Comparison of eCry3.1Ab Protein Produced in Event 5307-Derived Maize Plants and eCry3.1Ab Protein Produced in Recombinant *Escherichia coli*

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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 with the following exceptions:

1. Planting and cultivation of the seed test and control substances in the greenhouse was not conducted according to GLP standards.
2. Plant tissue was collected before the initiation of this study.
3. Software used during the conduct of this study was not validated.
4. Characterization of the seed test and control substance (07MG005417 and 07MG002998, respectively) and Taqman and SQC analysis of the plants generated from this seed were not conducted according to GLP.
5. The characterization of purchased standards was not conducted according to GLP.
6. The peptide mass mapping analysis conducted by Syngenta Analytical Sciences and the N-terminal sequence analysis conducted by Proseq, Inc., Protein Sequencing Services were not conducted under GLP.

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QUALITY ASSURANCE STATEMENT

Study Title: Comparison of eCry3.1Ab Protein Produced in Event 5307-Derived Maize Plants and eCry3.1Ab Protein Produced in Recombinant *Escherichia coli*

Study Director: Andrea Nelson

Study Number: 5307-08-05

Report Number: SSB-002-09

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	September 15 – 16, 2008	September 16, 2008
In-Progress Inspection	October 15, 2008	October 16, 2008
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LIST OF ACRONYMS AND ABBREVIATIONS

BAS	Beacon Analytical Systems
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bis-Tris	bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane
BSA	bovine serum albumin
CI	confidence interval
DIG	digoxigenin
ELISA	enzyme-linked immunosorbent assay
GLP	Good Laboratory Practice
IAP5307	eCry3.1Ab purified from extract of leaf material from 5307 maize
IgG	immunoglobulin G
LC_{50}	50% lethal concentration
LP5307	eCry3.1Ab extracted from leaf material of 5307 maize
LP-NEG	Nontransgenic, near-isogenic maize leaf tissue extract
MES	morpholinoethanesulfonic acid
MOPS	3-(N-morpholino)propane-sulfonic acid
NBT	nitro blue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PVDF	polyvinylidene difluoride
PVP	polyvinylpolypyrrolidone
SDS	sodium dodecylsulfate
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tween 20	polyoxyethylene sorbitan monolaurate

SUMMARY

In order to demonstrate the biochemical and functional equivalence between the eCry3.1Ab protein produced in a recombinant *Escherichia coli* overexpression system and the eCry3.1Ab protein produced in Event 5307-derived maize, both the microbially-produced protein and the plant produced protein were compared with respect to identity, integrity, insecticidal activity, and glycosylation status.

The eCry3.1Ab protein from Event 5307 maize (LP5307 and IAP5307) and microbially-produced eCry3.1Ab (ECRY3.1AB-0208) had the expected molecular weight of approximately 73.7 and 74.8 kDa respectively. Both plant produced and microbially-produced eCry3.1Ab cross-reacted with the same antibodies, as shown by Western blot analysis. In bioassays against first-instar Colorado potato beetle *(Leptinotarsa decemlineata)*, both the plant produced protein and microbially-produced protein were insecticidally active with comparable LC_{50} values. The 144-hour LC_{50} for each of the 3 independently conducted bioassays were 1.316, 1.669, and 2.888 µg eCry3.1Ab/ml for plant produced eCry3.1Ab, and 1.780, 1.113, and 3.226 µg eCry3.1Ab/ml for the microbially-produced eCry3.1Ab in the presence of nontransgenic, near-isogenic leaf matrix. There was no evidence of post-translational glycosylation of eCry3.1Ab from the immunopurified plant produced protein or the microbially produced protein. Peptide mass mapping identified 76% and 87% of the predicted amino acid sequence of eCry3.1Ab for the plant produced protein and microbially-produced protein, respectively, confirming the identity of the insecticidal protein from both sources. In addition, the expected N-terminal peptide was confirmed for both eCry3.1Ab proteins.

The results of this study support the conclusion that eCry3.1Ab produced in recombinant *E. coli* (ECRY3.1AB-0208) is biochemically and functionally equivalent to the eCry3.1Ab produced in Event 5307-derived maize. The eCry3.1Ab produced in recombinant *E. coli* (ECRY3.1AB-0208) can therefore serve as a suitable surrogate for eCry3.1Ab produced in Event 5307-derived maize.

INTRODUCTION

The purpose of this study was to determine whether eCry3.1Ab protein present in the microbially-produced test substance ECRY3.1AB-0208 is biochemically and functionally equivalent to eCry3.1Ab produced in transgenic maize plants derived from Event 5307. The eCry3.1Ab protein in ECRY3.1AB-0208 is identical to that expressed in Syngenta's maize transformation Event 5307 maize (5307 maize) except that it contains one additional methionine and six histidine residues at the N-terminus. The eCry3.1Ab protein is an engineered chimera of mCry3A and Cry1Ab proteins which has insecticidal activity against significant plant pests including certain corn rootworm species (*Diabrotica spp.*) and Colorado potato beetle (CPB; *Leptinotarsa decemlineata*).

Three distinct preparations of eCry3.1Ab were evaluated in this study: (1) LP5307, eCry3.1Ab extracted from leaf material of 5307 maize plants; (2) IAP5307, eCry3.1Ab purified from an extract of leaf material from 5307 maize; and (3) eCry3.1Ab from the microbial test substance ECRY3.1AB-0208. Key biochemical and functional parameters were evaluated to assess whether the microbially-produced eCry3.1Ab in ECRY3.1AB-0208 was a suitable surrogate for the eCry3.1Ab produced in 5307 maize. The plant produced protein and microbially-produced protein were compared with respect to integrity, insecticidal activity, and glycosylation status. Additionally, the plant and microbially-produced proteins were analyzed by peptide mass mapping. The expected N-terminal peptide was confirmed for both plant produced and microbially-produced eCry3.1Ab proteins. By examining these biochemical and functional parameters, the use of the microbially-produced eCry3.1Ab (ECRY3.1AB-0208) in studies evaluating the safety of 5307 maize can be justified.

