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Preparation of Hapten and Immunogen for Producing Polyclonal Antibody Specific to Cypermethrin

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ABSTRACT

This study aimed at developing a method for the preparation of hapten and immunogen for producing polyclonal antibody specific to cypermethrin for the determination of insecticide residues in contaminated fruits and vegetables. 3-(2, 2-dichlorovinyl)-2, 2-dimethyl – (1-cyclopane) carboxylic acid (DCCA) was used as a spacer arm of hapten preparation. DCCA was linked to beta alanine methyl ester and conjugated with bovine serum albumin (BSA) as an immunogen and with oval albumin (OVA) as a coating antigen. BALB/c mice were immunized with immunogen. After three doses, sera from each mouse were collected and used to develop an ELISA. 0.05% Tween20/PBS with 20% methanol was used as diluents. The obtained polyclonal antibody (pAb) gave IC_{50} to cypermethrin at 5.2 µg/well. The lowest quantification limit of detection of the ELISA was 0.18 µg/well at 85% *B/B0* and the detection limit was 0.039 µg/well at 90% *B/B0*. The antibody will be applicable for developing ELISA for screening of cypermethrin residue in vegetable and fruit samples and enable to save cost of currently used analytical procedures. © 2012 Friends Science Publishers

Key Words: Cypermethrin; Hapten synthesis; Antibody; Immunoassay; ELISA

INTRODUCTION

Cypermethrin is one of the most popular synthetic pyrethroid insecticides in agriculture. The synthetic pyrethroids and their natural pyrethrins can be divided into two groups based on their chemical structures and mechanism of action on insect target sites. Type I compounds are simple cyclic alcohol esters of 2, 2-dimethyl-3-(2-methyl-1-propenyl)-cyclopropanecarboxylic acid, while type II compounds are esters of an arylcyanohydrin. The type II pyrethroids are more photostable than the type I pyrethroids (Lee *et al.*, 1998).

Cypermethrin also has been used for killing house fly at low dose comparing with the use in crop protection (Ahmed *et al.,* 2004). The risk of chemical residues on food and the environment to consumers depends on the residue level. Certain metabolites of cypermethrin, as well as other pyrethroids, may be of concern to consumers due to their endocrine activity (Tyler *et al.,* 2000; Chen *et al.*, 2002).

Current analytical methods for the detection of synthetic pyrethroid involve multisteps of sample cleanup procedures following using gas chromatography and detection by electron capture (GC-ECD) or mass spectrometry (GC-MS) (Ling & Huang, 1995; Ramesh & Balasubramanian, 1998; Akre & MacNeil, 2006; Khan *et al.*, 2007; Banerjee *et al.*, 2010). These methods are time-consuming, require expensive instruments and not suitable for large numbers of samples for screening. In contrast, immunoassays, as alternative techniques, would provide a sensitive, selective, and rapid method for the detection of this pyrethroid at trace levels (Shan *et al*., 1999a, b; Wing *et al*., 1978, 1979) using photometric detection for large monitoring programs (Sherry, 1992; Van Emon & Lopez-AÄ Vila, 1992; Meulenberg *et al*., 1995). This paper describes preparation method of hapten and immunogent for producing pAb specific to cypermethrin to be employed in immunoassay development.

MATERIALS AND METHODS

Chemicals and reagents: The chemicals and reagents used in this study were: methyl 3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-cyclopropane) carboxylate (Acros, Germany); 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide hydrochloride (EDC); β-Alanine methyl ester hydrochloride and oval albumin (OVA) (Fluka, Germany); magnesium sulphate, sodium hydroxide and tetrahydrofuran (THF) (Merck, Germany), methanol, ethyl acetate and hexane (J.T. Baker, Germany);silica gel 60F254 20 x 20 cm aluminum sheet (Merck, Darmstadt, Germany); Tween 20, ortho-phenylenediamine (OPD) and bovine serum albumin (BSA) (Sigma-Aldrich Chemie, Steinheim,

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Germany); cypermethrin, permethrin, cyfluthrin and deltamethrin (Laboratory of Dr. Ehrenstorfer, Augburg, Germany).

