Cotton
RESEARCH & DEVELOPMENT

Annual, Progress and Final Reports

REPORTS

Part 1 - Summary Details

Please use your TAB key to complete Parts 1 & 2.

CRDC Project Number: **DAN 161C (now DAN 172C)**

Annual Report: ☒ Due 30-September

Progress Report: ☐ Due 31-January

Final Report: ☐ Due 30-September
(or within 3 months of completion of project)

Project Title: Biochemical Mechanisms of resistance to *Bacillus thuringiensis* endotoxins in *Helicoverpa armigera*

Project Commencement Date: 1/7/02 **Project Completion Date:** 30/6/03

Research Program: Insect Management

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Part 3.2 – Annual Reports (due end of September)

(Maximum four pages)

1. What were your major project objectives, milestones and performance indicators for the past year? (Please list these and any project results).

AIMS OF THE PROJECT

- To investigate the mechanisms of the binding of *cry*1Ac to *H. armigera* esterase and identify Bt toxin structures which are most susceptible.
- Investigate protease qualities of *H. armigera* esterase with respect to metabolism of Bt toxins.
- Screen other Bt crystal proteins for the potential for esterase mediated resistance in *Helicoverpa* spp.

MILESTONES YEAR 1

- Identify, isolate and purify esterases that bind to CRY 1ac pro-toxin in *H. armigera*
- Investigate by biochemical and surface plasmon resonance techniques the kinetics of activated and non-activated CRY 1Ac binding to esterase in resistant *H. armigera*.
- Investigate the ability of *H. armigera* esterases to bind to other toxins.

RESULTS

(a) Bioassay

H. armigera populations on cotton have been exposed to BT toxin from sprays and transgenic cotton for a number of years and a CRY 1Ac tolerant strain the "Silver strain" was bred by Dr HT Dang, from the survivors of the CRY 1Ac resistance monitoring programme. There has been some controversy over the CRY 1Ac resistance status of the "silver strain". This controversy has been exacerbated by BT bioassay methods with a poor delivery of toxin to larvae and hence a poor dose response relationship. As this research project about the apparent ability of the "silver strain" to metabolise C RY 1Ac, is dependent on the assumption of resistance, we have done some research to improve BT bioassay methods and toxin delivery to the insect.

We used feeding bioassays on third instar larvae, where CRY 1Ac was incorporated into an artificial diet. Formulated CRY 1Ac (MVP®) was serially diluted with distilled water containing 0.1% Triton X-100 and pipetted onto the diet surface. Larvae were confined on the *Bt* treated diet at 25 ^{o}C for 4 days before being transferred to fresh, non- BT diet. Mortality was assessed 14 days after CRY 1Ac dosage. Dosage mortality data were analysed by probit analysis. We bioassayed the "Silver strain", a lab susceptible strain GR (from CSIRO). The silver strain was then selected once with MVP at the LC_{50} concentration and the F_1 progeny were bioassayed, as described above.

Table 1 Dosage mortality data showing response of CRY 1Ac resistant and susceptible *H. armigera* strains to formulated CRY 1Ac.

Strain	Slope	X^2	LC_{50} (95% fiducial limits) mg CRY 1Ac / 0.5 g diet.	Resistance Factor*	$LC_{99.9}$ (95% fiducial limits) mg CRY 1Ac / 0.5 g diet.	Resistance Factor*
Susceptible GR	3.1	2.0	0.000125 (0.00010 – 0.00015)	1	0.0012 (0.00069 – 0.00225	1
"Silver strain"	1.9	5.9	0.0018 (0.001-0.003	14	0.030 (0.0029 – 0.32)	25
Silver sel.	2.5	0.73	0.019 (0.010 – 0.0.24)	150	0.33 (0.15 – 0.69)	275

. Resistance factor is calculated from the ratio of resistant LC_{50} or $LC_{99.9}$ / susceptible LC_{50} or $LC_{99.9}$

Results are shown in Table 1. There was an excellent relationship between CRY 1Ac dose and *H. armigera* mortality in the susceptible strain (shown by slope value), indicating that our modifications to the bioassay methods were successful. Compared to the susceptible strain, the "silver strain" was, 4 and 25 fold resistant at the LC_{50} and LC_{99} levels respectively. A lower slope value of 1.9, compared to 3.1 in the susceptible strain very indicative of resistance in the silver strain. Further proof of CRY 1Ac resistance in the "Silver strain" was provided by the "Silver" selected" strain. Our results showed that one moderate selection with MVP considerably increased both resistance and slope value of the dose response curve (resistance factors of 150 and 275 fold respectively at the LC_{50} and $LC_{99.9}$ levels respectively and a slope value of 2.5). Conclusions, are therefore, that the "Silver strain", which originated from field survivors of the BT resistance monitoring programme, is indeed resistant to CRY 1Ac.