MATERIALS AND METHODS

Maize Leaf Tissue and Negative Control Maize Leaf Tissue

Prior to this study, plants from maize seed test substance 07MG005417 (5307 maize) and maize seed control substance 07MG002998 (nontransgenic, near-isogenic maize) were grown under standard greenhouse conditions. The pedigree code of the maize seed test substance 07MG005417 is NP2171 × BC5F3. The pedigree code of the maize seed control substance is 07MG002998 is NP2171 × NP2460.

Young leaves from greenhouse-grown 5307 maize (pedigree NP2171 × BC5F3) and nontransgenic, near-isogenic plants (pedigree NP2171 × NP2460) were collected 4-6 weeks after emergence, frozen at –80°C ± 10°C, and ground into a fine powder using a Grindomix Knife Mill (Retsch Cat. No. GM200).

Extracts of Event 5307 Maize Leaf Tissue and Negative Control Maize Leaf Tissue for Western Blot Analysis

Leaf powder was resuspended in extraction buffer containing 100 mM sodium borate (pH 10.0) (Sigma Cat. No. S9640), 0.2% PVP (Sigma-Aldrich Cat. No. PVP360), 7.69 mM sodium azide (Sigma-Aldrich Cat. No. S2002), 0.5% Tween 20 (Sigma-Aldrich Cat. No. P1379), and supplemented with 1 Complete Protease Inhibitor Cocktail tablet/50ml of buffer (Roche Cat. No. 11836145001). The mixture was homogenized with an Omni-prep (Omni International), incubated for 30 minutes on ice and centrifuged at 3,000 rpm for 15 minutes at 4°C (Sorvall Legend RT). The resulting supernatants were stored overnight at 2°C to 8°C and then stored at -20°C ± 8°C in 4X NuPage LDS Sample Buffer (Invitrogen Cat. No. NP0007) containing NuPage Sample Reducing Agent (Invitrogen Cat. No. NP0004) for subsequent Western blot analysis. The samples

extracted from the 5307 maize and nontransgenic, near-isogenic tissues were designated LP5307 and LP-NEG respectively.

Extracts of Event 5307 Maize Leaf Tissue and Negative Control Maize Leaf Tissue for Bioassay

Extracts for bioassays were prepared by resuspending leaf powder, from 5307 maize and nontransgenic, near-isogenic plants as described above, in a 10 mM ammonium bicarbonate (Fisher Cat. No. A643), pH 10.0 buffer. The extraction mixtures were homogenized in a blender (Waring Laboratory) for 45 seconds, incubated on ice for 1.5 hours and centrifuged (Sorvall RC5B) at 8,000 rpm for 30 minutes at 4°C. The resulting supernatant was filtered through cheesecloth, centrifuged for an additional 30 minutes at 8,000 rpm at 4°C, and filtered through cheesecloth again. The resulting clear supernatant was concentrated using centrifugal filter devices (Millipore Cat. No. UFC90324). The sample was then stored overnight at 2-8°C for subsequent diet incorporation and ELISA analysis. The samples extracted from the 5307 maize and nontransgenic, near-isogenic tissues were designated LP5307 and LP-NEG respectively.

Extracts of Nontransgenic, Near-isogenic Leaf Tissue Fortified with Microbially-Produced Test Substance ECRY3.1AB-0208 for Western Blot Analysis

In order to investigate if the plant matrix affects eCry3.1Ab mobility and immunoreactivity, ECRY3.1AB-0208 was added to nontransgenic, near-isogenic leaf extract. This sample allows for comparison of the microbially-produced eCry3.1Ab and plant produced eCry3.1Ab to be examined in the same matrix.

For Western blot analysis, ECRY3.1AB-0208 was added to LP-NEG, as prepared for Western blot analysis, such that the total protein and amount of eCry3.1Ab loaded on the gel was equivalent to that estimated for sample LP5307, as prepared for Western blot analysis. This sample was designated LP-NEG + ECRY3.1AB-0208.

Extracts of Nontransgenic, Near-isogenic Leaf Tissue Fortified with Microbially-Produced Test Substance ECRY3.1AB-0208 for Bioassays

In order to investigate if the plant matrix affects bioactivity, ECRY3.1AB-0208 was added to nontransgenic, near-isogenic leaf extract. This sample allows for comparison of the microbially-produced eCry3.1Ab and plant produced eCry3.1Ab to be examined in the same matrix.

For the bioassays, ECRY3.1AB-0208 was added to LP-NEG, as prepared for bioassays, such that when incorporated into the diet the concentration of eCry3.1Ab in the diet is equivalent to the eCry3.1Ab concentration in the diet containing only the ECRY3.1AB-0208 test substance. This sample was designated LP-NEG + ECRY3.1AB-0208.

Immunoaffinity-Purified Plant-Produced Protein

Leaf powder from 5307 maize, prepared as described above in **Maize Leaf Tissue and Negative Control Maize Leaf Tissue**, was resuspended in extraction buffer (pH 7.5) containing 100 mM sodium borate, (Sigma Cat. No. S9640), 0.2% PVP (Sigma-Aldrich Cat. No. PVP360), 7.69 mM sodium azide (Sigma-Aldrich Cat. No. S2002), 1.2% concentrated hydrochloric acid (Fisher Cat. No. A508), 0.5% Tween 20 (Sigma-Aldrich Cat. No. P1379), supplemented with 1 Complete Protease Inhibitor Cocktail tablet/50ml of buffer (Roche Cat. No. 11836145001). The mixture was homogenized with an Omni-prep and incubated for up to 2 hours on ice. The mixture was then centrifuged at approximately 2700 rpm for 10 minutes at 4°C (Sorvall Legend RT). The supernatant was filtered through cheesecloth and centrifuged at approximately 3100 rpm for 15 minutes at 4°C (Sorvall Legend RT). After a second centrifugation step (10,000 rpm for 12 minutes (Sorvall RC5B) the clarified supernatant was then loaded onto an equilibrated immunoaffinity column with mouse anti-mCry3A antibodies bound to the matrix. To remove any proteins not bound to the antibodies, the column was washed with a 50 mM sodium bicarbonate buffer pH 8.0 containing 150 mM sodium chloride. After an additional wash step with a 10 mM sodium phosphate buffer pH 6.8, eCry3.1Ab was eluted in 100 mM glycine buffer (pH 2.5), neutralized, and fractions were analyzed for eCry3.1Ab protein by ELISA. Fractions containing eCry3.1Ab protein were pooled, concentrated by ultrafiltration, and stored at 2°C to 8°C until further use. The resulting sample, designated IAP5307, was used as the source of purified plant produced eCry3.1Ab for Western blot, glycosylation, N-terminal sequencing and peptide mass mapping analysis.

