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Abbreviations and Definitions[1]

AA	Amino acid
APS	Analytical Protein Standard
BSA	Bovine serum albumin
B.t.	*Bacillus thuringiensis*
CEW	Corn earworm
COA	Certificate of Analysis
CTP	Chloroplast transit peptide
DTT	Dithiothreitol
EC_{50}	Effective protein concentration to inhibit the growth of the target insect by 50%
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenediaminotetraacetic acid
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
GLP	Good Laboratory Practice
HRP	Horseradish peroxidase
IUPAC-IUB	International Union of Pure and Applied Chemistry - International Union of Biochemistry
MALDI-TOF	Matrix assisted laser desorption and ionization - time of flight
mAb	Monoclonal antibody
MH+	Protonated mass ion
MS	Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut-off
N/A	Not applicable
NIST	National Institute of Standards and Technology
NFDM	Non-fat dried milk
NMWC	Nominal molecular weight cut-off
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.05% (v/v) Tween-20
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene difluoride
PVPP	Polyvinylpolypyrrolidone
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard operating procedure
Tween-20	Polyoxyethylenesorbitan monolaurate
US	United States

[1] Standard abbreviations, e.g. units of measure, concentration, mass, time etc., are used without definition according to the format described in "Instructions to Authors" in The Journal of Biological Chemistry.

1.0 Summary

Monsanto has developed corn, MON 89034, that produces Cry2Ab2 insecticidal protein and is protected from feeding damage caused by European corn borer (*Ostrinia nubilalis*) and other lepidopteran insect pests. Cry2Ab2 is a *Bacillus thuringiensis* (subsp. *kurstaki*) protein.

This report provides a detailed description of the physicochemical and functional properties of the Cry2Ab2 protein isolated from grain of MON 89034 and demonstrates the equivalence of the biochemical properties of the plant-produced protein to the previously characterized *E. coli*-produced Cry2Ab2 protein.

A panel of analytical techniques was used to characterize the plant-produced Cry2Ab2 protein. These analytical techniques were: 1) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and densitometry, 2) total protein concentration by BioRad protein assay, 3) western blot analysis, 4) intact mass and tryptic mass map analysis using matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, 5) N-terminal sequencing, 6) glycosylation analysis, and 7) a corn earworm (*Helicoverpa zea*, CEW) diet-incorporation insect bioassay. The stability of the plant-produced Cry2Ab2 protein was also assessed (using SDS-PAGE and densitometry) by estimating the purity and molecular weight of the full length Cry2Ab2 protein present after storage at different temperatures.

Purity of the plant-produced Cry2Ab2 protein was determined using densitometric analysis of a Colloidal Brilliant Blue G stained SDS polyacrylamide gel and was estimated to be 33%. The molecular weight of the full-length plant-produced Cry2Ab2 protein was estimated by SDS-PAGE analysis to be 61.3 kDa. A lower molecular weight fragment of the Cry2Ab2 protein was also identified and its estimated purity and molecular weight were 13% and 49.6 kDa, respectively. The mass of the plant-produced Cry2Ab2 protein was unable to be determined by MALDI-TOF mass spectrometry. The functional activity of the plant-produced Cry2Ab2 protein was determined using an insect bioassay with the larvae of a susceptible pest, CEW. The mean EC_{50} value of the plant-produced Cry2Ab2 protein was 0.16 µg/mL of diet, which was identical to the functional activity of the *E. coli*-produced Cry2Ab2 protein that was analyzed concurrently. The identity of the plant-produced protein was confirmed using western blot analysis and MALDI-TOF mass spectrometry. On the basis of western blot analysis, the electrophoretic mobility and immunoreactive properties of the plant-produced Cry2Ab2 protein were demonstrated to be comparable to those of the *E. coli*-produced Cry2Ab2 reference standard. MALDI-TOF mass spectral analysis of trypsinized plant-produced protein yielded peptide masses consistent with the masses predicted for the Cry2Ab2 protein. Approximately 47.7% of the expected 637 amino acid sequence comprising the *in planta* Cry2Ab2 protein was identified using MALDI-TOF mass spectrometry. As

expected, the N-terminus of the full length Cry2Ab2 protein was blocked and thus no N-terminal sequence was obtained. However, the presence of a heterogeneous population of Cry2Ab2 protein sequences yielded N-terminal sequences starting at the 24th and the 145th positions. The plant-produced Cry2Ab2 protein was not glycosylated. The 32-day storage stability of the plant-produced Cry2Ab2 protein was confirmed using SDS-PAGE and densitometry by estimating the purity and molecular weight of the full length Cry2Ab2 protein after storage in a 4 °C refrigerator, and –20 °C and –80 °C freezers.

The equivalence of the plant- and *E.* coli-produced Cry2Ab2 proteins was evaluated by comparing physicochemical and functional properties including electrophoretic mobility on an SDS polyacrylamide gel, immunoreactivity with anti-Cry2Ab2 antibodies, glycosylation status, and functional activity using pre-set acceptance criteria. Collectively, these data provided a detailed characterization of the Cry2Ab2 protein isolated from grain of MON 89034 and demonstrated the comparability of the plant-produced Cry2Ab2 protein to the *E. coli*-produced Cry2Ab2 protein.

2.0 Introduction

Monsanto has developed corn, MON 89034, that produces Cry2Ab2 insecticidal protein and is protected from feeding damage caused by European corn borer (*Ostrinia nubilalis*) and other lepidopteran insect pests. Cry2Ab2 is a *Bacillus thuringiensis* (subsp. *kurstaki*) protein. The nucleotide sequence of the *cry2Ab2* gene in MON 89034 was confirmed by molecular characterization (Rice *et al.*, draft).

Expression of the Cry2Ab2 protein in MON 89034 is targeted to the chloroplast, using a chloroplast transit peptide (CTP) that has a potential cleavage site (methionine) three amino acids upstream from the start of the Cry2Ab2 protein. Attempts to confirm the N-terminal sequence of the Cry2Ab2 protein *in planta* indicate that it is blocked and the cleavage site in the chloroplast cannot be determined. Therefore, the three additional amino acids from the CTP were incorporated at the N-terminus of the *E. coli*-produced Cry2Ab2 protein, which resulted in the production of a Cry2Ab2 protein of 637 amino acids with a calculated molecular weight of approximately 71.2 kDa.

Because the expression level of Cry2Ab2 protein in the grain of MON 89034 is low, it was necessary to express the protein in bacteria in order to produce sufficient quantities for safety assessment studies. Consequently, select analyses to confirm comparability between the plant-produced and *E. coli*-produced proteins were conducted.

3.0 Purpose

The purpose of this plan was to characterize the physicochemical and functional properties of the Cry2Ab2 protein produced in MON 89034, and to compare the physicochemical and functional properties of the plant-produced protein with the

previously characterized *E. coli*-produced Cry2Ab2 protein (APS lot 20-100071). This characterization was conducted in accordance with Monsanto's Biotechnology Regulatory Sciences SOPs BR-PO-0537-02 and BR-PO-0655-02. A list of applicable SOPs can be found in Appendix 1.

4.0 Materials

4.1 Description of the Test Substance

The plant-produced Cry2Ab2 protein was isolated from ground grain of MON 89034. The grain used for the isolation of Cry2Ab2 protein was LIMS number 04ZMGRO00393, produced under production plan 04-01-39-22 from seed lot number GLP-0404-14916-S. The identity of the grain sample containing MON 89034 was confirmed by event specific PCR. A copy of the certificate of analysis (COA) for grain identity confirmation is archived under lot 60-100075. The isolated plant-produced Cry2Ab2 protein was stored in a –80 °C freezer in a buffer solution containing 50 mM CAPS, 2 mM DTT, pH 11 at a total protein concentration of 0.25 mg/mL. Data supporting the extraction and isolation of Cry2Ab2 protein from MON 89034 grain conducted prior to the initiation of this plan are archived under APS lot 60-100075.

4.2 Description of the Reference Standard

The *E. coli*-produced Cry2Ab2 protein (APS lot 20-100071) was used as a reference standard in select analyses. These analyses included apparent molecular weight determination by SDS-PAGE, western blot analysis, glycosylation analysis, BioRad protein assay, and the functional activity assay. The *E. coli*-produced Cry2Ab2 protein reference standard has previously been characterized under APS lot 20-100071 and is referred to as "Cry2Ab2.820" on the Certificate of Analysis (Appendix 2).

4.3 Description of Assay Controls

Protein molecular weight standards were used to calibrate SDS polyacrylamide gels and verify protein transfer to PVDF membranes. The *E. coli*-produced Cry2Ab2 reference standard protein was used to estimate the total protein concentration in the BioRad protein assays and also used as the positive control in western blot analysis. Beta-lactoglobulin protein and PTH-amino acid standards were used to verify the performance of the amino acid sequencer. A peptide mixture and an analytical BSA standard were used to calibrate the MALDI-TOF mass spectrometer for tryptic mass analysis and molecular weight determination, respectively. Transferrin and *E. coli*-produced Cry2Ab2 protein were used as the positive and negative control, respectively, in glycosylation analysis.

