In vitro Digestibility of eCry3.1Ab Protein as Contained in Test Substance ECRY3.1AB-0208 Under Simulated Mammalian Intestinal Conditions

Amended Report No. 1

Data requirement:	Not applicable
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Report No.:	SSB-015-09 A1
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The following statement applies to submissions to the United States Environmental Protection Agency (US EPA).

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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 — September 29, 2010

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Regulatory Affairs Manager

Date

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES

This study was conducted in compliance with the relevant provisions of Good Laboratory Practice Standards (40 CFR Part 160, US EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act with the following exception:

- The characterization of purchased standards was not conducted according to GLP. This had no impact on the quality or integrity of the study.

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QUALITY ASSURANCE STATEMENT

Study Title: *In Vitro* Digestibility of eCry3.1Ab Protein as Contained in Test Substance ECRY3.1AB-0208 Under Simulated Mammalian Intestinal Conditions

Study Director: Laura Seastrum

Study Number: 5307-08-12

Report Number: SSB-015-09 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	August 21, 2008	August 21, 2008
In-Progress Inspection	March 30, 2009	March 30, 2009
Final Report Audit	October 31 & November 2, 2009	November 2, 2009

Prepared by: Connie L. Connor Date: Sept. 27, 2010

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LIST OF ACRONYMS AND ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bis-Tris	bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
IgG	immunoglobulin G
kDa	kiloDaltons
LDS	lithium dodecyl sulfate
LOD	limit of detection
MES	2-(*N*-morpholino)ethanesulfonic acid
mg	milligram
ml	milliliter
mM	millimolar
N	normal
NBT	nitro blue tetrazolium
ng	nanogram
PVDF	polyvinylidene difluoride
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SIF	simulated mammalian intestinal fluid
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
Tween 20	polyoxyethylene sorbitan monolaurate
µg	microgram

REPORT AMENDMENTS

Amendment No. 1: August 13, 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 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. The implications of the GLPS exception were described.

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 page has been added listing the Report Amendments.

On page 8, the Summary paragraphs, Introduction and Materials and Methods have been revised for clarity. The reference to Thompson (2008) was removed from the Test Substance ECRY3.1AB-0208 paragraph under Materials and Methods.

On page 11, the Results and Discussion section was revised for clarity.

On page 12, the LOD lanes were removed from Figure 1 due to inability to resolve all of the bands when the document is printed.

On page 13, the LOD lanes were removed from Figure 2 due to inability to resolve all of the bands when the document is printed. The conclusion paragraph was revised for clarity. A footnote was added regarding faint bands on the Western blot.

On page 14, position and department titles have been updated.

On page 15, the reference listing for Thompson (2008) was removed from the References section and the US EPA MRID number was added for the Nelson (2008) reference.

With the exception of page 1, the updated pages in this amended report SSB-015-09 A1 are indicated as "*REVISED.*"

SUMMARY

The susceptibility of eCry3.1Ab to proteolytic degradation in simulated mammalian intestinal fluid (SIF) containing pancreatin was evaluated using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Intact eCry3.1Ab (molecular weight 74.8 kDa) from recombinant *Escherichia coli* was rapidly degraded in SIF. No intact eCry3.1Ab was detected by Western blot analysis following incubation in SIF fluid for 1 minute. Immunoreactive fragments of eCry3.1Ab with molecular weights of approximately 56, 40, and 5 kDa were present at the conclusion of the 48 hour time course of the study.

The results of this study support the conclusion that the eCry3.1Ab protein produced in Event 5307 maize is sensitive to proteolysis by pancreatin and is rapidly degraded to constituent peptides.

INTRODUCTION

The purpose of this study was to assess the *in vitro* digestibility of eCry3.1Ab in simulated mammalian intestinal fluid (SIF). Syngenta has transformed maize (*Zea mays*) to produce Event 5307 maize, a new cultivar that has insecticidal activity against certain corn rootworm (*Diabrotica*) species. Maize plants derived from transformation Event 5307 (5307 maize) contain the gene *ecry3.1Ab* encoding the eCry3.1Ab protein. The eCry3.1Ab protein is an engineered chimera of mCry3A and Cry1Ab proteins.