Microbially Produced Test Substance ECRY3.1AB-0208

Test substance ECRY3.1AB-0208 was prepared from an *E. coli* overexpression system. The eCry3.1Ab protein 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* overexpression system. The modified *ecry3.1Ab* gene used for the microbial expression was linked to the bacterial *tac* promoter in a vector derived from pET24a (Novagen Cat. No. 69749-3) and transformed into *E. coli* strain DH5α (New England Biolabs Cat. No. C2988).

Prior to this study, 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) as described by Thompson (2008). 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. Test substance ECRY3.1AB-0208 was characterized in detail in a previous study and was determined to contain 89.6% eCry3.1Ab by weight and the intact mass of the eCry3.1Ab protein, as measured by mass spectrometry, was determined to be 74.8 kDa (Nelson 2008).

eCry3.1Ab Quantification

The Beacon Analytical Systems (BAS) eCry3.1Ab ELISA kit is a double-antibody sandwich assay. The eCry3.1Ab protein is captured in the wells of a microtiter plate using a monoclonal anti-mCry3A antibody that binds to the mCry3A domains of the eCry3.1Ab protein. A secondary, rabbit polyclonal anti-Cry1Ab antibody is then used to bind the Cry1Ab domain of the eCry3.1Ab protein. Detection of the eCry3.1Ab protein is accomplished through binding of a polyclonal anti-rabbit antibody conjugated with alkaline phosphatase enzyme, which catalyzes the conversion of the colorimetric substrate, *p*-nitrophenylphosphate (pNPP). eCry3.1Ab concentration is proportional to absorbance values. Samples are quantified relative to a standard curve of known eCry3.1Ab concentrations.

The BAS ELISA kit contains the antibody-coated plate, the secondary antibody solution and the substrate solution. The ELISA diluent, wash buffer, IgG enzyme conjugate and stop solution are not part of the BAS ELISA kit. Both the materials in Table 1 and Table 2 are needed to conduct an ELISA.

Table 1. Materials supplied in the ELISA kit

Kit Component	Description
Antibody-coated plate	96-well microtiter plate pre-coated with monoclonal anti-mCry3A antibody
Secondary antibody solution	Ready-to-use solution containing rabbit polyclonal anti-Cry1Ab antibody
Substrate Solution	Ready-to-use solution containing pNPP substrate

Table 2. Required materials not supplied in the ELISA kit

Component	Description
ELISA Diluent	Phosphate buffered saline, 1% BSA, 0.02% sodium azide, 0.05% Tween 20
Wash Buffer	10mM Tris, 0.02% sodium azide, 0.05% Tween 20
Alkaline Phosphatase-conjugated Donkey Anti-Rabbit IgG	Jackson ImmunoResearch Laboratories, Inc., Cat. No. 711-055-152
Stop Solution	3N sodium hydroxide

The eCry3.1Ab standard, derived from test substance ECRY3.1AB-0208, was serially diluted in ELISA diluent to generate a standard curve from 0.63 ng/ml–80 ng/ml eCry3.1Ab. Samples were diluted with ELISA diluent to generate concentrations of eCry3.1Ab that were within the accurate range of the standard curve. All samples and standard curve dilutions were assayed in triplicate (at a minimum) using the 96 well plates provided in the BAS eCry3.1Ab ELISA kits and applied to the pre-coated plates at a total volume of 100 µl/well. The plates were incubated at room temperature on a titer plate shaker at 400 rpm for 1 hour. The plates were washed five times with wash buffer using a BioTek ELx405 Microplate Washer. After washing the plates, the secondary, rabbit polyclonal anti-Cry1Ab antibody (provided in the kit) was then added to the plate at 100 µl/well. The plates were incubated at room temperature on a titer plate shaker at 400 rpm for 1 hour and washed five times as described above.

After the plates were washed, a tertiary donkey anti-rabbit antibody conjugated with alkaline phosphatase diluted to 0.5 µg/ml in diluent was added to each of the wells (100 µl/well) and incubated at room temperature on a titer plate shaker at 400 rpm for 1 hour. The plates were then washed five times as described above, and alkaline phosphatase substrate solution provided in the kit was added at a volume of 100 µl/well. The plates were incubated for 30 minutes at room temperature on a titer plate shaker at 400 rpm. The reaction was stopped by the addition of 3N sodium hydroxide (100 µl per well), and absorbance of the wells at a dual wavelength 405-492 nm was measured with a Tecan Sunrise Microplate Reader. The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2, using a four-parameter algorithm.

Total Protein Determination

Total protein in samples, LP5307, LP-NEG + ECRY3.1AB-0208, and LP-NEG as prepared for Western blot analysis, was quantified via the bicinchoninic acid method

(Hill and Straka 1988), using BSA as the reference protein standard. The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2 using a 4-parameter fit of the standard curve.

Immunoreactivity and Molecular Weight Determination

Western blot analysis was used to investigate the integrity of eCry3.1Ab in ECRY3.1AB-0208, LP5307, LP-NEG + ECRY3.1AB-0208 and IAP5307. Aliquots containing 10 ng of eCry3.1Ab prepared in NuPage LDS Sample Buffer (Invitrogen Cat. No. NP0007) were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel (Invitrogen Cat. No. NP0335BOX) using MOPS running buffer (Bio-Rad Cat. No. 161-0788). An aliquot of the nontransgenic, near-isogenic plant sample LP-NEG, equivalent in total protein to the amount loaded on the gel for LP5307 (29.6 µg total protein), was included in the analysis as a negative control. The molecular-weight standard was SeeBlue Plus2 pre-stained standard (Invitrogen Cat. No. LC5925). After electroblotting, the membrane was probed with polyclonal goat antibodies capable of detecting eCry3.1Ab protein. Alkaline-phosphatase-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Cat. No. 705-055-147) diluted to 1:3,000 in Tris-buffered saline with Tween 20 (Sigma-Aldrich Cat. No. T9039) and 5% normal donkey serum was used to bind to the primary antibody and was visualized by development with BCIP/NBT alkaline phosphatase substrate solution (Sigma-Aldrich Cat. No. B1911). The Western blot was examined for the presence of intact immunoreactive eCry3.1Ab or immunoreactive eCry3.1Ab fragments.

