syngenta

Effect of Temperature on the Bioactivity of eCry3.1Ab Protein as Contained in Test Substance ECRY3.1AB-0208

Amended Report No. 1

Data requirement:	Not applicable
Author:	Andrea Nelson
Date:	Study completed on October 8, 2010
Syngenta Study No.:	TKRS0000041
Report No.:	SSB-014-09 A1
Performing Laboratories:	Syngenta Biotechnology, Inc. Product Safety PO Box 12257 3054 East Cornwallis Rd. Research Triangle Park, NC 27709-2257, USA

STATEMENTS OF DATA CONFIDENTIALITY CLAIMS

The following statement applies to submissions to the United States Environmental Protection Agency (US EPA).

Statement of No Data Confidentiality Claim

No claim of confidentiality is made for any information contained in this report on the basis of its falling within the scope of Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Section 10 (d) (1) (A), (B), or (C).

Company: *Syngenta Seeds, Inc.*

Company Representative:

Demetra Vlachos — October 7, 2010

Demetra Vlachos
Regulatory Affairs Manager

Date

These data are the property of Syngenta Seeds, Inc. and, as such, are considered to be confidential for all purposes other than compliance with the regulations implementing FIFRA Section 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other provision of common law or statute or in any other country.

The following statement applies to submissions to regulatory agencies and other competent authorities other than the US EPA and all other viewers.

This Document Contains Confidential Business Information

This document contains information that is proprietary to Syngenta and, as such, is considered to be confidential for all purposes other than compliance with the relevant registration procedures.

Without the prior written consent of Syngenta, this information may (i) not be used by any third party including, but not limited to, any other regulatory authority for the support of regulatory approval of this product or any other product, and (ii) not be published or disclosed to any third party including, but not limited to, any authority for the support of regulatory approval of any products.

Its submission does not constitute a waiver of any right to confidentiality that may exist in any other country.

STATEMENT CONCERNING GOOD LABORATORY PRACTICES

This study was conducted in compliance with the relevant provisions of Good Laboratory Practice (GLP) Standards, 40 CFR Part 160 (U.S. EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act and subsequent revisions.

Study Director

Andrea Nelson — October 8, 2010
Date

Andrea Nelson
Technical Expert II
Product Safety
Syngenta Biotechnology, Inc.

Submitted by

Demetra Vlachos — October 8, 2010
Date

Demetra Vlachos
Regulatory Affairs Manager
Syngenta Seeds, Inc.
3054 East Cornwallis Rd.
PO Box 12257
Research Triangle Park, NC 27709-2257, USA

Representative of the Sponsor

October 8, 2010
Date

Gerson Graser
Technical Leader 1
Product Safety
Syngenta Biotechnology, Inc.

QUALITY ASSURANCE STATEMENT

Study Title: Effect of Temperature on the Bioactivity of eCry3.1Ab Protein as Contained in Test Substance ECRY3.1AB-0208

Study Director: Andrea Nelson

Study Number: TKRS0000041

Report Number: SSB-014-09 A1

Pursuant to Good Laboratory Practice Regulations (40 CFR Part 160), this statement verifies that the aforementioned study was inspected and/or audited and the findings reported to Management and to the Study Director by the Quality Assurance Unit on the dates listed below.

Inspection/Audit Type	Inspection/Audit Dates	Reporting Date
Audit Protocol	January 28, 2009	January 28, 2009
In-Progress Inspection	February 6, 2009	February 6, 2009
Final Report Audit (1st Audit)	September 14 – 15, 2009	September 15, 2009
Final Report Audit (2nd Audit)	September 23, 2009	September 23, 2009

Prepared by: Kimberly W. Hill Date: Sept. 23, 2010

Kimberly W. Hill
Staff QA Auditor
Quality Assurance Unit
Syngenta Crop Protection, Inc.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

GLP	Good Laboratory Practice
LC_{50}	50% lethal concentration
h	hours
Milli-Q	water purified using the Millipore sytem (Ultrapure Organex Cartridge)

REPORT AMENDMENTS

Amendment No. 1: September 23, 2010

This amended report has the following correction(s):

On page 1, the department title has been changed to Product Safety.