5.0 Methods

5.1 Protein Purification

The plant-produced Cry2Ab2 protein was purified from the grain of MON 89034 prior to initiation of this characterization. Although the purification procedure was not performed under a GLP study or plan, procedures were documented on worksheets and, where applicable, SOPs were followed. The Cry2Ab2 protein was purified from an extract of ground grain of MON 89034 using a combination of ammonium sulfate fractionation, anion exchange chromatography, and immunoaffinity chromatography.

The isolation of Cry2Ab2 protein from ground corn grain was performed in two 10 kg batches. Prior to extraction of Cry2Ab2 protein, each batch was extracted with 1X PBS buffer (1 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) to remove contaminant proteins. The PBS extraction procedure consisted of soaking ground grain in PBS for 2 hrs in a 4 °C cold room at approximately 1:10 sample weight to buffer volume ratio. The slurry was clarified by filtration using an Ertel Alsop filter press (Kingston, NY), and the PBS washed solid particle (cake) was retained. Subsequently, the Cry2Ab2 protein in the cake was extracted with extraction buffer containing 50 mM CAPS, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, 0.5 mM CHAPS, 1% (w/v) PVPP, pH 10.8 at approximately 1:10 sample weight to buffer volume ratio for 2-3 hrs. During extraction, lipids were removed from the extract by adding CelPure P65 diatomaceous earth (Advanced Minerals Corp, Goleta, CA) to the homogenate at ~7.5% (w/v) and allowed to mix for ~10 minutes. The slurry was clarified by filtration using the filter press and the resultant extract from both batches was pooled for a total volume of ~230 L. The pooled extract was concentrated using a 30,000 NMWC Hollow Fiber Cartridge (Amersham Biosciences, Piscataway, NJ) to a final volume of ~35 L. To remove plant genomic DNA, polyethyleneimine, 10% (w/v), was added to the concentrated extract to a final concentration of 0.05% (w/v), the extract was clarified by centrifugation to remove precipitated DNA, and the supernatant was retained. The Cry2Ab2 protein in the supernatant was precipitated by 0% – 35% ammonium sulfate saturation. The 35% ammonium sulfate pellet was recovered by centrifugation and the pellet was dissolved in 20 L of resuspension buffer containing 50 mM CAPS, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, pH 10.8 by mixing in 4 °C cold room. The suspension was clarified by centrifugation and 21 L supernatant was retained, concentrated, and buffer exchanged by diafiltration against Buffer containing 50 mM CAPS, 1 mM EDTA, 2.5 mM DTT, 1 mM PMSF, 2 mM benzamidine-HCl, pH 10.8 to remove any residual ammonium sulfate salt. The concentrated sample of 13 L was loaded

onto an anion exchange column in two batches, Run 1 (6L) and Run 2 (7L). The elution parameters were identical for both runs and thus only the Run1 column parameters are described below.

A portion of concentrated sample was loaded onto a 4.5 L (20 cm x 14.4 cm column) Q Sepharose Fast Flow anion exchange resin column, which was equilibrated with Buffer. The bound Cry2Ab2 protein was eluted with step gradients as follows: 0-20% buffer B (Buffer containing 1M NaCl) in 10 column volumes (CV), and then the gradient was held at 20% buffer B for 4 CV, and then the gradient was increased to 65% buffer B over 10 CV and finally the gradient was stepped up to 100% buffer B and held at 100% buffer B in 2 CV. Fractions, each ~ 4 L, containing Cry2Ab2 protein were identified by Cry2A QuickStix™ (Portland, ME) for further analysis. Based on western blot analysis and SDS-PAGE analysis, fraction 7 from Run 2 was selected for affinity purification.

Subsequently, fraction 7 was concentrated to 400 mL using a 30,000 NMWC Hollow Fiber Cartridge. Approximately one half of the aforementioned sample was buffer exchanged into EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.2) using centrifuge concentrators (30 kDa MWCO), resulting in a final volume of 200 mL. This sample, in two separate batches, was applied to an affinity column (1.0 cm × 2.7 cm) containing Protein G agarose conjugated with Cry2Aa-specific mAb and equilibrated with EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.2). The sample was re-circulated through the column for 2 hrs at 100 mL/hr. The column was then washed with 17-20 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.2), followed by 5-7 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 8.5). The bound Cry2Ab2 protein was eluted with 100 mM Triethylamine, pH 11.4, in five 1.5 mL fractions. Based on SDS-PAGE analysis, fractions containing Cry2Ab2 protein from batch one and two were concentrated and buffer exchanged into a storage buffer containing 50 mM CAPS, 2 mM DTT, pH 11.0, using centrifuge concentrators (30 kDa MWCO). Concentrated samples were then pooled, resulting in a final volume of 2 mL.

The affinity chromatography procedure was repeated beginning with 100 mL of fraction 7 from Run2. As previously described, the sample was concentrated and buffer exchanged into EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.05) to a final volume of 50 mL using centrifuge concentrators (30 kDa MWCO). This sample was re-circulated for 2 hr (60 mL/hr) through an affinity column (1.0 cm × 2.75 cm) prepared with Cry2Aa-specific mAb equilibrated with EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.05). The column was then washed with 15 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 7.05) followed by 5 CV of EPPS buffer (50 mM EPPS, 2 mM DTT, pH 8.5). The bound Cry2Ab2 protein was eluted with 100 mM Triethylamine, pH 11.4, in five 1.5 mL fractions.

Based on SDS-PAGE analysis, Cry2Ab2 enriched fractions were individually concentrated and buffer exchanged into a storage buffer containing 50 mM CAPS, 2 mM DTT, pH 11, using a centrifuge concentrator (30 kDa MWCO). The concentrated samples were pooled into a final volume of 2.4 mL. The aforementioned sample and the sample from the first run (2 mL) were pooled resulting in a final volume of 4.4 mL. This sample was a clear colorless solution and was assigned lot # Cry2Ab.820_040705 and transferred into the APS program and assigned Lot 60-100075.

5.2 Total Protein Concentration by BioRad Assay

The total protein concentration of the purified plant-produced Cry2Ab2 protein was estimated using a BioRad protein assay. The *E. coli*-produced Cry2Ab2 reference standard protein (Lot 20-100071; concentrations ranging from 0.05 to 0.5 mg/mL) was used to prepare a standard curve. The plant-produced Cry2Ab2 total protein concentration was estimated by comparison of absorbance values obtained for the sample to the values of the standard curve. Data were collected using a Bio-Tek Instruments, Inc. Powerwave Xi microplate scanning spectrophotometer (Winooski, VT) employing KC4 software version 3.3 revision 10. Readings were taken at a wavelength of 595 nm.

5.3 Western blot Analysis

Equivalence would be demonstrated if the full-length plant-produced Cry2Ab2 protein was identified by the anti-Cry2Ab2 antibody and exhibited similar electrophoretic mobility compared to the *E. coli*-produced Cry2Ab2 reference standard. Aliquots of the stock solutions of the plant-produced Cry2Ab2 and reference standard were diluted to a final purity-corrected protein concentration of 2 ng/µL in dilution buffer (50 mM CAPS, 2 mM DTT, pH 11) and 5× sample loading buffer [5x concentrated Laemmli buffer (312 mM Tris-HCl, 20% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) bromophenol blue, 50% (v/v) glycerol, pH 6.8)]. Samples were then heated to ~100 °C for 5 min and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well gel. The plant-produced Cry2Ab2 protein was loaded in duplicate at three different loadings of 20, 30, and 40 ng per lane. The *E. coli*-produced Cry2Ab2 reference standard was loaded at 20 ng per lane. Electrophoresis was performed at a constant voltage of 140 V for 20 min followed by a constant voltage of 200 V for 43 min. Pre-stained molecular weight markers included during electrophoresis (BioRad Precision Plus Dual Color, Hercules, CA) were used to verify electrotransfer of protein to the membrane and to estimate the molecular weight of the immunoreactive bands. Samples were electrotransferred to a

0.45 micron PVDF membrane (Invitrogen, Carlsbad, CA) for 60 min at a constant current of 300 mA.

The membrane was blocked for one hour with 5% (w/v) NFDM in PBST. The membrane was probed with a 1:3000 dilution of goat anti-Cry2Ab2 antibody (lot 7227632) in 2% (w/v) NFDM in PBST for one hour. Excess antibody was removed using three 10 min washes with PBST. The membrane was probed with peroxidase-conjugated rabbit anti-goat IgG (Sigma, St. Louis, MO) at a dilution of 1:10,000 in 2% (w/v) NFDM in PBST for one hour. Excess peroxidase-conjugate was removed using three 10 min washes with PBST. All procedures, including blocking, and all other incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (Amersham Biosciences, Piscataway, NJ) and exposed (30 sec, 1 min, 2 min, 3 min, and 7 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor.