Insecticidal Activity

The insecticidal activity of eCry3.1Ab was assessed in feeding assays with freshly hatched first-instar Colorado potato beetles *(L. decemlineata)* in three independent bioassays. The insect diet was prepared by blending a boiling mixture of 2.6 grams of agar and 169 ml of Milli-Q water with 28.1 grams of Colorado potato beetle diet powder mix and 1 gram of potassium hydroxide as per the manufacturer's instructions (Bio-Serv Cat. No. F9380B). The diet mixture was cooled to approximately 55°C in a water bath. To prevent bacterial and fungal growth, solutions of nystatin (Sigma Cat. No. N3503; 1.8 mg/ml), cefotaxime (Research Products International Cat. No. C51000; 1.8 mg/ml), aureomycin (Bio-Serve Cat. No. 7135; 1.8 mg/ml), streptomycin (Sigma Cat. No. S6501; 1.8 mg/ml) and preservative for plant tissue culture media (Plant Cell Technology Cat. No. 100; 0.2%) were each added to the cooled diet.

Bioassay treatments consisted of (1) microbially-produced test substance; ECRY3.1AB-0208, (2) eCry3.1Ab extracted from leaf material of 5307 maize; LP5307, (3) nontransgenic near-isogenic leaf extract fortified with the microbially-produced test substance; LP-NEG + ECRY3.1AB-0208 and (4) nontransgenic, near-isogenic maize leaf tissue extract; LP-NEG.

To prepare treatment 1, a stock solution of ECRY3.1AB-0208 was prepared by resuspending ECRY3.1AB-0208 in 10mM ammonium bicarbonate buffer (pH 10.0) to a concentration of 5 mg eCry3.1Ab/ml. From the stock solution, a 50 µg eCry3.1Ab/ml solution was prepared and serially diluted 1:1 (v/v) in 10 mM ammonium bicarbonate buffer (pH 10.0) to produce 8 solutions with concentrations ranging from 50 to 0.390 µg eCry3.1Ab/ml. The dilution series was then mixed 1:1 (v/v) with the freshly prepared Colorado potato beetle diet to produce 8 diets with concentrations of active ingredient ranging from 25 to 0.195 µg eCry3.1Ab/ml diet.

Additional treatments containing eCry3.1Ab extracted from leaf material of 5307 maize LP5307 (treatment 2), nontransgenic near-isogenic leaf extract fortified with the microbially-produced test substance LP-NEG + ECRY3.1AB-0208 (treatment 3) and nontransgenic, near-isogenic maize leaf tissue extract LP-NEG (treatment 4) as prepared for bioassays were also serially diluted 1:1 (v/v) in 10 mM ammonium bicarbonate buffer (pH 10.0) and subsequently mixed 1:1 (v/v) with freshly prepared Colorado potato beetle diet. Each of these treatments was analyzed as a series of 8 dilutions. Water and buffer controls were prepared in the same fashion for each bioassay. The water control was prepared by mixing 1:1 (v/v) of purified water with freshly prepared Colorado potato beetle diet. The buffer control was prepared by mixing 1:1 (v/v) of 10mM ammonium bicarbonate buffer (pH 10.0) with freshly prepared Colorado potato beetle diet.

The bioassays were conducted in 24-well Costar culture plates (Corning Cat. No. 3524). Each well contained one freshly hatched *L. decemlineata* insect larva and 100 µl of insect diet. Larvae were transferred to each well manually using a small paint brush. The wells were covered with silicone stoppers and stored at ambient laboratory conditions. Mortality readings were taken periodically starting at 72 hours and continued until at least 144 hours.

Glycosylation Analysis

To determine whether eCry3.1Ab in the microbially-produced test substance (ECRY3.1AB-0208) and eCry3.1Ab purified from extract of leaf material from 5307 maize (IAP5307) were glycosylated, aliquots equivalent to 1 and 2 µg of eCry3.1Ab were analyzed with the DIG Glycan Detection Kit (Roche Cat. No. 11142372001), in accordance with the manufacturer's instructions. The positive control was transferrin (a glycosylated protein) at 100, 50, 25 and 10 ng, and the negative control was creatinase (a nonglycosylated protein) at 2 µg. Samples were separated by SDS-PAGE with a NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel and NuPAGE MES SDS running buffer and electroblotted to nitrocellulose membrane (Invitrogen Cat. No. LC2009). While on the membrane, glycan moieties were oxidized with periodate, labeled with digoxigenin (DIG), and detected with an alkaline-phosphatase-linked anti-DIG antibody.

Peptide Mass Mapping Analysis

eCry3.1Ab purified from an extract of leaf material from 5307 maize (IAP5307) and from microbially-produced test substance (ECRY3.1AB-0208) were sent to Syngenta Analytical Sciences (Jealott's Hill International Research Centre, Bracknell, UK) for peptide mass mapping analysis (Green 2009). Aliquots containing 2.5 to 5 µg of eCry3.1Ab purified from extract of leaf material from 5307 maize (IAP5307) and from the microbially-produced test substance (ECRY3.1AB-0208) were subjected to SDS-PAGE using a NuPAGE 4-12% Bis-Tris polyacrylamide gradient gel and NuPAGE MES SDS running buffer. The gel was stained with Coomassie G250 (Invitrogen LC6065), the protein band corresponding to the molecular weight of eCry3.1Ab was excised from the gel, and the protein was reduced, alkylated with iodoacetamide, and independently digested with trypsin and chymotrypsin. The mass analysis of the eCry3.1Ab-produced peptides was performed using a quadrupole time-of-flight mass spectrometer (Waters/Micromass Q-TOF Premier) connected to a Waters CapLC capillary liquid chromatography instrument. The detected peptide masses were searched using Mascot Software (Matrix Science) against a protein database containing the eCry3.1Ab protein sequence. The Mascot search parameters included likely N-terminal modifications, which have previously been reported to occur in plants. Specifically, the modifications investigated included α-N-acetylation, protein N- formylation and protein N-methylation.