Preparation of hapten and hapten-protein conjugates: The structure of synthetic pyrethroid insecticides had two parts: an acid part and a benzene ring part. The target hapten was synthesized from chemical that had structure similar with acid part of cypermethrin (Fig. 1). A mixture of 200 mg methyl 3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-cyclopropane) carboxylate and 62.0 mg NaOH in 5 mL THF: MeOH: water (3:2:1, v/v/v) was stirred for 4 hours at room temperature to prepare the acid. The crude compound was purified by thin layer chromatography on silica gel using ethyl acetate and hexane (40:60, v/v) solvents. Then, a mixture of 50 mg β-alanine methyl ester and 65 mg EDC in 3 mL THF was stirred for 10 min. The 59 mg DCCA was slowly added to mixture solution on ice bath (0°C). The mixture solution was stirred for 10 h in heating box (50°C) and evaporated to dryness. The crude mixture after evaporation was re-dissolved in dichloromethane and then washed twice with 10 mL of cool HCl and twice with 10 mL water. The product was evaporated until dry over magnesium sulphate. The reaction of hapten synthesis is shown in scheme 1. The target hapten was confirmed by thin layer chromatography (TLC) and then purified by column chromatography on silica gel using ethyl acetate and hexane (10:1, v/v) solvents. Single compound fraction was collected and evaporated to dryness. The structure and molecular mass of the obtained compound was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectrometry and GC-MS, respectively. Hapten obtained was a white-yellow powder compound with R_f value of 0.26 (ethyl acetate:hexane, 10:1, v/v). The target haptens would be covalently attached to BSA as an immunogen and OVA as a coating antigen using the modified active ester method (Langone & Van Vunakis, 1982). Protein of immunogen and coating antigen were determined by Bradford protein assay using Bio-Rad dye (Bradford, 1976).

Polyclonal antibody production: BALB/c mice (8-10 weeks old) were immunized with immunogen (target hapten–BSA). The first dose consisted of 30 µg protein of immunogen emulsified with complete Freund's adjuvant per mouse injected subcutaneously. Mice were given the subsequent injections with immunogen emulsified in incomplete Freund's adjuvant at 3 week intervals. Blood was collected from the tail of mouse at 7 day interval and sera were stored at -20°C.

Determination of sensitivity and specificity of polyclonal antibody: The pAb was detected by non-competitive indirect ELISA and tested for specificity to cypermethrin by competitive indirect ELISA (Hongsibsong *et al*., 2010). In brief, 96 well Maxisorp Immunoplates were coated with 2 µg/mL target hapten-OVA in 50 mM carbonate-bicarbonate buffer (pH 9.6) at 4°C overnight. The wells were washed 4 times with 0.05% Tween 20/PBS, pH 7.2 as washing buffer and then blocked by incubation with 200 µL/well of 1% gelatin in PBS. Fifty µL of cypermethrin and other pyrethrods having similar structures was dispensed into each well at different concentration (50 µL/well; 1:2,500 diluted antibody gave final 1:5,000) and incubated at room temperature (25°C) for 90 min. Then, the wells were washed and 100 µL of 1:5,000 HRP-conjugated goat anti-mouse I gG in washing buffer was added to each well. After incubation at 25°C for 1 h, the plates were washed and 100 µL of OPD solution were added to the wells. The reaction was allowed to continue for 30 min and was stopped by adding 50 µL of 2 N H_2SO_4. The absorbance was read at 492 nm. One mouse giving the best sensitivity and specificity to cypermethrin was selected for further study. The sensitivity of pAb was detected and inhibition concentrations at 50% (IC_{50}) were fit to a four parameter logistic equation. The relative cross-reactivity (CR) was calculated by the following equation:

$$\% \text{ CR} = (IC_{50} \text{ of cypermethrin } / IC_{50} \text{ of related compound}) \times 100$$

Effect of diluents: Methanol is an organic modifier of the buffer formulation in the extraction of poorly hydrophilic cypermethrin from samples. To determine the optimal concentration of methanol in the PBS buffer, non competitive indirect ELISA was carried out using various percentages of methanol solution (10%, 20%, 30%, 40%, & 50%) with 0.05% Tween 20 (v/v). The assays were done in duplicate wells.

