 0.198 | 0.626  |
| BSA-hapten 1 + 1   | pAb-m1     | 1: 3200           | 1.344 | 0.925 | 0.754 | 0.458 | 0.174  |
|                    | pAb-m2     | 1: 3200           | 1.302 | 1.064 | 0.400 | 0.352 | 0.174  |

a Four antibodies, named pAb-1, pAb-2, pAb-m1, and pAb-m2 respectively, were determined. Absorbances were measured by a checkerboard pattern with several coating conjugate concentrations and several antibody dilutions, and measured after a 12-min incubation with TMB at 37 °C. For convenience, only data from a coating antigen concentration of 0.1 μg per well and an antibody dilution of 1:8,000 or 1:3,200 are shown. Titer values are the means of three replicates.

The results indicated that the antibodies induced by type I hapten (hapten 1) had cross-reactivity to type II hapten (hapten 2) and the reverse was also true. Considering the distinguishing titer difference between coating haptons with and without a cyano group, we thought that the cyano group had a
Molecules 2010, 15

strong effect on such a antibody/hapten reaction system. We also discovered significantly different
data for the combination of pAb-2/cAg-1 (0.573) and of pAb-2/cAg-4 (0.384), which indicated that the
ester group had also little effects on the reaction system.

The results of those antibodies against the mixture immunogen (BSA-hapten 1 and BSA-hapten 2)
showed that the combination of pAb-m1/cAg-4 had a high titer value (0.745) in addition to “semi-
homologous combinations”, such as pAb-m1/cAg-1, pAb-m1/cAg-2 and so on. Those combinations of
antibody/coating antigen, with enough affinity and possibility to develop an ELISA for both types of
synthetic pyrethroid insecticides, were selected for further studies.

**Effect of the haptens on ELISA sensitivity and specificity**

To investigate homo- and heterologous sensitivity, five antibody/coating conjugate combinations
were tested by a noncompetitive ELISA protocol. To screen the broadest specific combination, five
synthetic pyrethroid insecticides were select for cross-reactivity analysis. The sensitivity and
specificity data are shown in Table 2.

In the first combination of pAb-1/cAg-2, the ELISA was only sensitive to type I pyrethroids
(phenomethrin and permethrin). Nevertheless the ELISA showed more sensitivity to type II
datamethrin, cypermethrin, and cyhalothrin) than to type I in the second combination of pAb-2/cAg-1.
Considering that pAb-1 was prepared with type I immunogen and that pAb-2 was prepared with type
II, we could obviously see the cyano group on the type II immunogen had an important effect during
the immunization process. The result suggested that, with only one type pyrethroid immunogen,
whether type I or type II, it is difficult (even impossible) to obtain antibodies with equal sensitivity to
both types of pyrethroids.

Therefore, to obtain the antibodies with equal sensitivity to both types of pyrethroids, a mixed
immunogen with type I and type II pyrethroid conjugates was used for antibody preparation. In Table
2, the last three combinations were all against such mixed immunogen. The ELISA of pAb-m1/cAg-1
combination showed an I_{50} range of 0.1–0.3 µg mL^{-1}, pAb-m1/cAg-2 combination 0.2–0.6 µg mL^{-1},
and pAb-m2/cAg-4 combination 0.016–0.023 µg mL^{-1}. According to these results we found the last
combination of pAb-m2/cAg-4, with a heterologous ELISA system, had the most equal sensitivity (all
I_{50}s were around 0.02 µg mL^{-1}) to both types of pyrethroids, and the sensitivity was close to the
references (0.02–0.03 µg mL^{-1} for type I, 0.0015–0.013 µg mL^{-1} for type II) [7,31,32]. So the
combination of pAb-m2/cAg-4 was select for further research.