N-Terminal Amino Acid Sequence Analysis

To determine the N-terminal amino acid sequence of eCry3.1Ab from the microbially-produced test substance (ECRY3.1AB-0208) and eCry3.1Ab purified from extract of leaf material from 5307 maize (IAP5307) both were subjected to SDS-PAGE followed by electroblotting to a PVDF membrane. The blot was stained with amido black, and the band corresponding to eCry3.1Ab was excised and sent to Proseq, Inc., Protein Sequencing Services (Boxford, MA, USA) for N-terminal amino acid sequence analysis. ProSeq's methodology was developed specifically for proteins immobilized on PVDF membrane and optimized for automated Edman-based chemistry (Brauer et al. 1984).

Statistical Methods

The LC_{50} values determined in the insecticidal activity assay were calculated with the U.S. EPA Probit Analysis Program, version 1.5. Statistical analysis was not required for any other 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.

Immunoreactivity and Molecular Weight

Western blot analysis of eCry3.1Ab in all preparations: ECRY3.1AB-0208, LP-NEG + ECRY3.1AB-0208, LP5307, and IAP5307 revealed immunoreactive bands consistent with the predicted molecular weight[1]. A molecular weight of 74.8 kDa for samples containing eCry3.1Ab from ECRY3.1AB-0208 (Figure 1, lanes 2, 3 and 6) and 73.7 kDa for samples containing plant produced eCry3.1Ab. (Figure 1, lanes 4 and 5) was confirmed as predicted. The slight difference in molecular weight between the eCry3.1Ab protein in the microbially-produced test substance (ECRY3.1AB-0208) and the eCry3.1Ab in the plant produced samples (LP5307 and IAP5307) is expected due to the additional seven amino acids, one methionine and 6 histidines residues, added to the N-terminus of the microbially-produced protein. The additional amino acids (histidine tag) facilitate purification of the recombinant protein after microbial production. As expected, no immunoreactive bands were observed in the nontransgenic, near-isogenic maize leaf tissue extract LP-NEG (Figure 1, lane 7).

[1] Although the eCry3.1Ab protein band showed slightly higher mobility (and therefore an apparent lower molecular weight) in comparison to the molecular weight standards on the Western blot (Figure 1), the difference between the expected and observed molecular weights on the gels can be explained by the limitations of SDS-PAGE for accurate determination of molecular weight. Dube and Flynn (1988) have reviewed the reliability of SDS-PAGE for molecular weight determinations and concluded that the apparent molecular weight of a protein by this method is typically within 10% of its true molecular weight. This depends greatly on the similarity between the properties of the protein of interest and the proteins in the standard set (Sadeghi *et al*. 2003). Additionally, the intact mass of eCry3.1Ab in ECRY3.1AB-0208 was previously measured as 74.8 kDa (Nelson 2008).

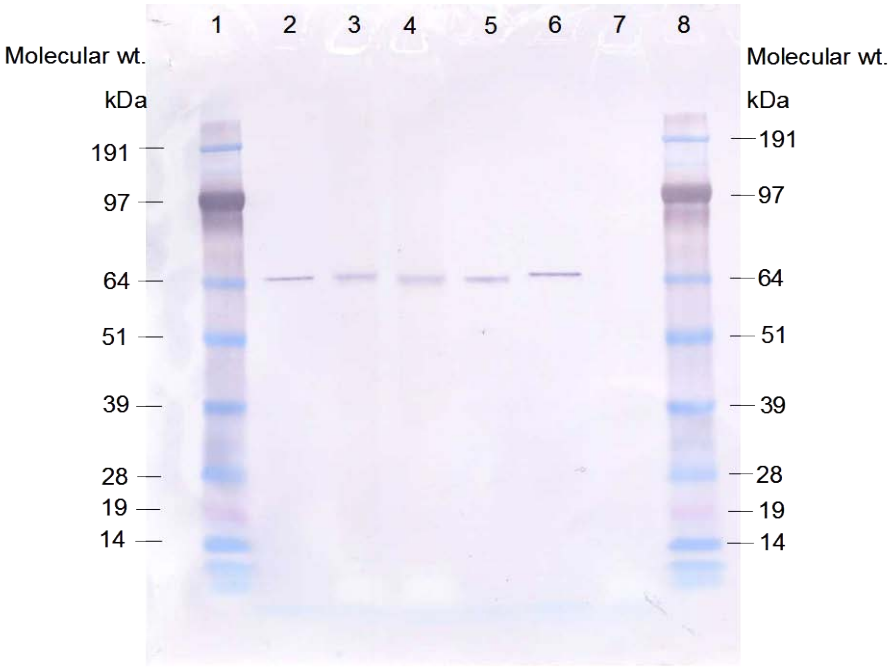

Figure 1. Western blot analysis of eCry3.1Ab in ECRY3.1AB-0208, LP-NEG + ECRY3.1AB-0208, LP5307, IAP5307 and the negative control LP-NEG

Lane 1: Molecular-weight markers.
Lane 2: Microbially-produced test substance ECRY3.1AB-0208 (10 ng eCry3.1Ab).
Lane 3: Nontransgenic, near-isogenic maize leaf tissue extract, LP-NEG (29.6 µg total protein) fortified with microbially-produced test substance ECRY3.1AB-0208 (10 ng eCry3.1Ab).
Lane 4: eCry3.1Ab extracted from leaf material of 5307 maize, LP5307 (10 ng eCry3.1Ab).
Lane 5: eCry3.1Ab purified from extract of leaf material from 5307 maize, IAP5307 (10 ng eCry3.1Ab).
Lane 6: Microbially-produced test substance ECRY3.1AB-0208 (10 ng eCry3.1Ab).
Lane 7: Nontransgenic, near-isogenic maize leaf tissue extract LP-NEG
Lane 8: Molecular-weight markers.