The effects of pH: pH of PBS was evaluated for effecting to pAb. The pAb in serum was dissolved in PBS buffer at pH values of 5, 6, 7, 8 and 9. The pH effect was tested with all other parameters of the assay fixed.

RESULTS AND DISCUSSION

Hapten and hapten-protein conjugates: The structure of target hapten was confirmed hydrogen atom by ^{1}H–NMR and showed peaks at (400 MHz, $CDCl_3$) δ 6.353 (t, ^{1}H, Cl_2CCH^{*}-R), δ 5.572 (d, ^{1}H, Cl_2CCH^{*}-R), δ 3.677 (s, ^{2}H, R-NH-CH_2^{*}-R), δ 2.519 (t, ^{2}H, R-CH_2^{*}-COO-R), δ 1.176 (s, 6H, $(CH_3)_2$-R). The carbon atom was confirmed by ^{13}C-NMR and shown peak (400 MHz, $CDCl_3$) at 173.17, 169.61, 169.53, 127.55, 125.71, 51.78, 36.87, 35.06, 34.82, 34.41, 33.96, 33.71, 33.12, 31.82, 31.00, 28.63, 27.75, 26.39, 22.71, 20.62, 20.04, 19.84, 15.00. Structure of target hapten had two isomers (*cis* & *trans*) (Dorman & Beasley, 1991); therefore, ^{1}H and ^{13}C-NMR exhibited 2 sets of data peaks. The molecular weight of target hapten was determined by GC-MS [MSD (EI) 5973, Hewlett Packard] gave homogeneous peak, t_R =13.045 min. The mass to charge (m/z) of target hapten was 281.4, which was corresponded to the calculated mass value of 281.15 for the molecular ion $M+H^{+}$ ($C_{11}H_{15}C_{l2}NO_3$). Molecular densities of target hapten on the carrier proteins were measured by UV spectroscopy and calculated following Brinkley (1992). The assuming hapten densities of immunogen and coating

Fig. 2: Polyclonal antibody titer from selected mouse at different day after 3 injections. pAb levels were determined by non-competitive indirect ELISA. Hapten-OVA was coating antigen

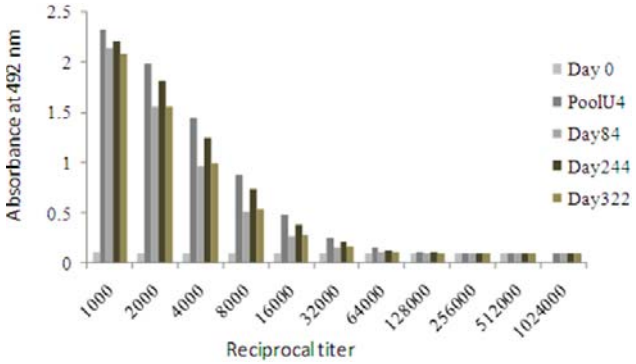


Fig. 3: (a) The effect of methanol and (b) pH of diluent to polyclonal antibody binding to coating antigen by non-competitive indirect ELISA