**Table 2. Specificity of antibody/coating antigen combinations.**

| Antibody/coating antigen | Pesticide      | I_{50} µg mL^{-1} | Cross-reactivity % \(^a\) |
|--------------------------|----------------|-----------------|---------------------------|
| pAb-1/cAg-2              | phenomethrin   | 0.29            | 100                       |
|                          | permethrin     | 0.42            | 69.0                      |
|                          | datamethrin    | - \(^c\)        | <0.1                      |
|                          | cypermethrin   | - \(^c\)        | <0.1                      |
|                          | cyhalothrin    | - \(^c\)        | <0.1                      |
| pAb-2/cAg-1              | phenomethrin   | 0.204           | 100                       |
|                          | permethrin     | 0.325           | 62.8                      |
|                          | datamethrin    | 0.052           | 392.3                     |
|                          | cypermethrin   | 0.049           | 416.3                     |
|                          | cyhalothrin    | 0.058           | 351.7                     |

\(^a\) Pesticide concentration at half-maximal sensitivity.
\(^b\) Cross-reaction percentage where 100% refers to the specificity of the respective test.
Table 2. Cont.

|                | Phenomethrin | Permethrin | Datamethrin | Cypermethrin | Cyhalothrin |
|----------------|--------------|------------|-------------|--------------|-------------|
| Phenomethrin   | 0.278        | 0.333      | 0.136       | 0.098        | 0.165       |
| Permethrin     | 100          | 83.5       | 204.4       | 283.7        | 168.5       |
| Datamethrin    | 100          | 76.2       | 49.9        | 39.1         | 64.0        |
| Cypermethrin   | 100          | 77.3       | 73.9        | 89.5         | 106.2       |
| Cyhalothrin    | 100          | 35.4       | 9.1         | 13.6         |             |
| Tetramethrin   | >10          | <0.1       |             |              |             |
| Bifenthrin     | >10          | <0.1       |             |              |             |
| 3-Phenoxybenzoic acid | >1       | <1        |             |              |             |

*a The coefficient of variation(CV) was below 12%; b The cross-reactivity of phenomethrin in all combinations was regarded as 100%, and those of other pyrethroids were calculated as follows: cross-reactivity (%) = [I50 (phenomethrin) /I50 (other pyrethroid)] × 100; c The data was not calculated because no significant inhibition was observed.

Average competitive curve and spiked sample analysis

In the above selected ELISA system, the coating antigen cAg-4 and the polyclonal antibody pAb-m1 were used. A series of concentrations of each standard pyrethroid: phenomethrin, permethrin, datamethrin, cypermethrin, and cyhalothrin was tested, whose results contributed to the average standard curve (Figure 2). Among the results, the average coefficient of variation (CV) was 16%, I50 was 0.02 µg mL⁻¹, and the dynamic range (I20–I80) was 0.002–0.084 µg mL⁻¹.

With the average standard curve, a spiking experiment was carried out for an elementary accuracy evaluation. The results showed the recoveries obtained by standard pyrethroids added to tap water samples were from 57% to 73% (Table 3), which indicated that the combination of pAb-m2/cAg-4 will be a useful screening test system for both type I and type II pyrethroids.

Theoretically, we also paid attention here to whether the total concentration of several different compounds with a same type could be calculated as that of just one analyte in a class specific ELISA curve. However, the results could not give clearly an answer to this, so we need to do more related work to answer this question.
Figure 2. Average standard inhibition curve of five pyrethroids. In an ELISA system, the coating antigen cAg-4 was used with a concentration of 2 µg mL⁻¹, and the polyclonal antibody pAb-m1 was used with a dilution of 1:4,000. Then a series concentrations of each standard pyrethroid phenomethrin, permethrin, datamethrin, cypermethrin, and cyhalothrin was tested, whose results contributed to the average standard curve (the average maximum absorbance was 0.952, and the slope value is -1.266). And the average coefficient of variation (CV) was 16%, I₅₀ was 0.02 µg mL⁻¹, and the dynamic range (I₂₀-I₈₀) was 0.002–0.084 µg mL⁻¹.