5.4 MALDI-TOF Tryptic Mass Map Analysis

MALDI-TOF mass spectrometry was used to confirm the identity of the plant-produced Cry2Ab2 protein. A protein can be typically identified when 40% of the mass fragments are identified from the analyzed protein (Jiménez *et al.*, 1998).

5.4.1 SDS-PAGE Separation of Proteins

An aliquot of the plant-produced Cry2Ab2 protein was diluted with 5× sample loading buffer to a final purity corrected protein concentration of 80 ng/µL and 2 µg was electrophoresed in each of five lanes. Broad Range molecular weight markers (BioRad, Hercules, CA) were used to estimate molecular weights. Samples were heated to ~99 °C for 5 min prior to electrophoresis on a pre-cast tris-glycine 4→20% SDS polyacrylamide gel at 140 V for 20 min followed by a constant voltage of 200 V for 46 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) glacial acetic acid for 30 min, stained 2 hr with Brilliant Blue G-Colloidal stain (Sigma, St. Louis, MO), destained 30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and followed by 25% (v/v) methanol for 1 hr. Two protein bands, band-1 and band-2, migrating at 61 kDa and 50 kDa, respectively, were identified for tryptic mass map analysis.

5.4.2 In-gel Protein Digestion

The plant-produced full length Cry2Ab2 protein, band-1 migrating at ~61 kDa, and band-2, a proteolytic fragment of the full length Cry2Ab2

protein migrating at ~50 kDa, were excised, destained, reduced, alkylated, and subjected to an in-gel trypsin (Promega, Madison, WI) digestion (Williams *et al.*, 1997). Each gel band was individually destained for 30 min by incubation in 100 µL of 40% (v/v) methanol and 10% (v/v) glacial acetic acid in its own microfuge tube. Following destaining, the gel bands were incubated in 100 µL of 100 mM ammonium bicarbonate buffer for 30 min at room temperature. Proteins were reduced in 100 µL of 10 mM dithiothreitol solution for 2 hrs at 37 °C. Proteins were then alkylated by the addition of 100 µL of buffer containing 200 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. The gel bands were incubated in 100 µL of 100 mM ammonium bicarbonate for 30 min at room temperature at which time 100 µL of acetonitrile was added and the incubation was continued for an additional 30 minutes. The ammonium bicarbonate/acetonitrile incubations were repeated two additional times to remove the reducing and alkylating reagents, and salts from the gel. The gel bands were dried in a SpeedVac concentrator (Savant, Holbrook, NY), rehydrated with 40 µL 25 mM ammonium bicarbonate containing 33 µg/mL trypsin, and the protein contained in the gel band was digested for 16 hours at 37 °C. Digested peptides were extracted for one hour at room temperature with 50 µL 70% (v/v) acetonitrile containing 0.1% (v/v) TFA per gel band. Supernatants for each extraction were combined into a single tube and dried in a SpeedVac concentrator. This process of extracting the peptides was repeated two more times. The final dried materials were reconstituted in 10 µL of 0.1% (v/v) TFA.

5.4.3 *Sample Preparation*

A portion (5 µl) of the digested samples was desalted (Bagshaw *et al.*, 2000) using Millipore (Bedford, MA) ZipTip®C18 pipette tips. Prior to desalting, the tips were wetted with methanol and equilibrated with 0.1% (v/v) TFA. Each sample was applied to a ZipTip®C18 and eluted with 5 µL of Wash 1 [0.1% (v/v) TFA], followed by 5 µL of Wash 2 [20% (v/v) acetonitrile containing 0.1% (v/v) TFA], followed by 5 µL of Wash 3 [50% (v/v) acetonitrile containing 0.1% (v/v) TFA], and finally with 5 µL of Wash 4 [90% (v/v) acetonitrile containing 0.1% (v/v) TFA].

5.4.4 *MALDI-TOF Instrumentation and Mass Analysis*

Mass spectral analyses were performed as follows: mass calibration of the instrument was performed using an external peptide mixture from a Sequazyme™ Peptide Mass Standards kit (Applied Biosystems). Samples (0.3 µL) from each of the desalting steps, as well as a sample of the solution taken prior to desalting, were co-crystallized with 0.75 µL

α-cyano-4-hydroxy cinnamic acid (Waters, Milford, MA) on the analysis plate. All samples were analyzed in the 500 to 5000 dalton range using 100 shots at a laser intensity setting of 2318-2460 (a unit-less MALDI-TOF instrument specific value). Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). GPMAW32 software (Applied Biosystems, version 4.23) was used to generate a theoretical trypsin digest of the expected Cry2Ab2 protein sequence deduced from the nucleotide sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks in the 500 to 1000 Da range if there were two or more isotopically resolved peaks, and in the 1000 to 5000 Da range if there were three or more isotopically resolved peaks in the spectra. Peaks were not assessed if the peak heights were less than approximately twice the baseline noise, or when a mass could not be assigned due to overlap with a stronger signal ±2 daltons from the mass analyzed. Known autocatalytic fragments from trypsin digestion were identified in the raw data. The identity of the Cry2Ab2 protein is confirmed if ≥ 40% of the protein sequence was identified by matching experimental masses for the tryptic peptide fragments to the expected masses for the fragments.

5.5 N-terminal Sequence Analysis

An aliquot of the plant-produced Cry2Ab2 protein was diluted with 5× sample loading buffer to a final purity corrected protein concentration of 80 ng/µL. Molecular weight markers (BioRad Precision Plus Dual Color, Hercules, CA) were used to confirm the transfer of protein to the PVDF membrane. The plant-produced Cry2Ab2 protein was electrophoresed in eight lanes at 2 µg per lane. The Cry2Ab2 containing samples were heated to ~99 °C for 5 min prior to electrophoresis on a pre-cast tris-glycine 4→20% SDS polyacrylamide gel at 140V for 20 min followed by 200 V for 43 min. The gel was then electroblotted to a 0.2 micron PVDF membrane for 60 min at a constant current of 300 mA in a solution containing 10 mM CAPS, 10% (v/v) methanol, pH 11. Protein bands were stained by briefly soaking the membrane with Coomassie Blue R-250 stain (BioRad) and visualized by destaining with a Coomassie R-250 destain solution (BioRad).

The protein bands with molecular weights of approximately 61.3, 49.6 and 27 kDa, (also referred to as band-1, band-2, and band-3, respectively) were excised from the membrane. N-terminal sequence analysis was performed using automated Edman degradation chemistry (Hunkapillar *et al.*, 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and

785 Programmable Absorbance Detector and Procise™ Control Software (version 2.1) was used. Chromatographic data were collected using Atlas[99] software (version 2003R1.1, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to calibrate the instrument for each analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 picomole β-lactoglobulin, Applied Biosystems) was analyzed before and after the analysis of the three protein bands to verify that the sequencer met performance criteria for repetitive yield and sequence identity.

5.6 Molecular Weight and Purity Estimation by SDS-PAGE

Aliquots of the test substance and reference standard protein were each diluted with sample dilution buffer and 5× sample loading buffer to a final protein concentration of 0.2 µg/µL. Molecular weight markers (BioRad Broad-Range, Hercules, CA) that were used to estimate the apparent molecular weight of the test substance, were diluted to a final total protein concentration of 0.9 µg/µL. The plant-produced Cry2Ab2 protein was analyzed in duplicate at 1, 2, and 3 µg total protein loads per lane. The *E. coli*-produced Cry2Ab2 reference standard (APS lot 20-100071) was analyzed at 1 µg total protein. All samples were heated at ~102 °C for 5 min and applied to a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel (Invitrogen, Carlsbad, CA). Electrophoresis was performed at a constant voltage of 150 V for 82 min. Proteins were fixed by placing the gel in a solution of 40% (v/v) methanol and 7% (v/v) glacial acetic acid for 30 min, stained 16 hr with Brilliant Blue G-Colloidal stain (Sigma, St. Louis, MO), destained 30 sec with a solution containing 10% (v/v) acetic acid and 25% (v/v) methanol, and finally destained with 25% (v/v) methanol for 6 hr.

Analysis of the gel was performed using a BioRad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). Values for the markers supplied by the manufacturer were used to estimate the apparent molecular weight of each observed band. All visible bands within each lane were quantified using Quantity One software. For the plant-produced Cry2Ab2 protein, purity was estimated as the percent optical density of the ~61 kDa band relative to all bands detected in the lane. Apparent molecular weight and purity were reported as an average of all six loadings containing the plant-produced Cry2Ab2 protein.