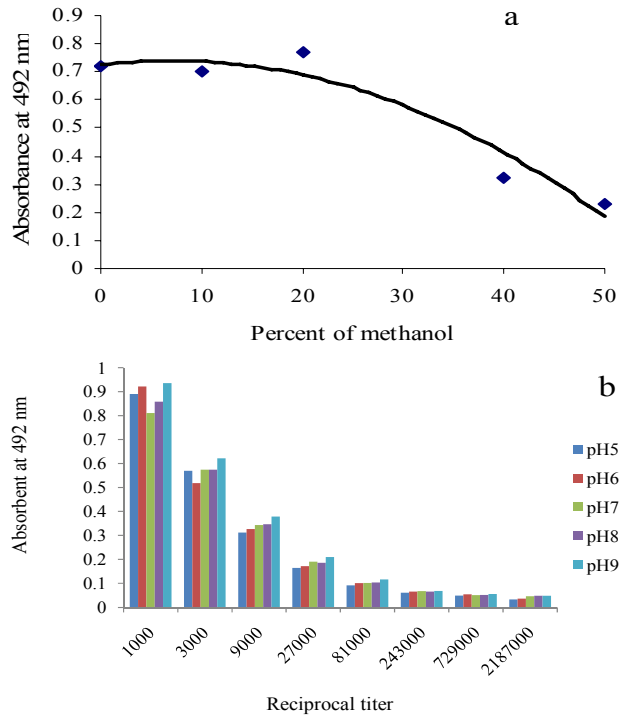
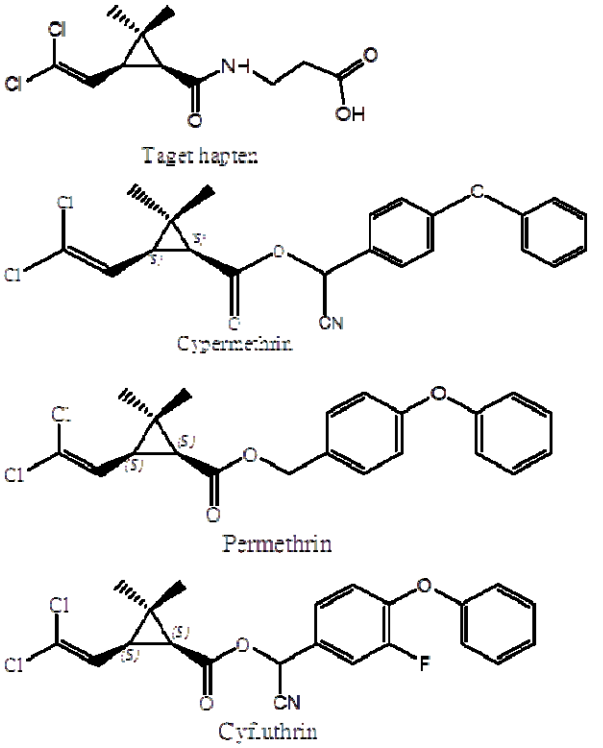
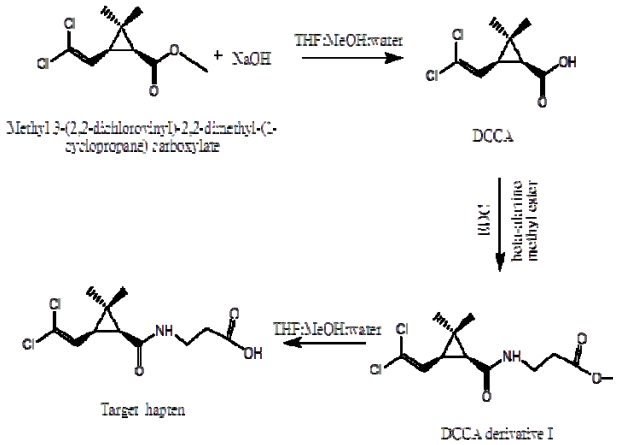


Fig. 1: Structure of target hapten and synthetic pyrethroid insecticides that target hapten have similar structure