A purified preparation of microbially produced eCry3.1Ab was used as a source of test protein in this study. SDS-PAGE and Western blot analysis were used to assess the digestibility of eCry3.1Ab in SIF over a 48 hour time course at 37°C.

MATERIALS AND METHODS

Test Substance ECRY3.1AB-0208

Test substance ECRY3.1AB-0208 was prepared from an *E. coli* over-expression system. The eCry3.1Ab protein in test substance ECRY3.1AB-0208 is identical to that encoded in Syngenta's Event 5307 maize, with the exception that it contains seven additional amino acids at the N-terminus which consist of one methionine and six histidine residues. The modified *ecry3.1Ab* gene used for 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, test substance ECRY3.1AB-0208 was prepared from pooled batches of *E. coli* cell paste by Syngenta Protein Science (Jealott's Hill International Research Centre, Bracknell, UK). 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, and eluted using an imidazole step gradient. Fractions containing the

eCry3.1Ab protein were then further purified via anion exhange 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 test substance 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. Test substance ECRY3.1AB-0208 was characterized in detail in a previous study and was determined to contain 89.6% eCry3.1Ab (molecular weight 74.8 kDa) by weight (Nelson 2008).

To prepare the test protein solution for this study, the lyophilized test substance ECRY3.1AB-0208 was resuspended in 10 mM ammonium bicarbonate (pH 10.0) to produce a 5 mg eCry3.1Ab/ml solution which was subsequently used in the SIF assessment.

Simulated Mammalian Intestinal Fluid

Simulated mammalian intestinal fluid (USP 2000) was freshly prepared, containing 50 mM potassium phosphate monobasic, 38 mM sodium hydroxide and 10 mg/ml pancreatin (Sigma-Aldrich Cat. No. P7545). The solution was adjusted to pH 7.5 ± 0.05 using 0.2 N sodium hydroxide. Immediately before use, the proteolytic activity of pancreatin was confirmed using azoalbumin as a substrate. SIF without pancreatin (intestinal fluid control solution) was prepared for use as a negative control.

Time Course of Digestion in SIF

A 5 mg eCry3.1Ab/ml solution was prepared as described above. Except in the case of the time-zero samples, the test protein solution was mixed with the SIF solution to give a final ratio of approximately 38 µg of pancreatin per 1 µg of eCry3.1Ab and incubated at 37 ± 2°C. The SIF-test protein mixture was sampled after 1, 2, 5, 10, 30 and 60 minutes and 2, 3, 6, 24 and 48 hours of incubation. At each sampling point, an aliquot of SIF-test protein mixture was removed, and the reaction was terminated by adding the aliquot to a solution containing 4X LDS NuPage Sample Buffer and 10X Sample Reducing Agent that was preheated at 95 ± 5°C for 2 minutes. The sample was subsequently heated at 95 ± 5°C for 10 minutes. For the time-zero sample an aliquot of the SIF solution and an aliquot of the test protein solution (at the same ratio of eCry3.1Ab to SIF as described above) were added directly to preheated 4X LDS NuPage Sample Buffer and 10X Sample Reducing Agent and incubated at 95 ± 5°C for 10 minutes.

As a negative control, an aliquot of the test protein solution added to the SIF without pancreatin was prepared in order to monitor the stability of the test protein over the 48 hour time course. Additionally, an aliquot of 10 mM ammonium bicarbonate (pH 10.0), which was the buffer used to produce the test protein solution, was added to the SIF in order to monitor the stability of pancreatin over the 48 hour time course. Both controls were sampled at 0, 2 and 48 hours and further treated as described above.

Samples produced from the above reactions were stored at –20°C ± 8°C until used for SDS-PAGE and Western blot analyses.

SDS-PAGE Analysis

Aliquots equivalent to 0.5 µg of eCry3.1Ab based upon the initial concentration in the control and reaction samples were subjected to SDS-PAGE with a Criterion XT 4%–12% Bis-Tris polyacrylamide gradient gel and Bio-Rad XT MES SDS running buffer. SeeBlue Plus2 Pre-Stained Standard (Invitrogen) was used as the molecular weight standard. The gel was stained with GelCode Blue Stain Reagent and examined for the presence of bands corresponding to intact eCry3.1Ab and eCry3.1Ab fragments.