On page 2, the Statements of Data Confidentiality Claims have been updated. The Regulatory Manager's name has been updated.

On page 3, the Regulatory Manager and Sponsor names have been updated, and position and department titles have been updated.

On page 4, a new Quality Assurance page has been issued.

On page 5, the Table of Contents has been updated.

On page 7, a new section has been added listing the Report Amendments.

On page 9, the reference to Thompson (2008) was removed from the Test Substance ECRY3.1AB-0208 paragraph under Materials and Methods.

On page 12, position and department titles have been updated.

On page 13, the reference listing for Thompson (2008) was removed from the References section and the US EPA MRID numbers were added for the Nelson (2008) and Nelson (2009) reference.

The "CONFIDENTIAL" mark was removed from the header or the footer of each page of the report. The corrected pages in this amended report SSB-023-08 A1 are indicated as "*REVISED*"

SUMMARY

The purpose of this study was to investigate the effect of temperature on the insecticidal activity of eCry3.1Ab by incubating solutions of test substance ECRY3.1AB-0208 for 30 minutes at various temperatures (25°C, 37°C, 65°C and 95°C) and assessing bioactivity against *Leptinotarsa decemlineata* (Colorado potato beetle) larvae. An additional sample of eCry3.1Ab solution, incubated at 4°C, was used to determine the baseline bioactivity for the assay.

After temperature treatment at 25°C, 37°C and 65°C, eCry3.1Ab retained bioactivity as shown by the estimated LC_{50} values and 95% confidence intervals of 1.802 µg eCry3.1Ab/ml (1.066-2.714 µg eCry3.1Ab/ml), 1.814 µg eCry3.1Ab/ml (0.411-4.714 µg eCry3.1Ab/ml) and 4.682 µg eCry3.1Ab/ml (1.321-10.092 µg eCry3.1Ab/ml), respectively. After temperature treatment at 95°C, LC_{50} values and 95% confidence intervals could not be estimated due to the low mortality in these samples at all concentrations tested. The data presented in this study support the conclusion that eCry3.1Ab becomes denatured and therefore, inactivated after heat treatment for 30 minutes at 95°C.

INTRODUCTION

The purpose of this study is to investigate the effect of temperature on the insecticidal activity of eCry3.1Ab as assessed by bioassay against *Leptinotarsa decemlineata* (Colorado potato beetle) larvae. Test substance ECRY3.1AB-0208 was prepared from a recombinant *Escherichia coli* strain. The eCry3.1Ab protein in test substance ECRY3.1AB-0208 is identical in amino acid sequence to that expressed in Syngenta's maize transformation Event 5307 except that it contains one additional methionine and six histidine residues at the N-terminus.

Syngenta has transformed maize (*Zea mays* L.), to produce Event 5307 maize which contains the *ecry3.1Ab* gene encoding the eCry3.1Ab protein. The eCry3.1Ab protein is an engineered chimera of mCry3A and Cry1Ab proteins, which has insecticidal activity against significant plant pests including some corn rootworm (*Diabrotica*) species and Colorado potato beetle (*Leptinotarsa decemlineata*).

In this study, eCry3.1Ab in test substance ECRY3.1AB-0208 was incubated at 4°C (control), 25°C, 37°C, 65°C and 95°C for 30 minutes and the loss of bioactivity, as compared to the control sample, was determined by bioassay against *L. decemlineata* larvae.

MATERIALS AND METHODS

Test Substance ECRY3.1AB-0208

Test substance ECRY3.1AB-0208 was prepared from an *Escherichia coli* over-expression system. The eCry3.1Ab in test substance ECRY3.1AB-0208 is identical to that expressed in Syngenta's maize transformation Event 5307 except that it contains one additional methionine and six histidine residues at the N-terminus. The intended additional 7 amino acids aid in purification from the *E. coli* over-expression system. The modified *ecry3.1Ab* gene used for the microbial expression was linked to the bacterial *tac* promoter in a vector derived from pET24a and transformed into *E. coli* strain DH5α.

