CHARACTERIZATION OF NAPHTHYL ESTERASE AND ACETYLCHOLINESTERASE ENZYME SYSTEMS IN *LYGUS HESPERUS*

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Abstract

We have begun to evaluate the roles of naphthyl esterase and insensitive acetylcholinesterase enzymes in conferring resistance to insecticides in California populations of *Lygus hesperus*. Initial work has focused on an alfalfa population in Moreno Valley which has served as a useful source of insects for developing the assays we hope to use in more extensive studies during the Summer of 2001. Naphthyl esterases were characterized using well established methods of polyacrylamide gel electrophoresis. Our preliminary data has shown that the resolution of *Lygus* naphthyl esterases using a barbitone buffer system was poor; this is in contrast to the excellent resolutions obtained with the same protocol for other insects such as whiteflies and aphids. We identified one form of acetylcholinesterase (AChE) in this population and this has now been well characterized using spectrophotometric techniques. Km and Vmax for this AChE have been determined and we have preliminary data showing it to be highly sensitive to inhibition by the organophosphorus (OP) insecticide, chlorpyrifos-oxon. For toxicological assessments, a filter-paper bioassay method was developed and tested. Its use enabled direct comparisons between bioassay LD_{50} data and manufacturers' recommended field application rates.

Introduction

In San Joaquin Valley cotton, *Lygus hesperus* has been controlled with organochlorine, organophosphate and carbamate insecticides for more than 30 years (Grafton-Cardwell *et al.*, 2000). However, resistance to these chemicals has now been reported in many populations, with the result that there has been increased dependence on the pyrethroid bifenthrin (Capture). Resistance to the pyrethroid was first reported in 1996 (Grafton-Cardwell *et al.*, 1997) and the incidence has been steadily increasing ever since. The problem is compounded by the use of pyrethroids for the control of other key cotton pests such as aphids. With so many conventional insecticides threatened or already rendered ineffective by resistance, newer products with novel modes of action are assuming major importance as components of pest control programs. For *Lygus*, however, growers must still largely depend on the older compounds, and it is, therefore, imperative that there are effective means of assessing their toxicological efficacy, in order to identify those compounds which have lost their efficacy due to resistance so that they can be removed from management schemes.

We advocate using a combined toxicological and biochemical approach to understanding and managing resistance problems. The close integration of the two disciplines provides a greater understanding of the levels of protection afforded to insects by specific mechanisms (Byrne et al., 1994, 2000; Byrne and Toscano, 2001). With careful assessment, this approach can provide valuable information on the complexity of resistance, leading ultimately to improved pesticide recommendations for management programs.

One widely used method for determining the contact toxicity of chemical insecticides against *Lygus* is the plastic ziploc bag bioassay (Brindley *et al.*, 1982). The inside of the bag is treated with technical grade insecticide dissolved in acetone and, after the acetone has dried, adults are placed inside. Mortality is normally assessed after 8 hours. A similar method has been developed which uses treated glass vials (Knabke and Staetz, 1991). Coating a known surface area with a specific concentration of insecticide is a feature which makes these techniques very attractive as far as relating laboratory bioassay data with field efficacy is concerned. One can easily extrapolate from treated surface area to field scale. However, one of the major drawbacks of these bioassay systems is their poor compatibility with formulated materials due to problems with adequate drying. We are developing a bioassay system for *Lygus* which overcomes some of the problems associated with the bag and vial bioassay procedures. Filter paper disks in petri dishes are impregnated with formulated insecticide, and once the filter papers have dried, the *Lygus* are confined on the filter paper surface for toxicological assessments. Impregnated filter paper bioassays have been used for many years for detecting resistance in stored products pests (Champ, 1968; Lorini and Galley, 1998), and they are an ideal system for testing *Lygus* at all growth stages using both formulated and technical grade materials.