b) Biochemical resistance mechanism studies on the "Silver strain"

Dr HT Dang observed that that in MVP treated survivors of discriminating dose bioassays, little CRY 1Ac could be recovered, compared to that recovered from dead, susceptible larvae, which suggested that *H. armigera* were metabolising CRY 1Ac. As esterase isoenzymes are already known mediate resistance in *H. armigera*, by sequestering a range of insecticide, we studied these enzymes in the "silver strain"

Our studies showed that "silver strain" *H. armigera* have greatly increased esterase isoenzyme activity compared to the BT lab susceptible strain (GR) and normal field *H. armigera* (Figure 1). The additional enzyme activity is inherited from generation and is not induced by CRY 1Ac exposure. Increased esterase activity was not shown in CRY 1Ac resistant lab strains (BX and VIC RATS) obtained from CSIRO (Figure 1).

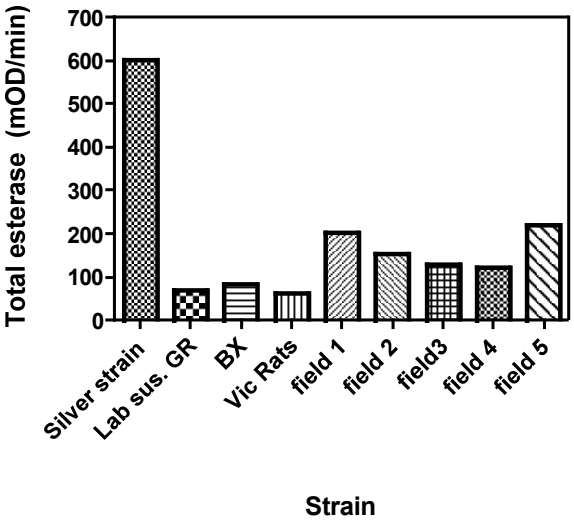


Figure 1 Total esterase activity (3-4 mg larvae) in "Silver strain", CRY 1Ac lab susceptible (GR,)BX, VIC RATS and *H. armigera*. field strains.

(i) *In vitro* esterase binding to CRY 1Ac

We have shown that esterase in the "silver strain binds to both CRY 1Ac pro-toxin and activated toxin (Figs 2 and 3) and that the binding ability is semi-domminant. No binding to CRY 1Ac occurs in esterase from susceptible, BX or VIC RATS strains. These finding of esterase binding to CRY 1Ac are very significant because sequestration is recognised by BT research workers as a potential resistance mechanism.

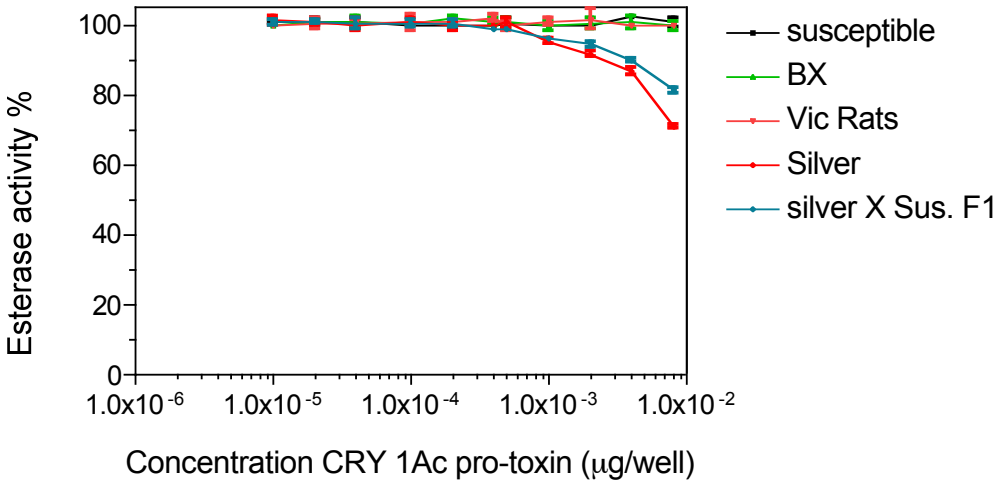


Figure 2 *In vitro* esterase inhibition by CRY 1Ac pro-toxin in strains of *H. armigera*