The limit of detection (LOD) of a method is defined as the lowest amount of analyte in a sample which can be detected but not accurately quantitated. The limit of detection for SDS-PAGE was determined by subjecting serial dilutions of eCry3.1Ab to SDS-PAGE. Amounts tested were 32, 8, 2, 0.5 and 0.25 ng eCry3.1Ab. The gel was stained with GelCode Blue Stain Reagent and the lowest amount of eCry3.1Ab visible on the gel was designated the LOD of eCry3.1Ab for SDS-PAGE.

Western Blot Analysis

Aliquots equivalent to 10 ng of eCry3.1Ab, based upon the initial concentration in the control and reaction samples, were subjected to SDS-PAGE with a Criterion XT 4%–12% Bis-Tris polyacrylamide gradient gel and Bio-Rad MES SDS running buffer. SeeBlue Plus2 Pre-Stained Standard was used as the molecular weight standard. Proteins were transferred to a PVDF membrane via electroblotting, and the membrane was subsequently probed with immunoaffinity-purified polyclonal rabbit antibodies capable of detecting the eCry3.1Ab protein. Alkaline phosphatase–conjugated donkey anti-rabbit IgG diluted to 1:3000 in Tris-buffered saline solution with Tween 20 was used to bind to the primary antibody and was visualized by development with BCIP/NBT alkaline phosphatase substrate solution. The Western blot was examined for the presence of intact immunoreactive eCry3.1Ab and other immunoreactive eCry3.1Ab fragments.

The LOD for Western blot analysis was determined by subjecting serial dilutions of eCry3.1Ab to SDS-PAGE. Amounts tested were 1.0, 0.5, 0.25, 0.10, 0.05 and 0.025 ng eCry3.1Ab. Proteins were transferred to a PVDF membrane and then probed with the same antibody used to monitor the SIF assay. The lowest amount of eCry3.1Ab visible on the membrane was designated the LOD for eCry3.1Ab for the Western blot analysis.

Statistical Analysis

No statistical analysis was required for any parameter evaluated in this study.

RESULTS AND DISCUSSION

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

Following exposure of eCry3.1Ab protein to SIF for 1 minute, no intact eCry3.1Ab (74.8 kDa) was evident, as assessed by SDS-PAGE and Western blot analysis (Figures 1 and 2, Lane 9). However, a protein band corresponding to a molecular weight of approximately 56 kDa appeared in the 1 minute sample; it remained stable over the 48-hour sample time course as shown by the Western blot analysis (Figure 2, Lanes 9 - 19). A band corresponding to a molecular weight of approximately 62 kDa also appeared in the 1 minute sample (Figure 2, Lane 9); it diminished in intensity over the time course and disappeared after 30 minutes. Because these bands cross-reacted with the antibodies capable of detecting eCry3.1Ab, they were identified to be degradation products of eCry3.1Ab protein. An additional immunoreactive band corresponding to a molecular weight of approximately 40 kDa was observed on the Western blot in the 1 hour sample and increased in intensity over the remaining time course (Figure 2, Lanes 14 - 19). This band most likely corresponded to a breakdown product of eCry3.1Ab. The SIF with pancreatin contains proteins that co-migrate with eCry3.1Ab degradation products so it is difficult to follow the breakdown of eCry3.1Ab on the SDS PAGE gel. The Western blot more clearly shows the degradation of eCry3.1Ab.

An immunoreactive protein band with a molecular weight of approximately 6 kDa was visible starting in the 1 minute sample (Figure 2, Lane 9). This band diminished in intensity over the time course and disappeared after 1 hour (Figure 2, Lanes 9 – 14). Another immunoreactive band with a molecular weight of approximately 5 kDa appeared after 5 minutes (Figure 2, Lane 11) and remained visible over the remaining time course (Figure 2, Lanes 12 – 19). These two bands most likely represent breakdown fragments of the eCry3.1Ab protein.

The hydrolytic activity of the SIF was also visible in the control assays (SIF alone) which showed a strong change in the protein pattern (all bands decreased in intensity and some completely disappeared) as shown by SDS-PAGE over the 48 hour time course (Figure 1, Lanes 1 - 3). SIF controls without pancreatin showed no significant degradation of eCry3.1Ab over the 48 hour time course (Figures 1 and 2, Lanes 4 - 6).