Biochemical methods have been developed for studying the roles of naphthyl esterases and acetylcholinesterase insensitivity in conferring resistance to insecticides in a wide range of insects. For naphthyl esterases, polyacrylamide gel electrophoresis is used to compare enzyme profiles of susceptible and resistant populations. Both qualitative and quantitative changes in enzymes have been observed in resistant strains (Byrne et al., 2000; Oppenoorth and van Asperen, 1960; Devonshire and Moores, 1982; Mouches *et al.*, 1987). Changes in the sensitivity of AChEs to inhibition by OPs and carbamates have been confirmed as the major cause of resistance to these insecticides in many pests (Byrne and Devonshire, 1993; Byrne and Toscano, 2001). Improved microplate reader technology has enabled the detection of modified AChE forms in fractions of individual insects (Byrne and Devonshire, 1993), making it possible to determine resistance gene frequencies in field populations.

In this paper, we report on our preliminary experiments in which we evaluated conventional assay methods for studying naphthyl esterases and AChEs in adult *Lygus*.

Materials and Methods

Insects

Insects were collected from an alfalfa crop in Moreno Valley, California, and transported live to the laboratory where they were immediately used for toxicological assessments. Surplus insects were stored at –20 ^{o}C until they were required for biochemical studies.

Insecticides

Technical grade chlorpyrifos-oxon was a gift from Dow AgroSciences (Indianapolis, IN). Formulated insecticides were kindly provided by their manufacturers – Capture 2EC and Thiodan 3EC (FMC Corp., Philadelphia, PA), and Lorsban 50W (Dow AgroSciences).

Toxicological Bioassays

Bioassays were performed in inverted plastic petri dishes (60 x 15 mm). Whatman filter papers (No. 1, 55mm) were placed in the lids and then impregnated with 500μl of formulated pesticide (administered by pipette). A range of doses was tested for each chemical with four replicates at each dose. Papers were allowed to dry in the petri dishes by leaving them to stand for 2 hours in a fume hood. When they were completely dry, five adult *Lygus* were placed in each dish, the base of the dish being used as the lid. To prevent the insects from settling on the upper part of the dish, the sides of the chamber were coated with Teflon. The dishes were kept in an incubator at 25 ^{o}C for 8 hours, after which mortality was assessed. Insects incapable of normal locomotory activity (the ability to walk at least one body length before falling over) were scored as dead. For controls, similar dishes were set up in which filter papers were treated with water. Probit

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analyses of concentration-dependent mortality were done using POLO-PC (LeOra Software, 1987).

Polyacrylamide Gel Electrophoresis of Naphthyl Esterases

Homogenates of individual *Lygus* heads were prepared in 0.1 % Triton X-100 containing 10 % sucrose. Fractions of these were loaded onto 7.5% polyacrylamide gels (70 x 75 x 2.5 mm) and then electrophoresed at 150 V using a modification of the discontinuous buffer system of Williams and Reisfeld (1964), in which the stacking gel contained the same buffer as the running gel, but only 2.5 % polyacrylamide. Naphthyl esterases were visualized with a solution of 0.5 mM 1-naphthyl butyrate in 0.2 % Fast Blue RR salt at pH 6.0 (0.2 M phosphate buffer). Staining was terminated by replacing the staining solution with 7 % acetic acid.

Acetylcholinesterase Assays

AChE activity was measured in microplate assays using acetylthiocholine iodide as substrate (Ellman *et al.*, 1961). Homogenates of 10 adult heads were prepared in 0.1 M phosphate buffer, pH 7.5, containing 0.1 % Triton X-100, and then centrifuged at 10,000 g for 15 min. To derive Km and Vmax, the AChE activity in 0.5 head equivalents was measured for 1 min at 25 ^{O}C over a range of substrate concentrations in a SpectraMax 250 microplate reader (Molecular Devices Corp., Sunnyvale, CA). All kinetic analyses were performed using EnzFitter software (Biosoft, Cambridge, U.K.).

For inhibition studies, mass homogenates were prepared as described above, and from these 0.02 head equivalents were co-incubated with substrate and OP (Byrne and Devonshire, 1993). Activity was monitored for 30 min at 25 ^{O}C in the SpectraMax microplate reader.