Table 3. Recovery test of synthetic pyrethroids in drinking water.

| pyrethroid insecticide | spiked (µg mL⁻¹) | theoretical (µg mL⁻¹) | found (µg mL⁻¹) | average recovery ± SD (%) |
|------------------------|------------------|-----------------------|-----------------|--------------------------|
| permethrin              | 0.01             | 0.006                 | <0.002          | 73.3 ± 14.2              |
| datamethin             | 0.02             |                       | 0.004           |                          |
| permethrin              | 0.01             | 0.006                 | 0.004           | 56.9 ± 10.6              |
| datamethin             | 0.02             | 0.010                 | 0.006           |                          |
| cyhalothrin            | 0.02             |                       |                 |                          |
| permethrin              | 0.02             |                       |                 | 72.9 ± 6.9               |
| datamethin             | 0.04             | 0.040                 | 0.029           |                          |
| cyhalothrin            | 0.04             |                       |                 |                          |
| phenomethrin           | 0.10             |                       |                 |                          |
| permethrin              | 0.02             |                       |                 | 66.3 ± 13.2              |
| datamethin             | 0.04             |                       |                 |                          |
| cyhalothrin            | 0.04             | 0.080                 | 0.053           |                          |
| phenomethrin           | 0.10             |                       |                 |                          |
| cypermethrin           | 0.20             |                       |                 |                          |
Experimental

Reagents and materials

Chemical reagents for hapten synthesis and pesticide standards used for cross-reactivity studies were supplied by China Redsun Group Nanjing No.1 Pesticide Co., Ltd. (Nanjing, China) and 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), tetramethylbenzidine (TMB), peroxidase-labeled goat anti-rabbit immunoglobulins (GAM-HRP), bovine serum albumin (BSA), ovalbumin (OVA), and complete and incomplete Freund’s adjuvants were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were analytical grade. Glass sheets precoated 0.3-mm silica gel 60 F 254 for thin-layer chromatography (TLC) and silica gel (60–230 mesh) for column chromatographic purifications were purchased from Qingdao Haiyang Chemical Co., Ltd (Qingdao, China).

Instruments

Mass spectra were obtained on a HPLCMS-LTQ XL spectrometer (Thermo-Fisher, USA). 1H-nuclear magnetic resonance (NMR) spectra were obtained with an INOVA-600 MHz spectrometer (Varian, Palo Alto, CA, USA). Chemical-shift values were given in parts per million (ppm) downfield from the 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 Beckman 640 spectrophotometer. Polystyrene 96-well microtiter plates were from Costar (Corning, MA, USA). A Wellwash 4MK-2 microplate strip washer (Thermo Electron Corporation.) was used to wash ELISA plates. Absorbance (A) was measured using a microplate reader (Wallac 1420 Victor 3, Perkin Elmer Inc.).

Hapten synthesis and verification

Five haptens were mentioned in this paper. The synthetic routes for haptens 1, 2, 4, and 5 are illustrated in Scheme 1. The structure of our previously prepared hapten XQ [35] is also shown in Scheme 1.

4-Oxo-4-(3-phenoxybenzyloxy)butanoic acid (hapten 1): Succinic anhydride (0.7 g, 6 mmol) was added to a solution of (3-phenoxyphenyl)methanol (1.2 g, 6 mmol) in dichloromethane (100 mL). After stirring at room temperature overnight, the solution was washed with water and then dried over anhydrous sodium sulfate. Finally, the solution was concentrated and gave hapten 1 (1.6 g, 94%) as a white solid. 1H-NMR (CD3Cl) δ: 2.66-2.68 (t, 2H, CH2), 2.69-2.70 (t, 2H, CH2), 6.94-7.36 (m, 9H, Ar); MS (ESI) m/z (%): 285 (M-H+, 89).

4-(Cyano(3-phenoxyphenyl)methoxy)-4-oxobutanoic acid (hapten 2): The aldehyde 3-phenoxybenzaldehyde (1.2 g, 6 mmol), in THF (9 mL) and water (1 mL), was cooled in ice. Powdered potassium cyanide (0.2 g, 6 mmol) was added into the solution of the aldehyde. With stirring, 24 N HCl (0.4 mL) was dropped slowly into the mixture. The reaction went on for 40 min, and then the
mixture was acidified with 3 N HCl and extracted with ether, and the organic phase was washed with water, dried over anhydrous magnesium sulfate, and evaporated to give the cyanohydrin, 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile, as a brown oil. Subsequently the brown oil was used to synthesize hapten 2 by the same method as used for hapten 1. Finally, 1.6 g of hapten 2 was obtained as a white solid, giving a yield of 87%. $^1$H-NMR (CDCl$_3$) $\delta$: 2.68-2.70 (t, 2H, CH$_2$), 2.72-2.75 (t, 2H, CH$_2$), 5.30 (s, 1H, Ar-CH), 7.03-7.41 (m, 9H, Ar); MS (ESI) $m/z$ (%): 310 (M-H$^+$, 62).