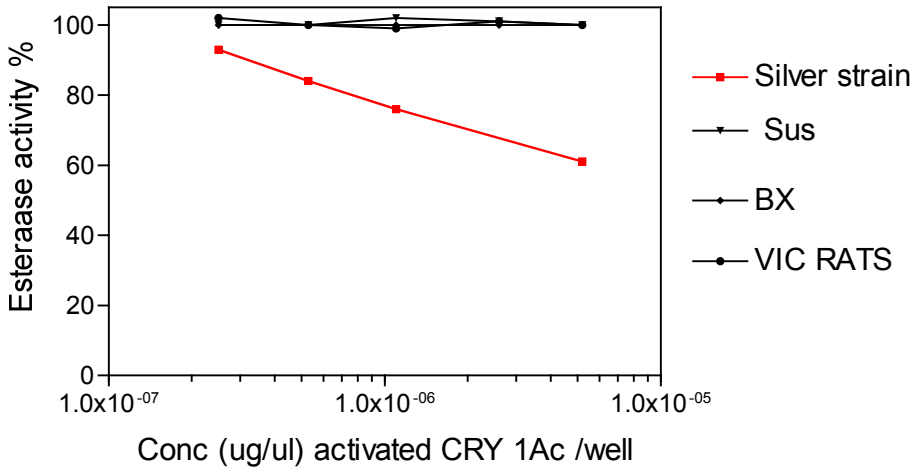


Figure 3 *In vitro* esterase inhibition by activated CRY 1Ac toxin in strains of *H. armigera*

Esterase isoenzymes from the resistant "silver strain" were purified by anion exchange chromotography and we identified three esterase bands which bind to CRY 1Ac toxin. These esterase bands are greatly over-produced. Up to 60% esterase inhibition by CRY 1Ac was achieved with purified esterase. Our conclusion are, that given the greatly increased esterase in the "silver strain", silver strain esterase has the ability to bind-to and probably detoxify considerable quantities of CRY 1Ac.

To further investigate the possibility of such a metabolic mechanism conferring resistance to *Bt* toxins in the "silver strain", we utilised novel Surface Plasmon Resonance (SPR) techniques using a BIACORE (at the Child Health Research Institute, Adelaide and at the University of Reading, UK.), to study *H. armigera* esterase / CRY1Ac interactions. Approximately 1800 response units (RU) of activated CRY1Ac toxin in 10mM sodium acetate (pH 4.0) was bound to a CM5 carboxymethyl surface using EDC-NHS (1-ethyl-3-{3-dimethylaminopropyl}-carbodiimide and N-hydroxy-succinimide) chemistry. Purified *H. armigera* esterases from the CRY 1Ac "selected silver strain" were then passed across this surface at concentrations ranging 1.74µM – 27.8µM in 10 mM disodium tetraborate, 1 M NaCl, pH 8.5 (to approximate mid-gut conditions).

Biacore data was captured in real-time and the 'inline' blank surface response was subtracted automatically from the binding curves. These were then plotted and association and dissociation rates were fitted globally across the concentration range using the Langmuir model (see example Figure 2). The resulting kinetic data confirm binding between analyte and ligand. The resultant K_D values are in the µM range (K_D (av) = 1.2µM) demonstrating that the esterase has a strong affinity for the toxin. Although no appreciable hydrolysis of the protein by resistant esterase may take place, the very large molar amounts of esterase in some resistant *H. armigera* populations would be sufficient to sequester quantities of the toxin, thus rendering it harmless before reaching the target-site.

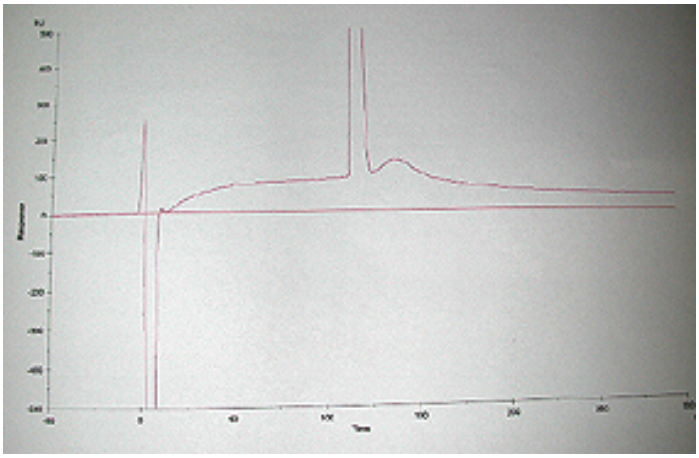
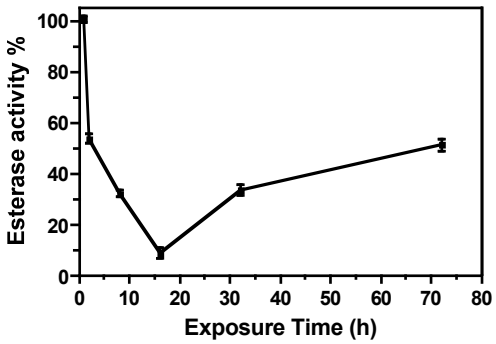


Figure 2: Plot of association and dissociation curve of purified "silver strain" esterase to activated CRY1Ac toxin.

