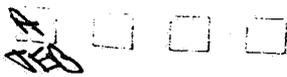


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DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist *MTW*

Secondary Reviewer: John L. Kough, Ph.D., Biologist *JLK*

STUDY TYPE:	Protein Binding
MRID NO:	447343-01
TEST MATERIAL:	CRY9C Protein
PROJECT NO:	TOX 98012
SPONSOR:	AgrEvo USA Company, Wilmington, DE
TESTING FACILITY:	AgrEvo UK Limited, Saffron Walden Essex, England & Plant Genetic Systems N.V., Gent, Belgium
TITLE OF REPORT:	BT CRY9C Protein: Investigative Study of the Potential for Binding to Mouse Intestinal Brush Membrane Vesicles
AUTHOR(S):	Konrad Purdy
STUDY COMPLETED:	November 5, 1998
CONCLUSION:	This study indicates that AgrEvo believes that the addition of unlabeled Cry9C resulted in displacement of the labeled Cry9C in the insect BBMVs, but did not result in displacement of the labeled Cry9C in the mouse BBMVs. Such a result would indicate that Cry9C binds specifically and saturably to <i>Ostrinia nubilalis</i> BBMVs and not to mouse intestinal BBMVs. However, the figures of the chemiluminescence results contained in the submission are of poor quality. Therefore, it is not possible to make an accurate assessment of the results without submission of the original, or much higher quality reproductions of the x-ray films. In addition, further discussion should be provided to support the conclusion drawn by AgrEvo regarding the displacement of labeled Cry9C proteins by unlabeled proteins. It is unclear from the data and discussion provided how such a conclusion has been reached.
CLASSIFICATION:	SUPPLEMENTAL. Can be upgraded to ACCEPTABLE with submission of adequate figures and supporting justification which correlate with the above-mentioned interpretation.
GOOD LABORATORY PRACTICE:	This study was performed in accordance with Good Laboratory Practice guidelines

185

I. STUDY DESIGN

Test Material: CRY9C protein derived from *Bacillus thuringiensis* subsp. *tolworthi* (batch # Cry9Ca-351-0198) and Cry1A(b) protein derived from *B. thuringiensis* subsp. *kurstaki*. Both proteins were produced in genetically altered *E. coli*.

Test Animals: Stock CD-1 mice were obtained from Charles River UK Ltd., Margate, Kent, UK. The animals were placed into cages in groups of five, after examination for any signs of ill health.

Methods:

Preparation of Biotinylated Cry9C:

Cry9C was incubated with Biotinyl-N-hydroxysuccinamide ester (BNHS). Following incubation, the biotinylated protein was separated from free BNHS using a Sephadex G-25 column.

Preparation of Insect Mid-Gut BBMVs:

Brush Border Membrane Vesicles (BBMVs) were prepared from fifth instar *Ostrinia nubilalis* larvae midguts by homogenization and ultracentrifugation for isolation of the vesicle fraction.

Preparation of Mouse Intestinal BBMVs:

Mouse intestinal BBMVs were prepared by eversion of intestines from CD-1 mice after sacrifice. The interior surface of the intestines was washed and scraped using a glass microscope slide. The scraped preparation was suspended in buffer (2.4 mM Tris, pH 7.1) containing Mannitol (60 mM) and EGTA (1 mM), homogenized and the vesicle fraction was isolated by ultracentrifugation. Chemical pathology assays were performed on the initial homogenate and the BBMVs preparation to confirm isolation of the appropriate vesicle fraction. The chemical assays included:

- Total Protein
- Alkaline Phosphatase
- Gamma Glutamyl Transferase
- Leucine Aminopeptidase

Assessment of Binding of Cry9C to BBMVs:

BBMVs from insect mid-guts and mouse intestines were incubated for one hour at room temperature in each of the following mixtures:

Mixture A: 10 μ g BBMVs + 10 ng Biotinylated Cry9C

Mixture B: 10 μ g BBMVs + 10 ng Biotinylated Cry9C + 2 μ g unlabeled Cry9C

The proteins were then separated using SDS-PAGE electrophoresis and electroblotted onto nitro-cellulose membranes. The membranes were incubated with streptavidin-horseradish peroxidase, followed enhanced chemiluminescence western blotting reagents (Amersham). The resulting chemiluminescence was detected using photographic emulsion.

II. RESULTS

Chemical Pathology - Assay results to confirm the correct isolation of the mouse intestinal BBMV fraction:

Fraction	Original Homogenate	BBMV Preparation
Total Protein (mg/ml)	1.76	0.47
Leucine Aminopeptidase (U/mg protein)	90	479
Alkaline Phosphatase (U/mg protein)	998	6464
γ -Glutamyl Transferase (U/mg protein)	44	232

The results indicate that the enrichment of the BBMV fraction is approximately 5-fold compared to the original homogenate.

Binding of Cry9C to BBMVs:

Insect BBMVs - A distinct band of the expected molecular weight (70 kDa) was detected from Mixture A and an extremely faint band was detected from Mixture B showing, according to AgrEvo, that the addition of the unlabeled Cry9C into the incubation solution resulted in displacement of biotinylated Cry9C.