3-(3-Phenoxybenzoyloxy)propanoic acid (hapten 4): To a solution of (3-phenoxyphenyl)methanol (1.0 g, 5 mmol) in dry acetone (30 mL) were added anhydrous potassium carbonate (0.6 g, 5 mmol) and ethyl bromoacetate (0.8 g, 5 mmol). After refluxing for 15 h, the mixture was filtered and the solvent was evaporated under reduced pressure. The residue was dissolved in ethyl acetate, washed with cold water, 1 M NaOH and 4 M NaCl, and dried over anhydrous sodium sulfate. Removal of the solvent gave ethyl 3-(3-phenoxybenzoyloxy)propanoate as a yellow oil. The ester was dissolved in THF (2 mL) and 1 M NaOH (12 mL) was added. After refluxing for 2 h, the mixture was extracted with CH$_2$Cl$_2$. The aqueous layer was acidified to pH 3-4 by careful addition of concentrated HCl and then extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and concentrated to give hapten 4 (0.7 g, 52%) as a white solid. $^1$H-NMR (acetone-$_d_6$) $\delta$: 2.01-2.02 (t, 2H, CH$_2$), 2.16-2.17 (t, 2H, CH$_2$-C), 3.38 (s, 2H, Ar-CH$_2$), 6.99-7.39 (m, 9H, Ar); MS (ESI) $m/z$ (%): 285 (M-H$^+$, 66).

3-(3-Phenoxybenzamido)propanoic acid (hapten 5): In a solution of anhydrous DMF (3 mL), 3-phenoxybenzoic acid (1.1 g, 5 mmol), NHS (0.6 g, 5 mmol) and DCC (1.0 g, 5 mmol) were added. With stirring, the reaction went on for 3 h at room temperature. The mixture was stored at 4 °C overnight, then the supernatant containing the active ester, 2,5-dioxopyrrolidin-1-yl 3-phenoxybenzoate, was separated and slowly added dropwise to a solution of 3-aminopropanoic acid (0.5 g, 5 mmol) in PBS (12 mL). The reaction was carried on for 1 h at room temperature and then overnight at 4 °C. After removal of the solvent, the residue was dissolved in ethyl acetate, washed with cold water, 1 M NaOH and 4 M NaCl, and dried over anhydrous sodium sulfate. Final removal of the solvent gave hapten 5 (0.8 g, 57%) as a white solid. $^1$H-NMR (acetone-$_d_6$) $\delta$: 2.24-2.27 (m, 1H, CH$_2$), 2.02-2.06 (t, 2H, CH$_2$-C), 7.05-7.79 (m, 9H, Ar), 10.83 (s, 1H, NH); MS (ESI) $m/z$ (%): 284 (M-H$^+$, 32).

Preparation of immunogens and coating antigens

The conjugations of the five haptens above and the estimations of hapten densities were carried out according to Zhang [36]. To generate immunogens, haptens 1 and B were covalently attached through their carboxylic acid moieties to the lysine groups of BSA using the active ester method. Using the same method, haptens 1, 2, 4, 5, and XQ were coupled to OVA to obtain coating antigens. The immunogens and coating antigens were purified by dialysis in phosphate buffer (PB: 0.02 mol L$^{-1}$, pH 6.8). The conjugates were stored at -20 °C until use. UV-Vis spectral data were used to confirm the structures of the final conjugates. Assuming that the molar absorptivity of haptens was the same for the free and conjugated forms, the hapten densities (the number of hapten molecules per molecule of protein) of the conjugates were estimated directly by the mole absorbance $\varepsilon$: 

\[ \varepsilon = \frac{A}{c} \]

where $A$ is the absorbance at a given wavelength and $c$ is the concentration of the conjugate.
Hapten density = \( \frac{\varepsilon_{\text{conjugation}} - \varepsilon_{\text{protein}}}{\varepsilon_{\text{hapten}}} \)