5.7 Molecular Weight Determination-*MALDI-TOF MS.*

Determination of the intact mass was attempted for the plant-produced Cry2Ab2 protein using MALDI-TOF mass spectrometry analysis. This analysis was not

specified in the characterization plan and is therefore a plan deviation. However, there is no impact on the protein characterization.

Prior to analysis, the plant-produced Cry2Ab2 protein and BSA reference protein (NIST, Gaithersberg, MD) were desalted using drop dialysis (Görisch, 1988). A portion of each protein sample (0.3 µL) was spotted on an analysis plate, mixed with 0.75 µL sinapinic acid solution and air-dried. Each sample was analyzed in triplicate. Mass spectral analysis was performed using an Applied Biosystems Voyager DE-Pro Biospectrometry Workstation MALDI-TOF instrument with the supplied Data Explorer software (version 4.0.0.0, Foster City, CA). Mass calibration of the instrument was performed using desalted BSA reference protein.

5.8 Functional Activity Assay

The purpose of this analysis was to compare the biological activity between plant-produced Cry2Ab2 protein and the *E. coli*-produced Cry2Ab2 protein by determining EC_{50} values in a CEW diet-incorporation insect bioassay. The EC_{50} value is defined as the concentration of Cry2Ab2 protein in the diet that results in 50% growth inhibition. In order to assess the functional activity of the plant-produced Cry2Ab2 protein and to compare its activity to the *E. coli*-produced Cry2Ab2 reference standard, aliquots of the plant-produced Cry2Ab2 protein and *E. coli*-produced Cry2Ab2 reference standard protein were transferred to the Product Safety Center (Ecological Technology Center, Monsanto). These aliquots were used to estimate the effective protein concentration necessary to inhibit the growth of the target insect by 50% as described in Appendix 4. The plant- and *E. coli*-produced Cry2Ab2 proteins will be determined to have comparable functional activity if the difference in mean EC_{50} values between plant- and *E. coli*-produced proteins is less than or equal to three fold.

5.9 Glycosylation Analysis

Glycosylation analysis was used to determine whether the plant-produced Cry2Ab2 protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the plant-produced Cry2Ab2 protein, the *E. coli*-produced Cry2Ab2 reference standard (in this instance, a negative control), and the positive control transferrin (Amersham Biosciences, Piscataway, NJ), were each diluted in dilution buffer and in 5× sample loading buffer to a final purity corrected (total protein for transferrin) concentration of 50 ng/µL. These samples were heated to ~100 °C for 5 min, and loaded along with Precision Plus Dual Color pre-stained protein molecular weight markers (BioRad , Hercules, CA) on a pre-cast tris-glycine 4→20% polyacrylamide gradient 10-well mini-gel. All three samples were loaded at a single loading of 0.5 and 1 µg protein per lane.

Electrophoresis was performed at a constant voltage of 140 V for 20 min followed by a constant voltage of 200 V for 47 min. After electrophoresis, proteins were electrotransfered to a 0.45 micron PVDF membrane for one hour at a constant current of 300 mA.

Carbohydrate detection was performed directly on the PVDF membrane containing the 0.5 and 1.0 µg sample loads using the ECL detection system (Amersham Biosciences, Piscataway, NJ). After the electrotransfer of the proteins, the PVDF membrane was incubated in PBS for 10 min, and transferred to a solution of 100 mM acetate buffer, pH 5.5, containing the oxidation reagent, 10 mM sodium metaperiodate. The membrane was incubated in the dark for 20 minutes. The oxidation solution was removed from the membrane by two brief rinses followed by three sequential 10 min washes in PBS. The membrane was transferred to a solution of 100 mM acetate buffer, pH 5.5, containing 25 nM biotin hydrazide and incubated for 60 minutes. Biotin hydrazide solution was removed by washing in PBS as previously described. The membrane was blocked with 5% blocking agent in PBS for 60 minutes. The blocking solution was removed by washing in PBS as previously described. The membrane was incubated with streptavidin-HRP conjugate (diluted 1:6000) in acetate buffer for 30 min to detect carbohydrate moieties bound to biotin. Excess streptavidin-HRP was removed by washing in PBS as previously described. Bands were visualized using the ECL detection system (Amersham Biosciences, Piscataway, NJ). Films were exposed (1 min, 3 min, and 6 min) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor.

5.10 Storage Stability

The stability of the plant-produced Cry2Ab2 protein was evaluated by purity and molecular weight analyses at day 32 of the experimental phase. Aliquots of the plant-produced Cry2Ab2 protein were stored in a 4 °C refrigerator, and –20 °C and –80 °C freezers, starting on day 0 of the experimental phase. At day 32, the sample was removed from each storage condition and the purity and molecular weight were estimated by SDS-PAGE analysis, as previously described in Section 5.6. The plant-produced Cry2Ab2 sample was diluted to 0.2 µg/µl in 5× sample loading buffer and loaded in duplicate at 1, 2, and 3 µg of total protein per lane. The protein samples will be considered to have undergone degradation if a >20% decrease in purity and/or molecular weight was observed relative to the value determined on day 0.

6.0 Data Rejected or Not Reported

Some data throughout the experimental phase of this characterization plan was rejected because it did not meet the assay criteria, and/or because of equipment malfunction.

On day 0 of the experimental phase, one set of purity and molecular weight analyses was rejected because the stained SDS polyacrylamide gel was not completely destained. As a result, the high background of the stain interfered with the densitometric analysis of the gel.

One N-terminal sequencing analysis was rejected due to an N-terminal sequencer system malfunction. The sequence collected on the Cry2Ab2 protein for the second run is reported herein and is shown in Table 5.

The first glycosylation analysis was rejected because of the unexpected low signal observed for the transferrin positive control.

7.0 Control of Bias and Quality Measures

Controls and standards were included with each analysis. A protein standard (β-lactoglobulin) was sequenced before and after N-terminal sequence analysis to assure the performance of the sequencing instrument. Additionally, instrument repetitive yield data for the analysis of β-lactoglobulin were calculated and found to meet specifications (>94% per cycle). A four-peptide mixture from the Sequazyme Peptide Mass Standards kit (Applied Biosystems) was used to calibrate the MALDI-TOF mass spectrometer for masses observed between 500-5000 daltons. BSA (NIST) was used to calibrate the MALDI-TOF mass spectrometer for native protein molecular weight determinations. Replicate analyses were used for the BioRad protein concentration assay, insect bioassay, western blot, and glycosylation analyses. For the glycosylation analysis, transferrin and *E. coli*-produced Cry2Ab2 proteins were used as the positive and negative controls, respectively.

8.0 Results and Discussion

8.1 Total Protein Concentration

The total protein concentration of the plant-produced Cry2Ab2 protein was estimated to be 0.25 mg/mL using a BioRad protein assay. The protein assay standard curve was generated using the *E. coli*-produced Cry2Ab2 reference standard protein. The total protein concentration of the reference standard protein (Lot 20-100071) was estimated using amino acid analysis. However, the lower protein concentration of the test substance limited the use of amino acid analysis.

8.2 Protein Identity

The identity of the plant-produced Cry2Ab2 protein (lot 60-100075) was confirmed using three analytical methods: western blot analysis using goat anti-Cry2Ab2 antibody, MALDI-TOF tryptic mass map analysis, and N-terminal sequence analysis.

8.2.1 Western Blot Analysis Using Anti-Cry2Ab2 Antibodies

Western blot analysis was performed using an affinity-purified goat IgG (lot 7227632). The goat anti-Cry2Ab2 antibody was previously characterized and the data were archived under lot 7227632. The plant-produced Cry2Ab2 protein was loaded in duplicates at 20, 30, and 40 ng (purity corrected) per lane and the reference standard was loaded at 20 ng (purity corrected) per lane. Two major immunoreactive bands migrating at approximately 61 kDa (band-1) and 50 kDa (band-2) were observed (Figure 3, lanes 4-9) in the plant-produced Cry2Ab2 sample. As expected, the immunoreactive signal increased with increased loading levels of the plant-produced protein. A band at the expected molecular weight of ~61 kDa was observed in the *E. coli*-produced Cry2Ab2 reference standard (Figure 3, lane 3). The N-terminal sequence analysis of the full-length plant-produced Cry2Ab2 protein (migrating at ~61 kDa) indicates that some population of Cry2Ab2 protein N-terminus sequence begins with amino acid 24. The microheterogenity may reflect the *in planta* protein itself or may be the product of endogenous proteases that are released from vesicles and/or are activated during the protein purification procedure. In addition to the two major immunoreactive bands, proteins with weaker signals were observed, migrating as high as 130 kDa and as low as ~15 kDa. These weaker signals are not uncommon in western blot analysis and are likely due to non-specific binding of either secondary or primary antibodies. The purity value of ~33% for the plant-produced Cry2Ab2 protein further substantiates the presence of contaminant plant proteins (Figure 4).