Mouse BBMVs - A band of the expected molecular (70 kDa), and an additional band of lower molecular weight were detected from Mixture A. A band of the expected molecular weight was detected from Mixture B, showing that the addition of unlabeled Cry9C into the incubation solution, did not result in the displacement of the biotinylated Cry9C, again according to AgrEvo.

III. DISCUSSION

The study author indicates that the addition of unlabeled Cry9C in the displacement of the labeled (biotinylated) Cry9C, demonstrating that the Cry9C binds specifically and saturably to *Ostrinia nubilalis* BBMVs. In addition, the addition of unlabeled Cry9C did not result in the displacement of the labeled Cry9C, demonstrating that Cry9C shows only a small amount of non-specific binding, and does not bind specifically and saturably to mouse intestinal BBMVs.

However, the copy of the electrophoretogram provided is not of very good quality, and therefore, it is impossible for BPPD to reach these same conclusions. The picture of the insect BBMVs has a very dark background, and it is not possible to determine if more than one band is present, specifically the "faint" band that would represent Mixture B. Also, the picture of the mouse BBMVs has virtually no background, and therefore it is also not possible to determine if additional "faint" bands might be present. Neither of the figures have molecular weight markers to

verify the 70 kDa weight of the proteins or control samples for comparison. The original, or a much higher quality copy of these films, should be provided in order for an accurate assessment to be made regarding these conclusions. In addition, there is no explanation provided, nor a molecular weight for the additional band present in the mouse samples. This information should also be provided.

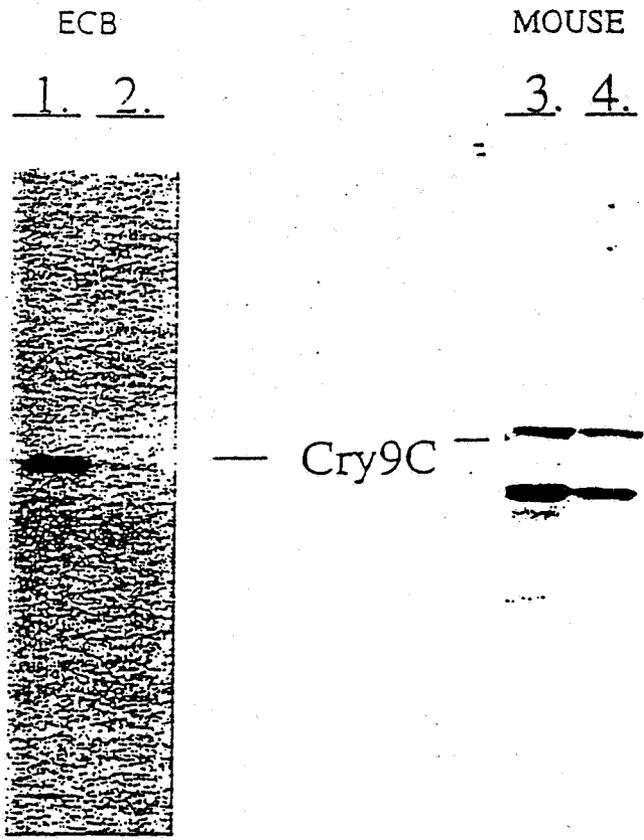
Assuming that clear figures are available, it is also unclear to BPPD what AgrEvo means by "displacement" by the unlabeled Cry9C in the insect BBMV samples. The term "displacement" indicates an active process where there is a mechanism responsible for dislodging labeled proteins and allowing these displaced proteins to be replaced by the unlabeled Cry9C proteins, or some similar process. There is no discussion in this submission which addresses the displacement issue. Further explanation/discussion should be provided.

CLASSIFICATION: SUPPLEMENTAL. This submission can be upgraded to ACCEPTABLE with submission of adequate figures and additional information to support the conclusion reached regarding the binding potential of Cry9C to the tissues tested. Supplemental data/information should include:

1. Adequate figures (originals or high quality reproductions) of the chemiluminescent results that will allow for clear differentiation between protein signals and background "noise".
2. Molecular weight markers to clearly identify the molecular weights of the identified protein bands. Also, identification of any "control" protein samples used for comparison to test samples.
3. Explanation/identification of the additional bands present in the Mouse BBMV samples.
4. Further discussion and justification regarding the "displacement" of the labeled Cry9C proteins by the unlabeled proteins.