Prior to this study, ECRY3.1AB-0208 was prepared from pooled batches of *E. coli* cell paste. Briefly, *E. coli* cells were ruptured and the cell debris removed by centrifugation. The soluble material was filtered, applied to an immobilized metal affinity column (GE Healthcare Nickel Sepharose Fast Flow column), and eluted using an imidazole step gradient. Fractions containing the eCry3.1Ab protein were then further purified via anion exchange chromatography and eCry3.1Ab was eluted with a sodium chloride gradient. The eluted eCry3.1Ab containing fractions were pooled, concentrated and the buffer was exchanged. The solution was lyophilized and designated ECRY3.1AB-0208. The test substance was sent on dry ice to Syngenta Biotechnology, Inc. (Research Triangle Park, NC, USA), where it was stored at -20°C ± 8°C until further use.

The characterization of ECRY3.1AB-0208 is described in a separate study report (Nelson 2008). In that study, ECRY3.1AB-0208 was determined to contain 89.6% eCry3.1Ab by weight. In another study, eCry3.1Ab from ECRY3.1AB-0208 was shown to have insecticidal activity against *L. decemlineata* and it was demonstrated that ECRY3.1AB-0208 was a suitable surrogate for eCry3.1Ab produced in 5307 maize (Nelson 2009).

Temperature Treatment

For the temperature treatment, test substance ECRY3.1AB-0208 was resolubilized in 10 mM ammonium bicarbonate buffer (pH 10.0) at a concentration of 1 mg eCry3.1Ab/ml. Aliquots of the eCry3.1Ab solution were incubated in duplicate at 25°C ± 2°C, 37°C ± 2°C, 65°C ± 2°C and 95°C ± 2°C for 30 minutes. For the baseline control, duplicate aliquots of the eCry3.1Ab solution were incubated at 4°C ± 2°C for 30 minutes. Following temperature treatment, the samples were placed on ice and subsequently incorporated into diet for bioassays.

Bioassays

After temperature treatment, the insecticidal activity of eCry3.1Ab in the duplicate aliquots of the test solution was assessed in bioassays using freshly hatched *L. decemlineata* larvae. The bioassays were conducted in 24-well plates. *L. decemlineata* diet (Bio-Serve) was prepared by blending a boiling mixture of 6.6 grams of agar and

422.5 ml of Milli-Q water with 70.15 grams of *L. decemlineata* diet powder mix per the manufacturer's directions. The diet mixture was cooled to approximately 55°C in a water bath. To prevent bacterial and fungal growth, solutions of potassium hydroxide (3.6 mg/ml), nystatin (1.8 mg/ml), cefotaxime (1.8 mg/ml), aureomycin (1.8 mg/ml), streptomycin (1.8 mg/ml) and preservative for plant tissue culture media (0.2%) were each added to the cooled diet. Each aliquot of eCry3.1Ab solution treated at 4°C, 25°C and 37°C was incorporated into the *L. decemlineata* diet at eight nominal concentrations ranging from 0.1953 to 25 µg eCry3.1Ab/ml. To compensate for a loss in eCry3.1Ab bioactivity in the higher temperature treatments (65°C and 95°C), the nominal concentration range was increased and examined at eight nominal concentrations ranging from 0.7813 to 100 µg eCry3.1Ab/ml.

A 150 µl aliquot of diet was added to each well of the bioassay plate and infested with one larva. Each treatment consisted of 48 larvae in total (i.e. 24 larvae × 2 temperature treated aliquots per concentration). Diets incorporated with Milli-Q water and 10 mM ammonium bicarbonate buffer (pH 10.0) were used as negative controls. The wells were covered with silicone stoppers and maintained at room temperature under ambient laboratory conditions. Mortality was assessed starting at 72 hours and continued daily for up to 144 hours after infestation. The total percent mortality of the 48 larvae taken 144 hours after infestation was reported.

Statistical Methods

The LC_{50} values determined in the *L. decemlineata* bioassay were calculated using the EPA Probit Analysis Program, Version 1.5.

RESULTS AND DISCUSSION

No circumstances occurred during conduct of this study that would have adversely affected the quality or integrity of the data generated.