N-terminal sequence analysis of the lower immunoreactive band migrating at ~50 kDa (also referred to as band-2) clearly indicates the presence of a truncated form of the full length Cry2Ab2 protein whose N-terminal sequence begins at amino acid position 145 (Table 5). Collectively, the western blot analysis showed that both the plant- and *E. coli*-produced Cry2Ab2 proteins are immunoreactive against goat anti-Cry2Ab2 antibody and also exhibited near identical electrophoretic mobility.

8.2.2 MALDI-TOF Tryptic Mass Map Analysis

The identity of the two major immunoreactive bands cross-reacting with anti-Cry2Ab2 antibodies during western blot analysis was further assessed by MALDI-TOF mass spectrometry. Observed tryptic peptides were considered a match to the expected tryptic mass when differences in molecular weight of less than one dalton were found between the observed and predicted fragment masses. Such matches were made without consideration for potential natural amino acid modifications. In practice, protein identity can be determined when tryptic peptide fragment masses derived from >40% of the amino acid sequence length of the protein of interest are matched with the predicted tryptic peptide fragment masses.

Using this criterion for band-1 migrating at ~61 kDa, a total of 32 observed mass fragments matched the expected tryptic digest mass fragments from the deduced amino acid sequence of the Cry2Ab2 protein (Table 1). These identified masses were used to assemble a coverage map indicating the matched peptide sequences for the entire protein sequence (Figure 1). Peptide sequence coverage equal to 47.7% of the 637 amino acid length of the Cry2Ab2 protein was obtained and is considered sufficient to confirm the identity of the plant-produced Cry2Ab2 protein. During this analysis, a tryptic mass was matched to an expected mass with >1 Da mass difference, resulting in a characterization plan deviation. This fragment originated from positions 144-164 of the full length Cry2Ab2 protein (Table 1). The increase in observed mass difference of 1 Dalton was attributed to the N-terminal asparagine residue becoming deamidated during mass spectrometry analysis. The instability of asparaginyl residues with respect to hydrolytic deamidation under various experimental conditions has been observed in proteins (McKerrow and Robinson, 1971). Inclusion of the fragment from positions 144-164, increased the tryptic mass map coverage from 44.4% to 47.7%. Nevertheless, both percentages were above the set criteria outlined in the characterization plan.

For band-2 migrating at ~50 kDa, a total of 24 observed mass fragments matched the expected tryptic digest mass fragments from the deduced amino acid sequence of the Cry2Ab2 protein (Table 2). These identified masses yielded a coverage map equal to 47.7% of the 493 amino acid length of the proteolytic fragment of Cry2Ab2 protein and is considered sufficient to confirm the identity of the plant-produced ~50 kDa Cry2Ab2 protein fragment (Figure 2). Collectively, both major bands were identified as Cry2Ab2 protein.

8.2.3 N-terminal Sequence Analysis

The results of the N-terminal sequence analysis of the plant-produced Cry2Ab2 protein are summarized in Table 5. Three protein bands were identified for N-terminal sequence analysis. Band-1 and –2 (migrating at ~61 and ~50 kDa) were immunoreactive, and were identified as the full length and proteolytic fragment of Cry2Ab2 protein, respectively. Based upon the SDS-PAGE analysis, band-3 represented ~21% of the total protein, with the apparent molecular weight of ~27 kDa.

N-terminal analysis of band-1 yielded no full-length sequence starting with methionine, but did yield a sequence starting with amino acid 24. N-terminal sequence analysis of band-2, clearly indicates the presence of a proteolytic fragment of the full-length Cry2Ab2 protein whose N-terminus begins with amino acid 145 (Table 5). Band-3, migrating at ~27 kDa, was identified as corn zein protein. The major form of storage protein in corn is composed of several types of prolamin proteins known as zeins (Gibbon and Larkins, 2005).

8.3 Molecular Weight and Purity Determination

The plant-produced Cry2Ab2 protein was separated using SDS-PAGE and stained with Brilliant Blue G-Colloidal stain (Figure 4). Purity and apparent molecular weight of the plant-produced Cry2Ab2 protein were estimated using densitometric analysis and the data are summarized in Table 3. The purity and molecular weight values were averaged from duplicate loads of 1, 2, and 3 µg total protein per lane (Figure 4, lanes 4-9). The apparent molecular weight of the protein band identified as the full length Cry2Ab2 protein was 61.3 kDa. Since this protein migrated with a near identical molecular weight as that of the *E. coli*-produced Cry2Ab2 reference standard (Appendix 2, and Table 4) analyzed concurrently, the plant-produced Cry2Ab2 protein was concluded to have the same MW as the *E. coli*-produced Cry2Ab2 protein. The average purity of the plant-produced Cry2Ab2 protein was estimated to be 33%.

8.4 Molecular Weight Determination – *MALDI-TOF MS*

Determination of the intact mass was attempted for the plant-produced Cry2Ab2 protein using MALDI-TOF mass spectrometry analysis. No mass was observed in the expected mass range of the Cry2Ab2 protein.

8.5 Functional Activity

The results of the functional activity assays are summarized in Appendix 4 and Figure 5. The biological activity of the *E. coli*- and the plant-produced Cry2Ab2

proteins against CEW was determined by measuring the EC_{50} values in a diet-incorporation insect bioassay. The bioassay was replicated three times on separate days with separate batches of insects. The mean EC_{50} values for the *E. coli*-produced Cry2Ab2 reference standard protein and the plant-produced Cry2Ab2 proteins were identical and were determined to be 0.16 µg Cry2Ab2 protein/mL diet, with standard deviations of 0.04 and 0.01 µg Cry2Ab2 protein/mL diet, respectively. The insect bioassay demonstrated that the *E. coli*-produced protein is functionally equivalent to the plant-produced protein.

8.6 Glycosylation Analysis

Many eukaryotic proteins are post-translationally modified with carbohydrate moieties (Rademacher *et al.*, 1988). These carbohydrate moieties may be complex, branched polysaccharide structures or simple monosaccharides. In contrast, prokaryotic organisms such as *E. coli* lack the necessary biochemical "machinery" required for protein glycosylation. To test whether potential post-translational glycosylation of the plant-produced Cry2Ab2 protein occurred, the isolated plant-produced Cry2Ab2 protein was analyzed for the presence of covalently bound carbohydrate moieties. The *E. coli*-produced Cry2Ab2 reference standard (negative control) and transferrin (positive control) were analyzed concurrently with the plant-produced Cry2Ab2 protein. The results of this analysis are presented in Figure 6. The positive control (transferrin) was detected at the expected molecular weight in a concentration-dependent manner at loadings of 0.50 and 1.0 µg/lane (Figure 6, lanes 3-4). No detectable signal was observed for the test or reference Cry2Ab2 proteins (Figure 6, lanes 5-8) on the films at any exposure time. However, a faint band, migrating at ~20 kDa was detected for MW markers, and *E. coli*-produced Cry2Ab2 protein which was a negative control. The faint band observed for the *E. coli*-produced protein and MW markers is likely due to a non-specific interaction between the detection reagent and protein mass bound to the blot and does not represent glycosylation of the protein. Thus, the plant-produced Cry2Ab2 protein is concluded to be equivalent to the *E. coli*-produced Cry2Ab2 reference standard with respect to the absence of glycosylation.

8.7 Storage Stability

The purity and apparent molecular weight of the plant-produced Cry2Ab2 protein was re-evaluated after storage for a 32-day period in a 4 °C refrigerator and −20 °C and −80 °C freezers to assess stability of the Cry2Ab2 protein. The purity and apparent molecular weight for the plant-produced Cry2Ab2 protein following different storage conditions were estimated using SDS-PAGE, Brilliant Blue G-Colloidal stain, and densitometry at the end of the experimental phase (Figures 7-9) and data are summarized in Table 6. The protein was considered to be stable at

–80 °C, –20 °C and 4 °C storage temperatures, since the purity and the apparent molecular weights of the protein present at day 32 relative to the purity and apparent molecular weights at day 0 each differed by less than 20%.

9.0 Conclusions

A panel of analytical techniques was used to characterize the plant-produced Cry2Ab2 protein. These analytical techniques were: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, densitometry, intact mass and tryptic mass map analysis using matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, N-terminal sequencing, glycosylation analysis, and a CEW diet-incorporation insect bioassay. Storage stability analysis of the plant-produced Cry2Ab2 protein was also conducted under the characterization plan. The total protein concentration of the plant-produced Cry2Ab2 protein sample was estimated to be 0.25 mg/mL using the BioRad protein assay.

The N-terminal sequence analysis of the full-length, (~61 kDa) plant-produced Cry2Ab2 protein was blocked, but sequence beginning at amino acid 24 was obtained. A protein band migrating at ~50 kDa was successfully sequenced and its sequence begins at the 145th position from the N-terminus of the full length Cry2Ab2 protein. Moreover, MALDI-TOF mass spectral analysis of the Cry2Ab2 protein identified peptides comprising 47.7% of the full length ~ 61 kDa Cry2Ab2 amino acid sequence and the ~50 kDa protein fragment.