Insecticidal Activity

eCry3.1Ab extracted from leaf material of 5307 maize (LP5307) and microbially-produced eCry3.1Ab in the presence of nontransgenic, near-isogenic leaf extract (LP-NEG + ECRY3.1AB-0208) showed LC50 values in very comparable ranges from 1.316-2.888 and 1.113-3.226 µg eCry3.1Ab/ml, respectively, as shown in three independent bioassays (Table 3 and 4). The fortification of nontransgenic, near-isogenic leaf extract with microbially-produced produced eCry3.1Ab allowed for a comparison of the microbially-produced eCry3.1Ab and plant produced eCry3.1Ab in the same matrix.

Table 3. Insecticidal activity of eCry3.1Ab in LP5307 (plant produced) and ECRY3.1AB-0208 (microbially-produced) in the presence of nontransgenic, near-isogenic leaf extract (LP-NEG + ECRY3.1AB-0208) against Colorado potato beetle larvae in three independent bioassays

Bioassay #1							
LP5307				LP-NEG + ECRY3.1AB-0208			
µg eCry3.1Ab/ml	Mortality[2] (%)	CPB Exposed	CPB Responding	µg eCry3.1Ab/ml	Mortality (%)	CPB Exposed	CPB Responding
10	83	24	20	25	96	24	23
5	58	24	14	12.5	71	24	17
2.5	54	24	13	6.25	67	24	16
1.25	54	24	13	3.125	50	24	12
0.625	33	24	8	1.5625	42	24	10
0.3125	33	24	8	0.78125	38	24	9
0.15625	29	24	7	0.390625	25	24	6
0.078125	33	24	8	0.1953125	42	24	10
LP-NEG control	3	192	5	144 hour reading			

[2] Mortality(%) is calculated by dividing the number of CPB responding by the number of CPB exposed and multiplying by 100.

Bioassay #2*							
LP5307				LP-NEG + ECRY3.1AB-0208			
µg eCry3.1Ab/ml	Mortality (%)	CPB Exposed	CPB Responding	µg eCry3.1Ab/ml	Mortality (%)	CPB Exposed	CPB Responding
11.025	83	24	20	12.5	79	24	19
5.5125	79	24	19	6.25	63	24	15
2.75625	38	24	9	3.125	42	24	10
1.378125	50	24	12	1.5625	63	24	15
0.6890625	33	24	8	0.78125	46	24	11
0.34453125	33	24	8	0.390625	54	24	13
0.172265625	33	24	8	0.1953125	38	24	9
LP-NEG control	5	168	8	144 hour reading			

*LC_{50} calculated with only 7 dilutions. High mortality seen in the 1:2 dilution of LP-NEG. Therefore the 1:2 dilution of all samples containing leaf extract matrix were eliminated from analysis.

Bioassay #3							
LP5307				LP-NEG + ECRY3.1AB-0208			
µg eCry3.1Ab/ml	**Mortality (%)**	**CPB Exposed**	**CPB Responding**	**µg eCry3.1Ab/ml**	**Mortality (%)**	**CPB Exposed**	**CPB Responding**
19.4	96	24	23	25	92	24	22
9.7	71	24	17	12.5	83	24	20
4.85	50	24	12	6.25	71	24	17
2.425	58	24	14	3.125	42	24	10
1.2125	25	24	6	1.5625	33	24	8
0.60625	46	24	11	0.78125	21	24	5
0.303125	21	24	5	0.390625	29	24	7
0.1515625	8	24	2	0.1953125	29	24	7
LP-NEG control	9	192	17	144 hour reading			

Table 4. LC50 and 95% confidence intervals for LP5307 and LP-NEG + ECRY3.1AB-0208

µg eCry3.1Ab/ml				
144 hour reading	LP5307		LP-NEG + ECRY3.1AB-0208	
Bioassay	LC_{50}	95% CI	LC_{50}	95% CI
#1	1.316	0.631-3.187	1.780	0.924-3.19
#2	1.669	0.875-3.279	1.113	0.113-4.001
#3	2.888	1.765-4.655	3.226	1.955-5.134

For each of the 3 independent bioassays, the microbially-produced eCry3.1Ab (ECRY3.1AB-0208) was analyzed alongside the other treatments. The microbially-produced eCry3.1Ab showed a weaker response in the insecticidal bioassays without the combination of the plant extract (LC50 value of 6.264 µg eCry3.1Ab/ml 95% CI=4.257 to 10.262 µg/ml, Table 5). This effect is most likely related to the plant matrix. The plant matrix could be serving as a feeding stimulant as the insect larvae developed much faster in the presence of the plant extract.

Table 5. Mean insecticidal activity of eCry3.1Ab as contained in ECRY3.1AB-0208 (microbially-produced) against Colorado potato beetle larvae

ECRY3.1AB-0208			
µg eCry3.1Ab/ml	**Mortality (%)**	**CPB Exposed**	**CPB Responding**
25	71	72	51
12.5	60	72	43
6.25	44	72	32
3.125	42	72	30
1.5625	28	72	20
0.78125	24	72	17
0.390625	29	72	21
0.1953125	15	72	11
Buffer control	0	72	0
144 hour reading			
LC_{50} = 6.264 µg eCry3.1Ab/ml		95% CI = 4.257-10.262 µg eCry3.1Ab/ml	

Combined mortality over the 3 independent bioassays calculated to determine one LC50 value for ECRY3.1AB-0208.