Scheme 1: Reaction of target hapten synthesis

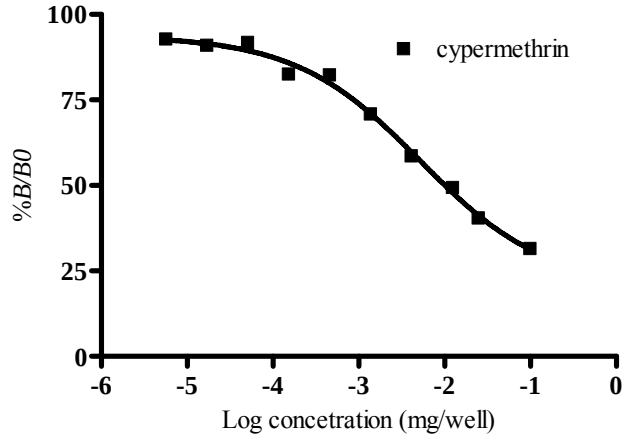


antigen were 8 and 11, respectively; which were in the range that can stimulate immune system to produce pAb with good titer (Wittmann & Hock, 1991).

The titer of polyclonal antibody after 3rd immunization: Sera from selected mouse collected at different time points post-immunization, which gave the similar pAb titers (reciprocal titer >128,000) were then pooled (Fig. 2). The obtained pool serum around 1.5 mL was aliquoted and stored at -20°C. From titer of pAb, pool serum could be used to develop immunoassays.

Optimization of ELISA: Pooled serum was used for optimizing the ELISA. The pAb was mixed with methanol (final dilution 20%) for analyzing the lipophilic synthetic pyrethroid cypermethrin. Few studies have reported methanol as a good solvent that could be used in ELISA system for lipophilic chemicals (Lee *et al.*, 2003; Park *et al.*, 2004). Effect of different concentrations of methanol (as a co-solvent for determination of cypermethrin) on ELISA system was investigated using non-competitive indirect ELISA. There was no effect of 20% methanol in PBS with 0.05% Tween 20 on pAb binding to coating antigen. Higher concentrations of methanol, however, decreased the

Fig. 4: Dose response curve of cypermethrin using obtained polyclonal antibody for specificity by competitive indirect ELISA



absorbance more than 50% compared with that without methanol (Fig. 3a). Though, high concentration of methanol helps in the solubility of hydrophobic cypermethrin, it could also affect the interaction of pAb and antigen, specially the low affinity of antibody and antigen. In the present study, 20% methanol was selected for further experiments. pH is another factor that can affect pAb – coating antigen. In the present study, pH 5 to 9 had no significant effect on pAb-coating antigen interaction (Fig. 3b). These results supported the previous findings that ELISA may be carried out on pH values ranging from 5.0 to 8.0 without affecting the sensitivity and specificity of the antibody (Shan *et al.*, 1999 a, b; Shan *et al.*, 2000; Park *et al.*, 2004).

Sensitivity and specificity: Sensitivity and specificity of pAb were assessed by competitive indirect ELISA using cypermethrin standard and other structures related to cypermethrin, including permethrin, cyfluthrin, deltamethrin, and DCCA as inhibitors. At optimized ELISA conditions, IC_{50} of cypermethrin (5.2 µg/well) was calculated by fitting dose response curve to a four parameter logistic regression equation. The lowest quantification limit of detection of the ELISA was 0.18 µg/well at 85% B/B0, and the detection limit was 0.039 µg/well at 90% B/B0 (Fig. 4). There was no cross reactivity at concentration more than 0.02 mg/well. In contrast to the present results, Pullen and Hock (1995) could not demonstrate cross-reactivity of monoclonal antibodies with cypermethrin probably due to shorter spacer arm. Therefore, length of spacer arm of hapten may have effect on the sensitivity and specificity in the process of antibody production.

CONCLUSION

The present study has demonstrated the process of preparing hapten and immunogen to produce pAb specific to cypermethrin in mouse. The obtained pAb is useful for developing immunoassays and may be further improved in sensitivity and specificity using phage display technique (Kim *et al.*, 2008, 2009). In addition, it can be employed to develop an immunoassay test kit for screening of cypermethrin residue in agricultural produces, which enable to save cost incurred on expensive and time consuming analytical procedures.

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