Purity of the plant-produced Cry2Ab2 protein was determined using densitometric analysis of a Colloidal Brilliant Blue G stained SDS polyacrylamide gel and was estimated to be 33%. The molecular weight of the plant-produced Cry2Ab2 protein was estimated to be 61.3 kDa. However, the intact mass of full length Cry2Ab2 was not observed by MALDI-TOF MS analysis. The functional activity of the plant-produced Cry2Ab2 protein was determined using an insect bioassay with the larvae of CEW. Furthermore, the insect bioassay demonstrated that the plant-produced Cry2Ab2 protein was as active as the *E. coli*-produced Cry2Ab2 protein, and thus, the *E. coli*-produced protein is functionally equivalent to the plant-produced protein with respect to the functional properties of the protein. The plant-produced Cry2Ab2 protein was stable for at least 32 days at –80 °C, –20 °C and 4 °C storage temperatures according to the criteria defined in the characterization plan.

The equivalence of the plant- and *E. coli*-produced Cry2Ab2 proteins was evaluated by comparing the apparent molecular weight of the full-length protein, immunoreactivity against Cry2Ab2 antibodies, glycosylation status, and functional activity. On the basis of western blot analysis, immunoreactive properties of the plant-produced Cry2Ab2 protein were comparable to those of the *E. coli*-produced Cry2Ab2 reference standard. The plant-produced Cry2Ab2 protein was not glycosylated. The insect bioassay demonstrated

the functional equivalence of the plant-produced Cry2Ab2 protein with the *E. coli*-produced Cry2Ab2 protein. Additional evidence of equivalence of the two proteins was demonstrated by the display of near identical electrophoretic mobility of both proteins on SDS polyacrylamide gels.

Collectively, these data provide a detailed characterization of the Cry2Ab2 protein isolated from grain of MON 89034 and demonstrate the equivalence of the plant-produced Cry2Ab2 protein to the *E. coli*-produced Cry2Ab2 reference standard with respect to immunoreactivity, molecular weight, functional activity, and absence of glycosylation.

10.0 References

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Table K Summary of the Tryptic Masses Identified Using MALDI-TOF Mass Spectrometry for the Plant-Produced Cry2Ab2 Protein (61 kDa).

Observed Mass[a] (Da)					Expected Mass (Da)	Δ[d]	AA[e] Position	Fragment Sequence(s)
No Desalting[b]	Desalting Wash 1[c]	Desalting Wash 2[c]	Desalting Wash 3[c]	Desalting Wash 4[c]				
				506.58	506.25	0.33	100-103	ETEK
		552.44	552.45	552.45	552.31	0.13	538-541	YTLR
553.35	553.40				553.27	0.08	218-221	NYTR
560.38	560.44	560.45			560.32	0.06	237-241	GLNTR
	646.39	646.48	646.47		646.32	0.07	439-443	NEDLR
	659.44		659.44		659.38[f]	0.06	563-568	VTINGR
					659.41[f]	0.03	69-75	VGSLVGK
677.47	677.53	677.54	677.54	677.54	677.37	0.10	104-108	FLNQR
709.46	709.52	709.54	709.54	709.54	709.36	0.10	410-416	SGAFTAR
		724.57			724.39	0.18	514-519	TFISEK
730.54		730.63	730.63	730.63	730.45	0.09	77-82	ILSELR
903.61		903.72	903.72	903.72	903.49	0.12	109-116	LNTDTLAR
993.62	993.70	993.73	993.73	993.73	993.48	0.14	520-528	FGNQGDSLR
		1022.73	1022.72		1022.45	0.28	380-388	SWLDSGSDR
1033.71		1033.83	1033.82	1033.82	1033.56	0.15	553-562	VISSIGNSTIR
1053.79		1053.91	1053.91	1053.91	1053.64	0.15	429-438	NISGVPLVVR
1060.21		1060.80	1060.79	1060.79	1060.52	0.31	242-249	LHDMLEER
1076.69		1076.80	1076.80	1076.80	1076.53	0.16	466-474	AYMVSVHNR
1080.67	1080.76	1080.79		1080.79	1080.51	0.16	529-537	FEQNNTTAR
	1163.92				1164.60	0.68	100-108	ETEKFLNQR
1184.78		1184.92	1184.91	1184.90	1184.60	0.18	453-465	NIASPSGTPGGAR

Table 1. Continued

Observed Mass[a] (Da)					Expected Mass (Da)	Δ[d]	AA[e] Position	Fragment Sequence(s)
No Desalting[b]	Desalting Wash 1[c]	Desalting Wash 2[c]	Desalting Wash 3[c]	Desalting Wash 4[c]				
1197.82	1197.94	1197.96	1197.95	1197.95	1197.65	0.17	444-452	RPLHYNEIR
1216.79		1216.92	1216.92	1216.91	1216.61	0.18	134-143	QVDNFLNPNR
1492.91		1493.13	1493.06	1493.06	1492.69[f]	0.22	417-428	GNSNYFPDYFIR
					1492.75[f]	0.16	211-221	TYRDYLKNYTR
1680.15		1680.31			1680.94	0.79	429-443	NISGVPLVVRNEDLR
1904.24	1904.45	1904.46	1904.43	1904.41	1903.95	0.29	117-133	VNAELTGLQANVEEFNR
1919.28			1919.47	1919.47	1919.01	0.27	83-99	NLIFPSGSTNLMQDILR
2311.25		2311.68			2311.06	0.19	569-590	VYTATNVNTTTNNDGVNDNGAR
			2334.77	2334.78	2333.23	1.54	144-164	NAVPLSITSSVNTMQQLFLNR
2339.51			2339.75	2339.69	2339.15	0.36	389-409	EGVATVTNWQTESFETTLGLR
2451.70			2451.98	2451.97	2451.34	0.36	320-343	LSNTFPNIVGLPGSTTHALLAAR

[a] Only experimental masses that matched to an expected mass are listed in the table. The composition of each wash step used to desalt samples is described in Section 5.4.3.

[b] Sample, 0.3 µL, was analyzed prioto desalting.

[c] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations [0, 20, 50, and 90% (v/v) acetonitrile], as described in the methods section (Section 5.4.3).

[d] A difference of less than one dalton between the observed (first column where this mass is documented) and expected mass was necessary for consideration as a match (Except for mass fragment 144-164).

[e] AA position refers to amino acid position within the predicted Cry2Ab2 sequence as depicted in Figure 1.

[f] Two expected fragments having nearly identical masses were matched to one observed mass.

Table 2. Summary of the Tryptic Masses Identified Using MALDI-TOF Mass Spectrometry for the Lower Molecular Weight Fragment (50 kDa) of the Plant-Produced Cry2Ab2 Protein.

Observed Mass[a] (Da)					Expected Mass (Da)	Δ[d]	AA[e] Position	Fragment Sequence(s)
		Desalting						
No Desalting[b]	Wash 1[c]	Wash 2[c]	Wash 3[c]	Wash 4[c]				
		552.44	552.45		552.31	0.13	394-397	YTLR
553.37	553.41				553.27	0.10	74-77	NYTR
560.40	560.45	560.45	560.46		560.32	0.08	93-97	GLNTR
		646.47	646.48		646.32	0.15	295-299	NEDLR
659.40	659.45			660.20	659.38	0.02	419-424	VTINGR
709.49	709.54	709.54	709.55	709.57	709.36	0.13	266-272	SGAFTAR
993.66		993.73	993.74	993.77	993.48	0.18	376-384	FGNQGDSLR
			1022.73		1022.45	0.28	236-244	SWLDSGSDR
1033.74		1033.84	1033.85		1033.56	0.18	409-418	VSSIGNSTIR
1053.83		1053.92	1053.93	1053.97	1053.64	0.19	285-294	NISGVPLVVR
1060.25		1060.80	1060.81		1060.52	0.27	98-105	LHDMLEFR
1072.30					1072.54	0.24	70-77	DYLRNYTR
1076.73		1076.81	1076.82	1076.84	1076.53	0.20	322-330	AYMVSVFDR
1080.71	1080.79	1080.79	1080.80		1080.51	0.20	385-393	FEQNNTTAR
1184.81		1184.91	1184.93		1184.60	0.21	309-321	NIASPSGTRGGAR
1197.87		1197.97	1197.98	1198.02	1197.65	0.22	300-308	RPLHYNEIR

Table 2. Continued

No Desalting[b]	Observed Mass[a] (Da) Desalting				Expected Mass (Da)	Δ[d]	AA[e] Position	Fragment Sequence(s)
	Wash 1[c]	Wash 2[c]	Wash 3[c]	Wash 4[c]				
1492.96		1493.09	1493.10	1493.12	1492.69[f]	0.27	273-284	GNSNYFPDYFIR
					1492.75[f]	0.21	67-77	TYRDYLKNYTR
1680.22		1680.31	1680.35		1680.94	0.72	285-299	NISGVPLVVRNEDLR
1844.28					1843.96	0.32	50-66	DVILNADEWGISAATLR
		1875.16			1874.79	0.37	78-92	DYSNYCINTYQSAFK
		2311.67			2311.06	0.61	425-446	VYTATNVNTTTNNDGVNDNGAR
			2339.79		2339.15	0.64	245-265	EGVATVTNWQTESFETTLGLR
2451.81			2452.02	2452.03	2451.34	0.47	176-199	LSNTFPNIVGLPGSTTTHALLAAR

[a] Only experimental masses that matched to an expected mass are listed in the table. The composition of each wash step used to desalt samples is described in Section 5.4.3.