Faint protein bands were noted on the Western blot with molecular weights higher than the band representing intact eCry3.1ab (i.e., higher than 74.8 kDa). These bands likely represented dimer and timer forms of eCry3.1Ab, and were consistent with the corresponding molecular weights of two or three eCry3.1Ab polypeptides, respectively, at approximately 150 kDa (Figures 1 and 2, Lanes 4, 5, 6 and 8) and 224 kDa (Figure 2, Lanes 4-6).

The LOD for SDS-PAGE was determined to be 8 ng. The LOD for Western blot analysis was determined to be 0.25 ng eCry3.1Ab.

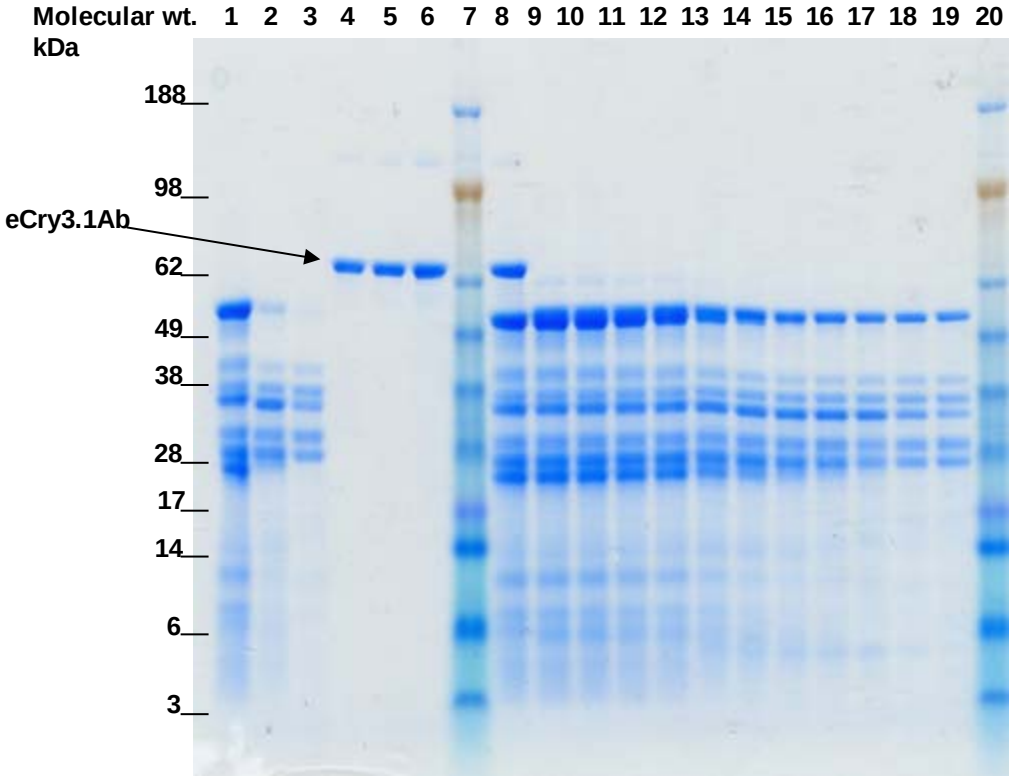

Figure 1. SDS-PAGE analysis of eCry3.1Ab following digestion in SIF.

Figure Note(s): Lanes 1 – 3: SIF-only controls; time points 0, 2 and 48 hours
Lanes 4 – 6: eCry3.1Ab incubated in SIF without pancreatin; Time points 0, 2 and 48 hours
Lanes 7 and 20: SeeBlue Plus2 molecular weight standard
Lanes 8 – 19: Reaction time points 0, 1, 2, 5, 10, 30 and 60 minutes, 2, 3, 6, 24 and 48 hours

The molecular weight of eCry3.1Ab is 74.8 kDa.