APPENDIX

ELECTROPHORETOGRAMS SHOWING LABELLED CRY9C FOLLOWING INCUBATION WITH INSECT AND MOUSE BBMVs



Labelled Cry9C	:	-	-	+	+
200 fold unlabelled Cry9C	:	-	-	-	+

Key:

ECB = Insect BBMVs	MOUSE = Mouse BBMVs
1. = Mixture A	3. = Mixture A
2. = Mixture B	4. = Mixture B

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DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist *MTW*

Secondary Reviewer: John L. Kough, Ph.D., Biologist *JK*

STUDY TYPE: Acute Intravenous Toxicity (152A-13)
 MRID NO: 447343-02
 TEST MATERIAL: *Bt* Cry9C and *Bt* Cry1A(b) Proteins
 PROJECT NO: TOX 98009
 SPONSOR: AgrEvo USA Company, Wilmington, DE
 TESTING FACILITY: AgrEvo UK Limited, Saffron Walden Essex, England
 TITLE OF REPORT: *Bt* Cry9C Protein Mouse Acute Intravenous Toxicity Study
 AUTHOR(S): N. Buss, M. McFarlane
 STUDY COMPLETED: October 7, 1998
 CONCLUSION: Male CD-1 mice were dosed with saline solutions containing CRY9C, CRY1A(b) and BSA proteins. The animals were observed for clinical effects over a 14 day period, and were examined by gross necropsy at the end of the observation period. A dose of 0.3 mg/kg body weight did not produce any apparent adverse effects in the mice tested.

CLASSIFICATION: ACCEPTABLE
 GOOD LABORATORY PRACTICE: This Study Was Performed in Accordance with Good Laboratory Standards guidelines.

I. STUDY DESIGN

Test Material: *Bt* Cry9C protein derived from *Bacillus thuringiensis* subsp. *tolworthi* and *Bt* Cry1A(b) protein derived from *B. thuringiensis* subsp. *kurstaki*, obtained by expression in *E. coli*. Control test material was bovine serum albumin (BSA), obtained from Sigma Aldrich Fluka.

Test Animals: Forty-five male CD-1 mice were received from Charles River UK Ltd., with body weights in the range of 16.5 g to 21.2 g. The animals were allowed one day of acclimatization, examined, and separated into four groups of five mice on the basis of body weights. The groups of mice had similar initial mean body weights and weight distributions, with a range between 21.3 and 22.8g.

Methods: Body Weights: Each animal was weighed upon receipt, at separation into groups, prior to the start of treatment, on Days 1 & 8, and at termination.

103

Dosing: The theoretical maximum daily intake (TMDI) was calculated based upon the assumption that the only crops containing Cry9C protein are maize, cotton, and rape and that the maximum levels present are estimated to be c. 18 ppm (18 mg/kg/day). Based upon this, the global TMDI was calculated to be 0.0247 mg/kg/day and based upon the European diet, the TMDI would be 0.0169 mg/kg/day.

AgrEvo has determined the possible human systemic exposure resulting from an oral exposure of an estimated TMDI (0.03 mg/kg/day) to be 0.2 ng/kg/day based on information from a bioavailability study in rats. From this calculation, the intravenous dose level chosen was 0.3 mg/kg, which provided a 150,000 fold margin of safety over the estimated systemic human level. Both BSA and CRY 1A(b) proteins were administered at the same dose level to allow for direct comparison.

Table 1. Treatment Groups

Group Number	Test Material	Dose Level (mg/kg)	Cage Number	Animal Number
1	0.9% Sodium Chloride (vehicle)	0	1	7631-7635
2	Bt Cry 9C	0.3	2	7636-7640
3	Bt Cry 1 A(b)	0.3	3	7641-7645
4	Bovine Serum Albumin	0.3	4	7646-7650

The test material was administered intravenously (in sterile 0.9% sodium chloride) through the tail vein at a constant volume of 10 ml/kg based upon body weights using in-line 0.2 μ m syringe filters to ensure sterility.

All surviving animals were euthanized on Day 14.

Clinical Signs: Observations were made frequently on the day of treatment, and at least once each morning thereafter. The animals were also observed on Monday and Friday afternoons.

Chemical Pathology: Blood samples for analysis of Cry9C and Cry 1A(b) proteins were collected on the day of necropsy (Day 14). All samples were obtained from the retro-orbital sinus under isoflurane anaesthesia. The blood samples were then snap frozen in liquid nitrogen at -70° C and sent for storage at Plant Genetic Systems.

283

Necropsy: Surviving animals were euthanized by carbon dioxide asphyxiation on Day 14 and subjected to gross *post-mortem* examination.

II. RESULTS

Mortality: No animals died during the 14 day observation period.

Clinical Signs: No treatment-related clinical signs were observed through the 14 day observation period.

Body Weight: None of the animals lost weight over the 14 day observation period. One animal in the BSA test group did not gain as much weight as the other animals in this, and other groups, and therefore the mean for this group was significantly different from the control. However, this result did not affect the outcome of the testing.

Gross Necropsy: There were no abnormal findings in any of the animals examined by gross necropsy at the end of the 14 day observation period.

Chemical Pathology: No analysis of these samples was reported.

III. DISCUSSION

The test substances used in these tests were proteins derived from two *B. thuringiensis* species and BSA. In the toxicity testing included in this submission, there does not appear to be toxic effects associated with any of the three proteins. A 14 day observation period after dosing did not reveal any abnormalities associated with injection of any of the proteins.

CLASSIFICATION: ACCEPTABLE