**Immunization**

Four female New Zealand white rabbits of about three months of age were immunized with the conjugates of BSA-hapten 1, BSA-hapten 2, or the mixture of equal amount of BSA-hapten 1 and BSA-hapten 2. The first dose consisted of 800 \( \mu \)g of conjugate injected as an emulsion of PBS and Freund’s complete adjuvant. Three subsequent injections emulsified in Freund’s incomplete adjuvant were given at three-week intervals. One week after the last injection, the rabbits were bled, and the production of Ab was made following the protocol reported by Shan [23]. The anti-hapten antibody titers of the sera were tested by indirect ELISA, and the analyte recognition properties were examined by competitive indirect ELISA. Four sera were obtained and tested: pAb-1 against BSA-hapten 1, pAb-2 against BSA-hapten 2, and pAb-m1 and pAb-m2 against the mixture immunogen.

**Titration of antisera**

The titers of antisera were determined by measuring the binding of serial dilutions of the antisera to the corresponding coating antigen (hapten-OVA) using noncompetitive ELISA protocol. Polystyrene microtiter plates were coated with the coating antigen (1 \( \mu \)g mL\(^{-1}\), 50 \( \mu \)L per well) in 50 mmol L\(^{-1}\) carbonate-bicarbonate buffer (pH 9.6) by 2 h incubation at 37 °C. The following steps were the same as the description of Zhang [33]. The coated plates were washed five times with PBST (PBS containing 0.05% Tween 20, pH 7.4) and blocked by incubation with 1% gelatin in PBS (100 \( \mu \)L per well) for 1.5 h at 37 °C. After another washing step, 50 \( \mu \)L per well of antiserum diluted with PBS (1:1,600–1:512,000) were added to the plate, and the plates were incubated for 1 h at 37 °C. After another washing step, 50 \( \mu \)L of a GAM-HRP conjugate diluted 1:10,000 with PBST were added to each well and incubated for 1 h at 37 °C. Next, the plates were washed again, and 50 \( \mu \)L of substrate solution (3.3 \( \mu \)L of 30% H\(_2\)O\(_2\) and 400 \( \mu \)L of 0.6% TMB in DMSO per 25 mL of acetate buffer, pH 5.5) were added to each well. Color development was stopped after 15-30 min with 25 \( \mu \)L per well of 2 mol L\(^{-1}\) H\(_2\)SO\(_4\). The absorbance was measured using the single-wavelength mode at 450 nm.

**Determination of the effect of the haptens on the affinity of antisera for coating antigens**

The affinity of each of the six antisera for each of the five coating antigens (hapten-OVA conjugates) was determined by noncompetitive indirect ELISA as follows: all incubations were performed at 37 °C, including the incubation of 2 h with the coating antigens. Microtiter plates were coated with the hapten-OVA conjugates (25, 50, 100, or 200 ng per well), and 50 \( \mu \)L of antiserum was diluted with PBST (1:1,600, 1:3,200, 1:6,400, 1:8,000, 1:12,800, or 1:16,000). The other steps were described as the above.

**Determination of the effect of the haptens on the ELISA sensitivity and specificity**

The effect of hapten heterology between the immunogen and the coating antigen on the ELISA sensitivity and specificity was investigated by competitive indirect ELISA for all possible combinations of antiserum and coating antigen. The assay procedure was presented previously [34].
All incubations were performed at 37 °C except for the incubation with the coating antigens. Microtiter plates were coated with hapten-OVA conjugate (1 µg mL$^{-1}$, 50 µL per well) in 50 mM carbonate-bicarbonate buffer (pH 9.6) by overnight incubation at 4 °C. The plates were washed five times with PBST and blocked by incubation with 100 µL per well of 1% gelatin in PBS for 1.5 h. After another washing step, 25 µL per well of serial dilutions of the analyte in 40% methanol-PBS were added, followed by 25 µL per well of antiserum diluted in PBST. After incubation for 1 h, the antibody binding was assessed as described above using HRP-conjugated goat anti-mouse IgG diluted in PBST. I$ _{50}$ values (the concentration at which the binding of the antibody to the coating antigen is inhibited by 50%) were determined using logistic equations. The specificities of the ELISAs against several pyrethroid pesticides were determined and calculated as follows: CR (%) = [I$ _{50}$ (fenthion)/I$ _{50}$ (test compound)] x 100.