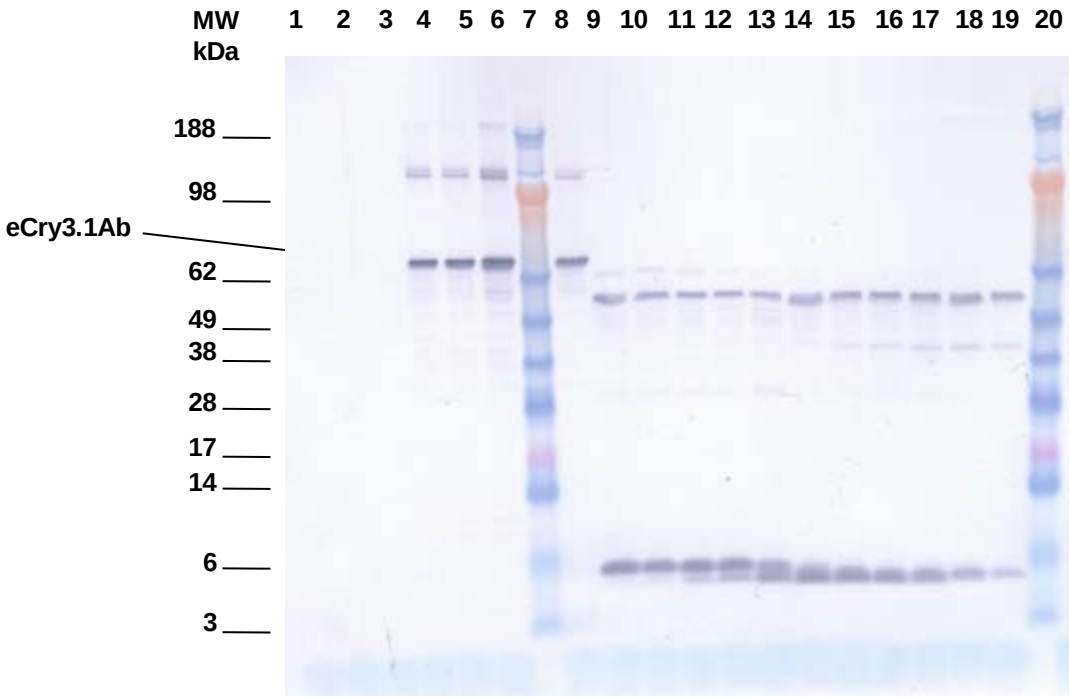

Figure 2. Western blot analysis of eCry3.1Ab following digestion in SIF.

Figure Note(s): Lanes 1 – 3: SIF-only controls; time points 0, 2 and 48 hours
Lanes 4 – 6: eCry3.1Ab incubated in SIF without pancreatin; Time points 0, 2 and 48 hours[1]
Lanes 7 and 20: SeeBlue Plus2 molecular weight standard
Lanes 8 – 19: Reaction time points 0, 1, 2, 5, 10, 30 and 60 minutes; 2, 3, 6, 24 and 48 hours

The molecular weight of eCry3.1Ab is 74.8 kDa.

CONCLUSIONS

The results of this study indicate that intact eCry3.1Ab produced in recombinant *E. coli* is readily degraded under simulated mammalian intestinal conditions. The eCry3.1Ab protein was degraded so rapidly that no intact eCry3.1Ab was detected upon sampling of the reaction mixture at 1 minute. However, immunoreactive fragments of eCry3.1Ab protein (molecular weights ca. 56, 40 and 5 kDa) were detected at the conclusion of the 48-hour time course of the study. The results of this study support the conclusion that the

[1] Due to the resolution limits of the scanner and the printer, the faint bands visible on the actual blot might not be visible on the printed image that appears here.

eCry3.1Ab protein produced in Event 5307 maize is sensitive to proteolysis by pancreatin and is rapidly degraded to constituent peptides.

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 Laura Seastrum, B.A. at Syngenta Biotechnology, Inc.

Reported by: Laura Seastrum Sept. 29, 2010

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CRITICAL DATES

Study initiation date:	September 15, 2008
Experimental start date:	March 25, 2009
Experimental end date:	October 7, 2009

REFERENCES

Unpublished Reports

Nelson A. 2008. *Characterization of Test Substance ECRY3.1AB-0208 and Certificate of Analysis.* Report No. SSB-010-08 (unpublished). Research Triangle Park, NC, USA: Syngenta Biotechnology, Inc. 17 pp. US EPA MRID 47734704.

Literature Citations

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

USP. 2000. *The United States Pharmacopeia, 24th rev. The National Formulary, 19th ed.* Rockville, MD: United States Pharmacopoeial Convention, Inc. p. 2235.