[b] Sample, 0.3 µL, was analyzed prior to desalting.

[c] The ZipTip was washed with 0.1% (v/v) trifluoroacetic acid containing acetonitrile at varying concentrations [0, 20, 50, and 90% (v/v) acetonitrile], as described in the methods section (Section 5.4.3).

[d] A difference of less than one dalton between the observed (first column where this mass is documented) and expected mass was necessary for consideration as a match.

[e] AA position refers to amino acid position within the predicted Cry2Ab2 sequence as depicted in Figure 2.

[f] Two expected fragments having nearly identical masses were matched to one observed mass.

Table 3. Molecular Weight and Purity Estimation Using SDS-PAGE Analysis for the Plant-Produced Cry2Ab2 Protein Isolated from Grain of MON 89034.

Total Cry2Ab2 Protein load	Full Length Cry2Ab2		Lower MW Fragment of Cry2Ab2	
	MW[a] (kDa)	% Purity[b]	MW[a] (kDa)	% Purity[b]
1 µg in lane 4	61.43	33.2	49.51	13.6
1 µg in lane 9	61.11	36.2	49.24	14.7
2 µg in lane 5	61.52	30.5	49.72	12.3
2 µg in lane 6	61.33	33.6	49.70	13.5
3 µg in lane 7	61.28	33.4	49.68	13.4
3 µg in lane 8	61.02	31.6	49.48	12.7
Average	**61.3**	**33**	**49.6**	**13**

[a] The apparent molecular weights were calculated from the molecular weight markers (Figure 4, Lanes 2 and 10) using the manufacturer's supplied molecular weight values. Molecular weights were rounded to one decimal place and purity was rounded to nearest whole number.

[b] Relative percent quantities (percent purity) of plant-produced Cry2Ab2 protein bands were derived from densitometric analysis of the SDS polyacrylamide gel shown in Figure 4, Lanes 4-9 (test substance).

Table 4. Molecular Weight Difference Between Full-Length Plant- and *E. coli*-Produced Cry2Ab2 Proteins.

Molecular Weight of Full-Length Plant-Produced Cry2Ab2 Protein[a]	Molecular Weight Difference from *E. coli*-Produced Cry2Ab2 Protein[b]	Percent Difference from *E. coli*-Produced Cry2Ab2 Protein[c]
61.3 kDa	0.2 kDa	0.3%

[a] The molecular weight of the full-length plant-produced protein was calculated as an average of six loadings in Table 3.

[b] The molecular weight of the full-length *E. coli*-produced reference standard is described in COA (Appendix 2).

[c] Percent difference was calculated as follows:

$$\left|\frac{(\text{MW of } E.coli \text{ Cry2Ab2}) - (\text{MW of Plant Cry2Ab2})}{(\text{MW of } E.coli \text{ Cry2Ab2})}\right| \times 100$$

Table 5. N-terminal Amino Acid Sequence Analysis of the Plant-Produced Cry2Ab2 Protein Isolated from Grain of MON 89034.

Amino acid residue # from the N-terminus →		24	25	26	27	28	29	30	31	. . .	145	146	147	148	149	150	151
Predicted Cry2Ab2 Sequence[a,d] →		A	H	D	P	F	S	F	Q	. . .	A	V	P	L	S	I	T
Band-1 →	Observed[b,c]	A	H	D	P	F	S	F	Q	. . .							
Band-2 →	Observed[b,c]									. . .	X	V	P	L	(S)	I	T

[a] The amino acid sequence of the Cry2Ab2 protein was deduced from the coding region of the full length *cry2Ab2* gene present in grain of MON 89034 (Rice *et al.*, draft). The N-terminus of the full length Cry2Ab2 protein, isolated from grain of MON 89034, was blocked and thus no N-terminal sequence was obtained.

[b] Presence of a heterogeneous population of Cry2Ab2 protein sequences [band -1 (61.3 kDa), and band-2 (49.6 kDa)] yielded N-terminal sequences starting at 24th and 145th amino acid positions, respectively.

[c] Undesignated amino acid assignments are shown as an "X", tentative assignments are shown in parentheses ( ), and amino acids are assigned using the single letter amino acid code:

[d] The single letter IUPAC-IUB amino acid code is **A,** alanine; **D**, Aspartic acid; **F**, phenylalanine; **H,** histidine; **I**, isoleucine; **L**, leucine; **Q**, glutamine; **P**, proline; **S**, serine; **T**, threonine; and **V**, valine

Table 6. Storage Stability (Day 32) of the Cry2Ab2 Protein Isolated from Grain of MON 89034.

Amount of total Cry2Ab2 protein load/lane	32-day Storage @ 4 °C		32-day Storage @ – 20 °C		32-day Storage @ – 80 °C	
	MW[a,b] (kDa)	% Purity[b]	MW[a,b] (kDa)	% Purity[b]	MW[a,b] (kDa)	% Purity[b]
1 µg load in lane 3	62.01	28.8	62.14	42.2	61.63	31.5
1 µg load in lane 4	61.31	29.8	61.79	30.6	61.46	31.4
2 µg load in lane 5	61.25	27.5	61.56	31.7	61.31	31.0
2 µg load in lane 6	60.70	27.6	61.35	31.7	61.24	31.3
3 µg load in lane 7	60.64	27.7	61.35	31.1	61.16	30.8
3 µg load in lane 8	61.35	27.5	62.13	32.0	61.73	31.3
Average[c]	**61.2**	**28**	**61.7**	**33**	**61.4**	**31**
% Difference (Day 32 vs. Day 0)[d]	**0.2**	**15**	**0.7**	**0**	**0.2**	**6**

[a] The apparent molecular weights were determined from a standard curve constructed from molecular weight markers using the manufacturer's supplied values (Figures 7 to 9, Lanes 2 and 9).

[b] The apparent molecular weight and relative purity were derived from densitometric analysis of the stained SDS polyacrylamide gels shown in Figures 7 to 9, Lanes 3-8.

[c] Average molecular weight and purity of the Cry2Ab2 protein band was calculated as the average value obtained from the six loads. Molecular weights were rounded to one decimal place, and purity was rounded to nearest whole number.

[d] % Difference (Day 32 vs. Day 0): According to the characterization plan, the protein will be considered to have undergone degradation if greater than 20% loss in purity and /or molecular weight is observed (Day 32) relative to the initially (Day 0; Table 3) determined values.

$$\%\ \text{Difference (Day 32 vs. Day 0) for MW} = \left|\frac{(\text{MW at Day 0}) - (\text{MW at Day 32})}{(\text{MW at Day 0})}\right| \times 100$$

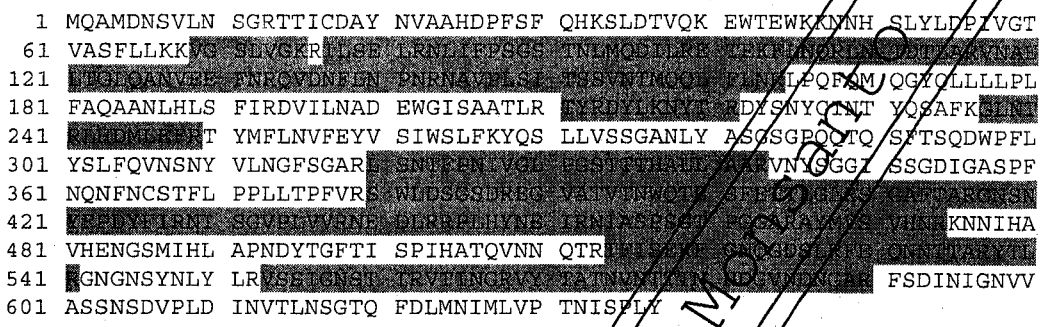

1 MQAMDNSVLN SGRTTICDAY NVAAHDPFSF QHKSLDTVQK EWTEWKKNNH SLYLDPIVGT
61 VASFLLKKVG SLVGKRILSE LRNLIFPSGS TNLMQDILRE TEKFLNQRLN TDTIARVNAE
121 LTGLQANVEE FNRQVDNFLN PNRNAVPLSI TSSVNTMQQL FLNRLPQFQM QGYQLLLLPL
181 FAQAANLHLS FIRDVILNAD EWGISAATLR TYRDYLKNYT RDYSNYCINT YQSAFKGLNT
241 RLHDMLEFRT YMFLNVFEYV SIWSLFKYQS LLVSSGANLY ASGSGPQQTQ SFTSQDWPFL
301 YSLFQVNSNY VLNGFSGARL SNTFPNLVGL PGSTTTHALL AARVNYSGGI SSGDIGASPF
361 NQNFNCSTFL PPLLTPFVRS WLDSGSDREG VATVTNWQTE SFETTLGLRS GAFTARGNSN
421 YFPDYFIRNI SGVPLVVRNE DLRRPLHYNE IRNIASPSGT PGGARAYMVS VHNRKNNIHA
481 VHENGSMIHL APNDYTGFTI SPIHATQVNN QTRTFISEKF GNQGDSLRFE QSNTTARYTL
541 RGNGNSYNLY LRVSSIGNSN IRVTINGRVY TATNVNTTVN NDGVNDNGAR FSDINIGNVV
601 ASSNSDVPLD INVTLNSGTQ FDLMNIMLVP TNISPLY