**Average competitive curve and water sample analysis**

In an ELISA system, the coating antigen cAg-4 was used with a concentration of 2 µg mL$^{-1}$, and the polyclonal antibody pAb-m1 was used with a dilution of 1:4000. Then a series concentrations (0.0001, 0.001, 0.01, 0.1, 1, and 10 µg mL$^{-1}$) of each standard pyrethroid phenomethrin, permethrin, datamethrin, cypermethrin, and cyhalothrin was tested, whose results contributed to the average standard curve.

To simply evaluate the above ELISA, several water samples spiked with different pyrethroid insecticides were prepared. The tap water was collected from local families in Wuhan, Hubei Province. For the spike-and-recovery test, five final concentrations (0, 0.03, 0.05, 0.2, 0.4 µg mL$^{-1}$) of pyrethroids of the above samples were prepared. These water samples were detected directly by the developed ELISA (the samples were diluted five times with PBS-methanol buffer).

**Conclusions**

Usually, a generic hapten was used for preparation of a class specific antibody against a type of analytes [37]. In this paper, with a mixture of conjugates of type I and type II pyrethroids, we prepared a polyclonal antibody against both types of pyrethroid insecticides. Therefore, use of a mixture immunogen, seems to provide a new behavior for preparation of class specific antibodies against a group of small molecular analytes. In addition, we found the best combination of pAb-m2/cAg-4 with equal high sensitivities (about 0.02 µg mL$^{-1}$) to both types of pyrethroid insecticides tested here, such as phenomethrin, permethrin, datamethrin, cypermethrin, and cyhalothrin. With the developed heterologous ELISA system, the recovery tests show that this assay can be used for screening water samples for pyrethroid multi-residues.

**Acknowledgements**

This work was supported by the Chinese National ‘863’ High-Tech. Research Program (2006AA10Z448), the Program of National Science Foundation of China (30800771), and by projects of Science and Technology of Wuhan (200820337086, 200950431224) and Special Foundation of President of the Chinese Agricultural Academy of Sciences.
References

1. Fabellar, L.T.; Heinrichs, E.A. Relative toxicity of insecticides to rice planthoppers and leafhoppers and their predators. *Crop Prot.* **1986**, *5*, 254–258.

2. Li, Y.; Luo, W.; Zhao, S. The variation of susceptibility of diamondback moth (*Plutella xylostella* (L.)) fed with different host plants to datamethrin and cypermethrin. *J. Shandong Agric. Univ.* **1996**, *27*, 269–274.

3. Chen, S.; Li, F.; He, J.; Chen, X.; Wang, D.; Wei, L.; Yang, H.; Guan, L. Experimental study on prevention of dog-sand fly contact by Datamethrin collar. *End. Dis. Bull.* **2001**, *16*, 17–19.

4. Arthur, F.H. Evaluation of a new insecticide formulation (F2) as a protectant of stored wheat, maize, and rice. *J. Stored Prod. Res.* **2004**, *40*, 317–330.

5. Shao, X.; Huang, Q.; Zhou, G. Study on the pest control with 2.5% control release micro-emulsion of datamethrin. *Chin. J. Vector Bio. Control* **2001**, *12*, 53–55.

6. Zhang, Y.; Meng, F.; Liu, Q.; Xu, F. The susceptibility of female adult house fly (*Musca domestica*) at different eclosion days to Datamethrin. *Chin. J. Vector Bio. Control* **2005**, *16*, 95–97.

7. Lee, N.; McAdam, D.P.; Skerritt, J.H. Development of immunoassays for type II synthetic pyrethroids hapten design and application to Heterologous and homologous assay. *J. Agric. Food Chem.* **1998**, *46*, 520–534.

8. Sharma, J.; Satya, S.; Kumar, V.; Tewary, D.K. Dissipation of pesticides during bread-making. *Chem. Health Saf.* **2005**, *12*, 17–22.