Glycosylation Analysis

The positive control protein transferrin at 10 ng generated a clearly visible band (Figure 2, lane 4). Transferrin has a molecular weight of approximately 80,000 Da and contains approximately 5% glycan moieties by weight. This corresponds to approximately 25 glucose equivalents per molecule (based on a calculated molecular weight of 162 Da for the glycan moiety). Of the 10 ng of transferrin loaded on the gel, 0.5 ng could be attributed to glycan moieties and was clearly detectable. The highest concentration of eCry3.1Ab from both plant and microbial sources (IAP5307 and ECRY3.1AB-0208) loaded on the blot was 2 µg (2,000 ng). If 0.5 ng of glycan were detected in eCry3.1Ab, this would correspond to 0.025% by weight (0.5/2,000 ng), or 0.115 glucose equivalents per molecule. In other words, if eCry3.1Ab bands were stained as strongly as 10 ng of transferrin in lane 4, this would indicate glycosylation of about 1 in 8.7 of the eCry3.1Ab molecules. No bands corresponding to glycosylated eCry3.1Ab were visible for the sample prepared from the microbially-produced ECRY3.1AB-0208 (Figure 2, lanes 9 and 10) or the immunopurified plant produced eCry3.1Ab protein IAP5307 (Figure 2, lanes 7 and 8). Therefore, the results indicate that neither the microbially-produced nor the plant produced eCry3.1Ab protein was glycosylated.

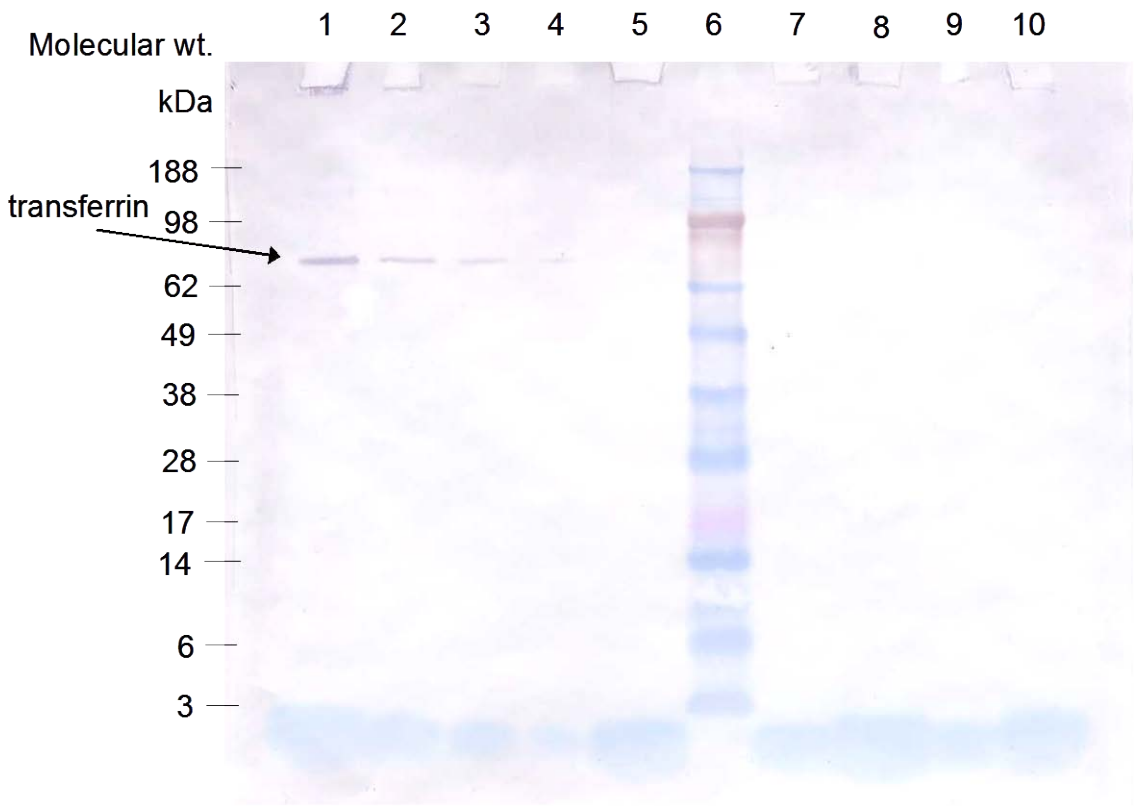

Figure 2. Glycosylation analysis of eCry3.1Ab in IAP5307 (immunopurified plant produced protein) and ECRY3.1AB-0208 (microbially-produced test substance)

Lane 1: Transferrin (positive control), 100 ng
Lane 2: Transferrin (positive control), 50 ng
Lane 3: Transferrin (positive control), 25 ng
Lane 4: Transferrin (positive control), 10 ng
Lane 5: Creatinase (negative control), 2 µg.
Lane 6: Molecular-weight markers.
Lane 7: eCry3.1Ab purified from extract of 5307 maize leaf tissue, IAP5307; 1 µg
Lane 8: eCry3.1Ab purified from extract of 5307 maize leaf tissue, IAP5307; 2 µg
Lane 9: eCry3.1Ab from microbially-produced test substance ECRY3.1AB-0208; 1 µg
Lane 10: eCry3.1Ab from microbially-produced test substance ECRY3.1AB-0208; 2 µg.

Peptide Mass Mapping

Analysis of the eCry3.1Ab purified from extract of 5307 maize leaf tissue IAP5307 yielded coverage equivalent to 76% of the total predicted eCry3.1Ab amino acid sequence, as shown in Figure 3. Analysis of the microbially-produced eCry3.1Ab (ECRY3.1AB-0208) yielded coverage equivalent to 87% of the total predicted eCry3.1Ab amino acid sequence, as shown in Figure 4. The identified peptides corresponded to regions throughout the sequence of eCry3.1Ab including the N-termini of both proteins. The results of the peptide mass mapping analysis confirmed the identification of the purified proteins from both sources as eCry3.1Ab.

In addition, the peptide mass analysis provided two additional results regarding the structure of the proteins. Firstly the intact N-terminus of the plant expressed protein could be identified. The analysis showed that the N-terminal methionine was removed leaving the penultimate amino acid, threonine, at the N-terminus of the eCry3.1Ab protein. This is a common process for many proteins occurring during translation (Buchanan 2000 and Walling 2006).

Secondly, the nature of the N-terminal block found for the plant produced protein as described under **N-Terminal Amino Acid Sequence Analysis** was identified. The analysis of the N-terminal peptide of the plant produced protein suggested the addition of an acetyl-residue at the primary aminogoup of the N-terminal threonine; a common modification known for plant expressed proteins (Martinez 2008).