The results of the *L. decemlineata* bioassay summarized in Table 1 and Table 2, demonstrate that eCry3.1Ab, in test substance ECRY3.1AB-0208, retains activity after temperature treatment at 25°C, 37°C and 65°C when compared to the activity of the control sample incubated at 4°C. The LC_{50} values and 95% confidence intervals at 4°C, 25°C, 37°C and 65°C were 0.918 (0.419-1.617), 1.802 (1.066-2.714), 1.814 (0.411-4.714) and 4.682 (1.321-10.092) µg eCry3.1Ab/ml, respectively. For the temperature treatment at 95°C, LC_{50} values and 95% confidence intervals could not be estimated due to low mortality at all concentrations tested (Tables 1 and 2). The negative controls (water and 10 mM ammonium bicarbonate buffer (pH 10.0) treated diets) showed low mortality, 14.6% and 8.3% respectively (Table 2).

Table 1. Effect of Temperature on Insecticidal Activity of eCry3.1Ab (ECRY3.1AB-0208) in Diet Incorporation Bioassay with *L. decemlineata* Larvae: LC_{50} Values and 95% Confidence Intervals at 144 h

Temperature [°C]	LC_{50} [µg eCry3.1Ab/ml]	95% Confidence Interval [µg eCry3.1Ab/ml]
4	0.918	0.419-1.617
25	1.802	1.066-2.714
37	1.814	0.411-4.714
65	4.682	1.321-10.092
95	>100	Not estimable

Table 2. Effect of Temperature on Insecticidal Activity of eCry3.1Ab (ECRY3.1AB-0208) in Diet Incorporation Bioassays with *L. decemlineata* Larvae: Percent Mortality Data

Concentration [µg eCry3.1Ab /ml]	Mortality at 144 h [%]				
	4°C	25°C	37°C	65°C	95°C
0.1953	43.8	33.3	27.1	nt[1]	nt
0.3906	45.8	33.3	43.8	nt	nt
0.7813	52.1	37.5	50.0	38.3	10.4
1.5625	52.1	47.9	45.8	42.6	4.2
3.125	68.8	58.3	54.2	45.8	12.5
6.25	68.8	72.9	60.4	43.8	6.3
12.5	81.3	89.6	75.0	70.8	19.1
25	93.8	97.9	97.9	72.9	8.3
50	nt	nt	nt	95.8	8.3
100	nt	nt	nt	95.8	4.2
Water control	14.6				
Buffer control[2]	8.3				

[1]nt: not tested

[2]10mM ammonium bicarbonate buffer (pH 10.0)

CONCLUSIONS

The data presented in this study supports the conclusion that eCry3.1Ab becomes denatured and therefore inactivated after heat treatment for 30 minutes at 95°C.

RECORDS RETENTION

Raw data, the original copy of this report, and other relevant records are archived at Syngenta Biotechnology, Inc., 3054 East Cornwallis Rd., Research Triangle Park, NC 27709-2257, USA.

CONTRIBUTING SCIENTISTS

The analytical work reported herein was conducted by Andrea Nelson, B.S. and Ian Kietzman, B.S. at Syngenta Biotechnology, Inc.

Reported by: Andrea Nelson — October 8, 2010 (Date)
Technical Expert II
Product Safety
Syngenta Biotechnology, Inc.

Approved by: Gerson Graser — October 8, 2010 (Date)
Technical Leader 1
Product Safety
Syngenta Biotechnology, Inc.

CRITICAL DATES

Study initiation date:	January 30, 2009
Experimental start date:	February 4, 2009
Experimental end date:	February 12, 2009

REFERENCES

Nelson A. 2008. *Characterization of Test Substance ECRY3.1AB-0208 and Certificate of Analysis.* Report No. SSB-010-08. Research Triangle Park, NC: Syngenta Biotechnology. MIRID No. 47734704.

Nelson A. 2009. Comparison of eCry3.1Ab Protein Produced in Event 5307-Derived Maize Plants and eCry3.1Ab Protein Produced in Recombinant Escherichia coli. Report No. SSB-002-09 (unpublished). Research Triangle Park, NC: Syngenta Biotechnology. MIRID No. 47734703.

U.S. EPA. 1989. Good Laboratory Practice Standards. 40 CFR Part 160.