9. Jimenez, J.J.; Bernal, J.L.; del Nozal, M.J.; Bernal, J.; Toribio, L. Persistence and degradation of metalaxyl, lindane, fenvalerate and datamethrin during the wine making process. *Food Chem.* **2007**, *10*, 4216–4223.

10. Bouwman, H.; Sereda, B.; Meinhardt, H.M. Simultaneous presence of DDT and pyrethroid residues in human breast milk from a malaria endemic area in South Africa. *Environ. Pollution* **2006**, *144*, 902–917.

11. Lu, X.; Wang, M. Development of hapten synthesis and ELISA for the pyrethroid pesticides. *Agrochem* **2007**, *10*, 653–655.

12. Erstfeld, K.M. Environmental fate of synthetic pyrethroids during spray drift and field runoff treatments in aquatic microcosms. *Chemosphere* **1999**, *39*, 1737–1769.

13. Velisek, J.; Jurcikova, J.; Dobsikova, R.; Svobodova, Z.; Piaackova, V.; Machova, J.; Novotny, L. Effects of datamethrin on rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Pharmacol.* **2007**, *23*, 297–301.

14. Akhtar, M.H. Gas chromatographic determination of datamethrin in biological samples. *J. Chromatogr. A* **1982**, *246*, 81–87.

15. Ding, H.; Bao, X.; Zheng, Z. Determination of 7 pyrethroids residues in tea. *J. Instrumental Analysis* **2000**, *19*, 31–34.

16. Li, Y.; Huang, Z.; Dai, H.; Zhang, Y. Determination of pyrethroids pesticides multi-residues in tea by gas chromatography / mass spectrometry. *Chin. J. Anal. Chem.* **2002**, *30*, 865–868.
17. Esteve-Turrillas, F.A.; Pastor, A.; de la Guardia, M. Determination of pyrethroid insecticide residues in vegetable oils by using combined solid-phases extraction and tandem mass spectrometry detection. *Anal. Chim. Acta* **2005**, *553*, 50–57.

18. Lopez-Lopez, T.; Gil-Garcia, M.D.; Martinez-Vidal, J.L.; Martinez-Galera, M. Determination of pyrethrins in vegetables by HPLC using continuous on-line post-elution photoirradiation with fluorescence detection. *Anal. Chim. Acta* **2001**, *447*, 101–111.

19. Vazquez, P.P.; Mughari, A.R.; Martinez, G.M. Solid-phase microextraction (SPME) for the determination of pyrethroids in cucumber and watermelon using liquid chromatography combined with post-column photochemically induced fluorimetry derivatization and fluorescence detection. *Anal. Chim. Acta* **2008**, *607*, 74–82.

20. Ferrer, C.; Gomez, M.J.; Garcia-Reyes, J.F.; Ferrer, I.; Thurman, E.M.; Fernandez-Alba, A.R. Determination of pesticide residues in olives and olive oil by matrix solid-phase dispersion followed by gas chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry. *J. Chromatogr. A* **2005**, *1069*, 183–194.

21. Knopp, D. Immunoassay development for environmental analysis. *Anal. Bioanal. Chem.* **2006**, *385*, 425–427.

22. Wengatz, I.; Stoutamire, D.W.; Gee, S.J.; Hammock, B.D. Development of an enzyme-linked immunosorbent assay for the detection of the pyrethroid insecticide fenpropathrin. *J. Agric. Food Chem.* **1998**, *46*, 2211–2221.

23. Shan, G.; Stoutamire, D.W.; Wengatz, I.; Gee, S.J.; Hammock, B.D. Development of an immunoassay for the pyrethroid insecticide esfenvalerate. *J. Agric. Food Chem.* **1999**, *47*, 2145–2155.

24. Shan, G.; Leeman, W.R.; Stoutamire, D.W.; Gee, S.J.; Chang, D.P.Y.; Hammock, B.D. Enzyme-linked immunosorbent assay for the pyrethroid permethrin. *J. Agric. Food Chem.* **2000**, *48*, 4032–4040.