M**TSNGRQCAGIRPY**DGRQQHR**GLDSSTTKDVIQK**GISVVGDLLGVVGFPFGGALVSFYT
NFLNTIWPSEDPW**KAFMEQVEALMDQK**IADYAKNKALAELQGLQNNVEDYVSALSSWQK
NPAAPFRNPHSQGRIRELFSQAESHFRNSMPSFAISGYEVLFLTTYAQAANTHLFLLKD
AQIYGEEWGYEKEDIAEFYKRQLK**LTQEYTDHCVKWYNVGLDKLRGSSYESWVNFNR**YR
R**EMTLTVLDLIALFPLYDVRLYPKEVKTELTRDVLTDPIVGVNNLRGYGTTFSNIENYI**
RKPHLFDYLHRIQF**HTRFQPGYY**GNDSFNYWSGNY**VSTRPSIGSNDIITSPFYGNKSSE**
PVQNLEFNGEKVY**RAVANTNLAVWPSAVYSGVTKVEFSQYNDQTDEASTQTYDSKR**NVG
AVSWDSIDQLPPETTDEPLEK**GYSHQLNYVMCFLMQGSRGTIPVLTWTHKSVDFFNMID**
SKKITQLPLTKSTNLGSGTSVVKGPGFTGGDILRR**TSPGQISTLRVNITAPLSQR**YRVR
IR**YASTTNLQFHTSIDGRPINQGNFSATMSSGSNLQSGSFR**TVGFTTPFNFSNGSSVF**T**
LSAHVFNSGNEVYIDRIEF**VPAEVTF**EAEY**DLERAQKAVNELFTSSNQIGLKTDVTDYH**
IDQV

Figure 3. Predicted amino acid sequence of eCry3.1Ab and sequence identified by peptide mass mapping analysis of eCry3.1Ab purified from extract of leaf material from 5307 maize IAP5307

Identified eCry3.1Ab protein fragments are bold and underlined.

MHHHHHHMTSNGRQCAGIRPYDGRQQHR**GLDSSTTKDVIQK**GISVVGDLLGVVGFPFGG
ALVSFYTNFLNTIWPSEDPW**KAFMEQVEALMDQK**IADY**AKNKALAELQGLQNNVEDYVS**
ALSSWQKNPAAPFRNPHSQGRIRELFSQAESHFRNSMPSFAISGYEVLFLTTYAQAANT
HLFLLKDAQIYGEEWGYEKEDIAEFYKRQLK**LTQEYTDHCVKWYNVGLDKLRGSSYESW**
VNFNRYRR**EMTLTVLDLIALFPLYDVRLYPKEVKTELTRDVLTDPIVGVNNLRGYGTTF**
SNIENYIRKPHLFDYLHRIQFHTRFQPGYYGNDSFNYWSGNY**VSTRPSIGSNDIITSPF**
YGNKSSEPVQNLEFNGEKVYR**AVANTNLAVWPSAVYSGVTKVEFSQYNDQTDEASTQTY**
DSKRNVGAVSWDSIDQLPPETTDEPLEKGYSHQLNYVMCFLMQGSRGTIPVLTWTHKSV
DFFNMIDSKKITQLPLTKSTNLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPL
SQRYRVRIR**YASTTNLQFHTSIDGRPINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFS**
NGSSVFTLSAHVFNSGNEVYIDRIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLK
TDVTDYHIDQV

Figure 4. Predicted amino acid sequence of eCry3.1Ab and sequence identified by peptide mass mapping analysis of eCry3.1Ab from microbially-produced test substance ECRY3.1AB-0208

Identified eCry3.1Ab protein fragments are bold and underlined.

N-Terminal Amino Acid Sequence Analysis

The N-terminal sequence results confirmed that eCry3.1Ab in the microbially-produced test substance (ECRY3.1AB-0208) had the predicted N-terminal amino acid sequence:

Predicted sequence: MHHHHHHMTS

eCry3.1Ab in ECRY3.1AB-0208: MHHHHHHMTS

N-terminal sequencing analysis of eCry3.1Ab purified from extract of leaf material from 5307 maize (IAP5307) revealed that the majority of the protein was naturally N-terminally blocked. However, the N-terminal peptide was identified by peptide mass mapping and confirmed the expected sequence for the eCry3.1Ab protein starting at threonine as described under **Peptide Mass Mapping**.

CONCLUSIONS

eCry3.1Ab from 5307 maize (LP5307 and IAP5307) and produced from a recombinant *E. coli* overexpression system (ECRY3.1AB-0208) had apparent molecular weights consistent with the predicted molecular weights of 73.7 and 74.8 kDa respectively and immunologically cross-reacted with antibodies capable of detecting the eCry3.1Ab protein. Both the plant produced and microbially-produced eCry3.1Ab showed insecticidal activity against Colorado potato beetle larvae. Plant produced eCry3.1Ab and microbially-produced eCry3.1Ab in the presence of nontransgenic, near-isogenic leaf

extract, had comparable LC50 values. There was no evidence of post-translational glycosylation of eCry3.1Ab purified from extract of leaf material from 5307 maize or from the microbially-produced test substance. Peptide mass mapping analysis provided confirmation of eCry3.1Ab as the insecticidal protein in 5307 maize and in the microbially-produced test substance. These results support the conclusion that eCry3.1Ab produced in recombinant *E. coli* (test substance ECRY3.1AB-0208) is biochemically and functionally equivalent to the eCry3.1Ab produced in 5307 maize. The eCry3.1Ab produced in recombinant *E. coli* (test substance ECRY3.1AB-0208) can therefore serve as a suitable surrogate for eCry3.1Ab produced in 5307 maize.

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 Laura Seastrum, B.S. and was conducted at Syngenta Biotechnology, Inc., except for the peptide mass mapping analysis, which was conducted by Syngenta Analytical Sciences, Jealott's Hill, Bracknell, Berkshire, UK. and the N-terminal amino acid sequence analysis, which was conducted by Proseq, Inc., Boxford, MA, USA.

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CRITICAL DATES

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Experimental start date:	October 7, 2008
Experimental end date:	January 23, 2009

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