Results

Filter Paper Bioassay

The filter paper bioassay was used to compare the efficacies of three insecticides (Table 1) against a *Lygus* population collected from an untreated alfalfa crop. The dose range tested for Thiodan (endosulfan) (data not shown in Table 1) was too high to derive an LD_{50} – the lowest dose of 250 ppm per paper disk gave 90 % kill. Full log dose-probit data were obtained for Capture (bifenthrin) and Lorsban (chlorpyrifos) (Table 1). Capture was slightly more effective than the OP.

Naphthyl Esterases

Naphthyl esterase activity could readily be detected in fractions as low as 0.05 of a single *Lygus* head. However, the resolution of naphthyl esterases was not good using the barbitone system of Williams and Reisfeld (1964). This system provides excellent separation of whitefly naphthyl esterases (Byrne and Devonshire, 1993), even when aliquots of *Lygus* and whitefly samples are mixed together and co-electrophoresed in the same well.

Acetylcholinesterase

One form of AChE was found in the *Lygus* population. Km and Vmax are summarized in Table 2. The AChE was highly sensitive to inhibition by the OP chlorpyrifos-oxon, with an I_{50} of approximately 0.01 μM.

Discussion

The filter paper bioassay worked well for both Capture and Lorsban. There was little difficulty in scoring the mortality of the test insects at the 8 h assessment period and we found the results to be consistent between tests. One essential feature of the bioassay is to treat the sides of the test chamber with Teflon to ensure that the insects remain on the treated papers for the duration of the assay. Apart from its ease of use, the assay has two clear advantages. Its adaptability for use with formulated products enables a direct comparison to be made between LD_{50} data and field rates. The LD_{50} of Capture was 166 ppm which corresponds to 83 μg a.i. per paper disk. This can easily be converted to a 'per acre' value (141g per acre) knowing that the disk area is 5.87 x 10^{-7} acres. 166 ppm is well above recommended field rates for Capture against Lygus on cotton (19.3 g – 47.5 g a.i. per acre). There are obvious operational differences that will hinder direct comparisons between laboratory and field data; however, LD_{50}s greater than the field rate might indicate the presence of resistance and should be investigated further. The validity of the test method will be established by widening the monitoring area to include areas where there are reports of poor control of *Lygus* populations. The simplicity of the method also makes it an ideal method of testing insects in the field. We recommend using freshly prepared filter papers and storing them while in transit to the field site in a cool environment.

Resolution of naphthyl esterases was poor when the electrophoresis protocol used for whitefly naphthyl esterases was tested for *Lygus* enzymes. While it was clear that there was considerable activity present, the activity did not resolve into sharp bands. We are currently investigating alternative buffer systems.

AChE activity was readily detected in small fractions of individual *Lygus* heads using established techniques. The enzyme identified during this study was particularly sensitive to chlorpyrifos-oxon, the active form of Lorsban. We will be monitoring populations throughout California in order to determine if there is a link between changes in toxicological response and changes in the levels of sensitivity of the enzyme to inhibition (Byrne and Toscano, 2001; Byrne et al., 1994).

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Table 1. Toxicity of Capture (bifenthrin) and Lorsban (chlorpyrifos) to *Lygus* adults in the filter paper bioassay.

Insecticide	**LD_{50} (μg/ml)**	**95 % Conf. Limits**	**Slope**	**S.E.**
Capture	166	130 - 208	5.2	1.3
Lorsban	297	242 - 359	3.1	0.8

Table 2. Km (μM) and Vmax (mOD/min/head) of AChE in adult *Lygus* for the natural substrate analog ATChI. The I_{50} data is for the inhibition of the enzyme by chlorpyrifos-oxon measured in the presence of the same substrate.

Km	141	(± 19)
Vmax	67	(± 3)
*I_{50} (μM)	0.01	-

Values are the means (± SD) of three independent determinations
*This is an approximate value only determined from one experiment