25. Lee, H.J.; Shan, G.; Watanabe, T.; Stoutamire, D.W.; Gee, S.J.; Hammock, B.D. Enzyme-linked immunosorbent assay for the pyrethroid datamethrin. *J. Agric. Food Chem.* **2002**, *50*, 5526–5532.

26. Lee, H.J.; Shan, G.; Ahn, K.C.; Park, E.K.; Watanabe, T.; Gee, S.J.; Hammock, B.D. Development of an enzyme-linked immunosorbent assay for the pyrethroid cypermethrin. *J. Agric. Food Chem.* **2004**, *52*, 1039–1043.

27. Ahn, K.C.; Watanabe, T.; Gee, S.J.; Hammock, B.D. Hapten and antibody production for a sensitive immunoassay determining a human urinary metabolite of the pyrethroid insecticide permethrin. *J. Agric. Food Chem.* **2004**, *52*, 4583–4594.

28. Johnson, J.C.; Van Emon, J.M.; Pullman, D.R.; Keeper, K.R. development and evaluation of antisera for detection of the O,O-diethyl phosphorothionate and phosphorothionothiolate organophosphorus pesticides by immunoassay. *J. Agric. Food Chem.* **1998**, *46*, 3116–3123.

29. Alcocer, M.J.C.; Dillon, P.P.; Manning, B.M.; Doyen, C.; Lee, H.A.; Daly, S.J.; O’Kennedy, R.; Morgan, M.R.A. Use of phosphonic acid as a generic hapten in the production of broad specificity anti-organophosphate pesticide antibody. *J. Agric. Food Chem.* **2000**, *48*, 2228–2238.

30. Jang, M.S.; Lee, S.J.; Xue, X.P.; Kwon, H.M.; Ra, C.S.; Lee, Y.T.; Chung, T. Production and characterization of monoclonal antibodies to a generic hapten for class-specific determination of organophosphorus pesticides. *Bull Korean Chem. Soc.* **2002**, *23*, 1116–1120.
31. Watanabe, T.; Shan, G.; Stoutamire, D.W.; Gee, S.J.; Hammock, B.D. Development of a class-specific immunoassay for the type I pyrethroid insecticides. *Anal. Chim. Acta* **2001**, *444*, 119–129.

32. Mak, S.K.; Shan, G.; Lee, H.J.; Watanabe, T.; Stoutamire, D.W.; Gee, S.J.; Hammock, B.D. Development of a class selective immunoassay for the type II pyrethroid insecticides. *Anal. Chim. Acta* **2005**, *534*, 109–120.

33. Luo, A.; Yu, X.; Zhang, C.; Zhu, S.; Liu, X. Development of enzyme immunoassays for pyrethriods. *Sci. Agri. Sinica* **2005**, *38*, 308–312.

34. Zhang, Q.; Wu, Y.; Wang, L.; Hu, B.; Li, P.; Liu, F. Effect of hapten structures on specific and sensitive enzyme-linked immunosorbent assays for N-methylcarbamate insecticide metolcarb. *Anal. Chim. Acta* **2008**, *625*, 87–94.

35. Kong, Y.; Li, P.; Zhang, Q.; Zhang, W.; Ding, X.; Huang, Y.; Tang, X. Synthesis and application of a novel hapten of pesticide datamethrin. *Chem. Reagents* **2009**, *31*, 245–249.

36. Zhang, Q.; Wang, L.; Ahn, K.C.; Sun, Q.; Hu, B.; Wang, J.; Liu, F. Hapten heterology for a specific and sensitive indirect enzyme-linked immunosorbent assay for organophosphorus insecticide fenthion. *Anal. Chim. Acta* **2007**, *596*, 303–311.

37. Glass, T.R.; Ohmura, N.; Morita, K.; Sasaki, K.; Saiki, H.; Takagi, Y.; Kataoka, C.; Ando, A. Improving an immunoassay response to related polychlorinated biphenyl analytes by mixing antibodies. *Anal. Chem.* **2006**, *78*, 7240–7247.

Sample Availability: Available from the authors.

© 2010 by the authors; licensee Molecular Diversity Preservation International, Basel, Switzerland. This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/3.0/).