(iii) In vivo esterase inhibition studies in CRY 1Ac

We studied the *in vivo* inhibition of esterase by CRY 1Ac in first instar "Silver strain" larvae, by feeding MVP in diet (at LC_{50} level) and sampling larvae at regular time intervals for esterase analysis. As Figure 3 shows, esterase inhibition (compared to non-MVP controls), was detected after 2 hours of feeding and continued to decline for 16 hours. Esterase activity remained significantly inhibited whilst the larvae had access to MVP treated food.

Figure 3 *In vivo* esterase inhibition in first instar ""Silver strain larvae fed with MVP diet.



Similar experiments were performed on first instar larvae with Ingard cotton (Figure 4). Esterase inhibition (compared to conventional cotton controls), was detected after 4-5 hours of feeding on Ingard cotton and continued to decline for the duration of the experiment and the esterase activity remained inhibited whilst the larvae had access to Ingard cotton. Our conclusions are, that esterase in "silver strain" *H. armigera* binds to CRY 1Ac in live, first instar larvae and this resistance mechanism could be selected for, on Ingard cotton, in the field.

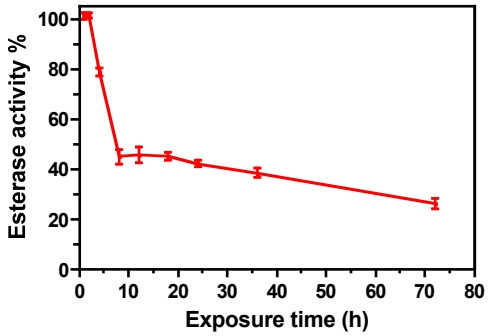


Figure 4 *In vivo* esterase inhibition in first instar Silver strain larvae on Ingard cotton

(iii) In vitro experiments with CRY 2Ab toxin

Our work showed that "silver strain" *H. armigera* esterase does not bind to CRY 2Ab toxin in either the pro-toxin or activated form. These data are consistent with Dr Dang's bioassays, which showed that the "silver strain" was susceptible to CRY 2Ab.

(iv) Biochemical assays

We are developing a biochemical assay, based on the ability of esterase to bind to CRY 1Ac as a rapid assay for this resistance mechanism. Using a diagnostic concentration of CRY 1Ac pro-toxin, we screened field populations of *H. armigera* from cotton in 2002/3 and are analysing the data to find a level of esterase inhibition that correlates to bioassay discriminating dose survival. Bioassays for BT toxins are very time consuming and technically difficult and there would be great benefits to cotton growers from rapid diagnosis of resistance.

2. Which of these have been achieved?

All objectives for the first year of project have achieved.

3. Which were not achieved and why? (Please provide detail of any problems you have had during the year and how you plan to address these problems).

N/A

4. Are there any aspects of your research project do you envisage having problems with in the coming year and what is your contingency plan?

There may be some problems in gaining access to CRY toxins in Australia and to overcome this, we have entered into a collaboration with an acknowledged BT research worker who has access to many CRY toxins (Prof Dennis Wright, Imperial College, UK).

5. What are your specific project objectives, milestones and performance indicators for the coming financial year? Have any of these changed?

Year 2:

- To determine whether "silver strain" *H. armigera* esterase to binds to other Bt toxins
- Study mechanisms of esterase/Bt toxin binding. Metabolism or sequestration or protease action. Initial studies will concentrate on *cry* 1Ac.
- Determine whether esterase inhibitors will prevent *cry*1Ac binding to esterase in *H. armigera*.

6. Are changes to the Intellectual Property Register required? (You may also submit a separate confidential report of information, which should be included in the report but which you reasonably consider is confidential information).

No

7. How do you plan to demonstrate that your research is addressing the Corporation's three outputs - Economic, Environmental and Social?

The CRDC's three outputs depend to a large extent on the success of transgenic cotton in controlling *H. armigera*, from the points of view of the cost of cotton production and the need to reduce insecticide use for environmental and social reasons. This research is contributing to the better management of, and the continued success of BT cotton in Australia.

8. To what extent have your research results to date been disseminated to other researchers, growers or the industry? Please provide details and list any publications.

Data concerning, BT resistance in *H. armigera* is rather controversial and at this stage, our findings have only been made available to other researchers and to the CRDC.

9. How do you intend to communicate the results or findings of your research.

Findings will be communicated to researchers and industry via the TIMS Committee, the CCA Annual General Meeting, the *Cotton Grower,* the British Crop Protection Council Annual Conference, International Congress of Entomology, the AGCRA Cotton Conference and via publication in scientific journals.

10. Were there major highlights in your work over the last twelve months? Please give a brief outline.

Developing a bioassay method to reliably detect CRY 1Ac resistance in *H. armigera* and the elegant demonstration of real-time CRY 1Ac binding to "silver strain" esterase, using Biacore technology, were the highlights of this years work.