Figure 1. MALDI-TOF MS Coverage Map of the Plant-Produced Full Length Cry2Ab2 Protein Isolated from Grain of MON 89034.

The amino acid sequence of the plant-produced Cry2Ab2 protein was deduced from the coding region of the full-length *cry2Ab2* gene present in grain of MON 89034 (Rice *et al.*, draft). Shaded regions correspond to 32 fragments of tryptic peptide masses that were identified from the 61.3 kDa Cry2Ab2 protein band (band-1), analyzed using MALDI-TOF MS.

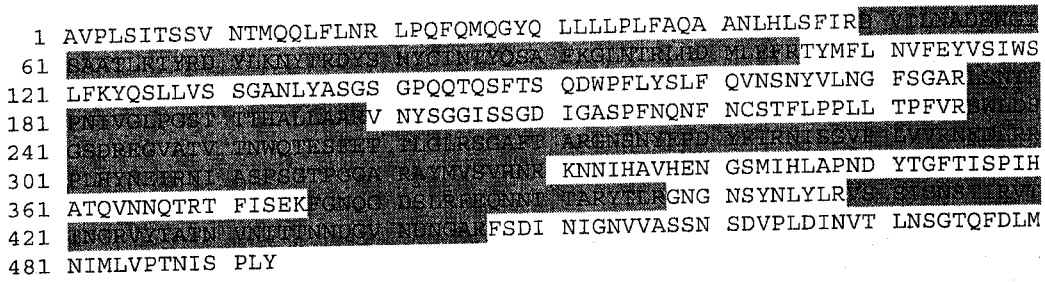
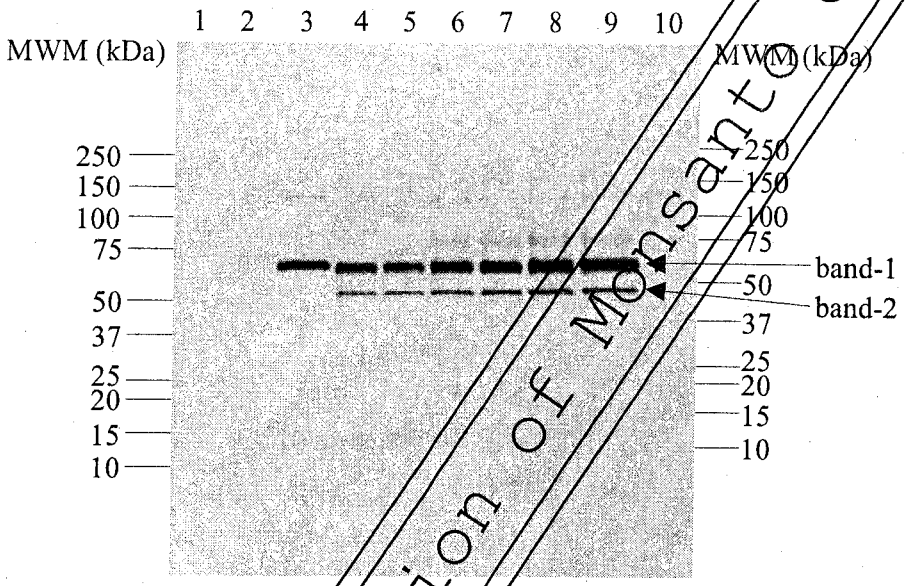


Figure 2. MALDI-TOF MS Coverage Map of the Lower MW Fragment (50 kDa) of the Cry2Ab2 Protein Isolated from Grain of MON 89034.

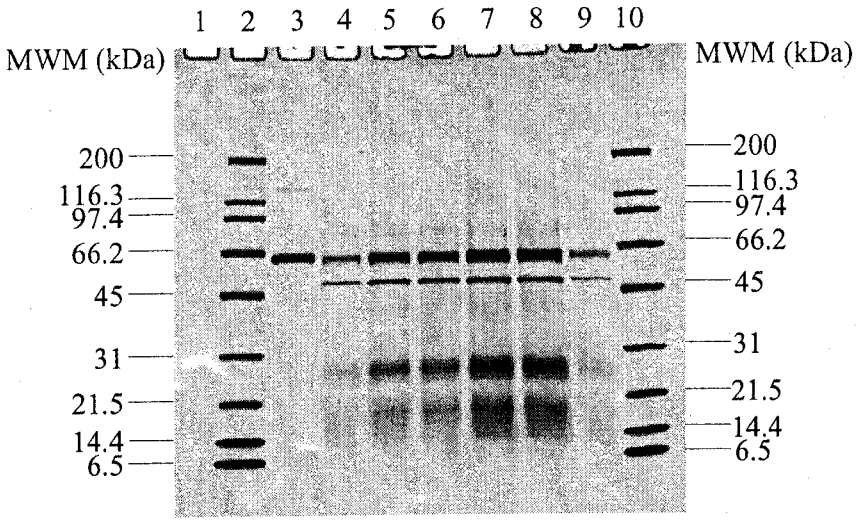
The amino acid sequence of the lower MW fragment of Cry2Ab2 protein was deduced from the coding region of the full-length *cry2Ab2* gene present in grain of MON 89034 (Rice *et al.*, draft). Shaded regions correspond to 24 fragments of tryptic peptide masses that were identified from the ~50 kDa protein band (band-2), analyzed using MALDI-TOF MS.



Lane	Sample	Amount of Cry2Ab2 (ng)
1	Empty Lane	N/A
2	MW Markers (Precision Plus Dual Color)	N/A
3	*E. coli*-produced Cry2Ab2 reference standard	20
4	Plant-produced Cry2Ab2 protein	20
5	Plant-produced Cry2Ab2 protein	20
6	Plant-produced Cry2Ab2 protein	30
7	Plant-produced Cry2Ab2 protein	30
8	Plant-produced Cry2Ab2 protein	40
9	Plant-produced Cry2Ab2 protein	40
10	MW Markers (Precision Plus Dual Color)	N/A

Figure 3. Immunoreactivity of the Plant-Produced Cry2Ab2 Protein and *E. coli*-Produced Cry2Ab2 Reference Standard with Anti-Cry2Ab2 Antibodies.

Samples of the plant-produced Cry2Ab2 protein and *E. coli*-produced Cry2Ab2 reference standard were separated by a tris-glycine 4→20% SDS polyacrylamide gel, electroblotted to a PVDF membrane, detected using goat anti-Cry2Ab2 antibody (lot 7227632), and developed using an ECL system (30 sec exposure shown). Amounts loaded correspond to purity corrected protein. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 10.



Lane	Sample	Amount (µg)
1	Empty Lane	N/A
2	MW Markers (Broad Range)	4.5
3	*E. coli*-produced Cry2Ab2 reference standard	1
4	Plant-produced Cry2Ab2 protein	1
5	Plant-produced Cry2Ab2 protein	2
6	Plant-produced Cry2Ab2 protein	2
7	Plant-produced Cry2Ab2 protein	3
8	Plant-produced Cry2Ab2 protein	3
9	Plant-produced Cry2Ab2 protein	1
10	MW Markers (Broad Range)	4.5

Figure 4. Purity and Molecular Weight Analysis Using SDS-PAGE of the Plant-Produced Cry2Ab2 Protein Isolated from Grain of MON 89034.

Samples of the plant-produced Cry2Ab2 protein, and the *E. coli*-produced Cry2Ab2 reference standard were separated by a tris-glycine 4→20% SDS polyacrylamide gel and stained with Brilliant Blue G-Colloidal stain. Amounts loaded correspond to total protein loaded per lane. Approximate molecular weights (kDa) correspond to the markers loaded in Lanes 2 and 10.

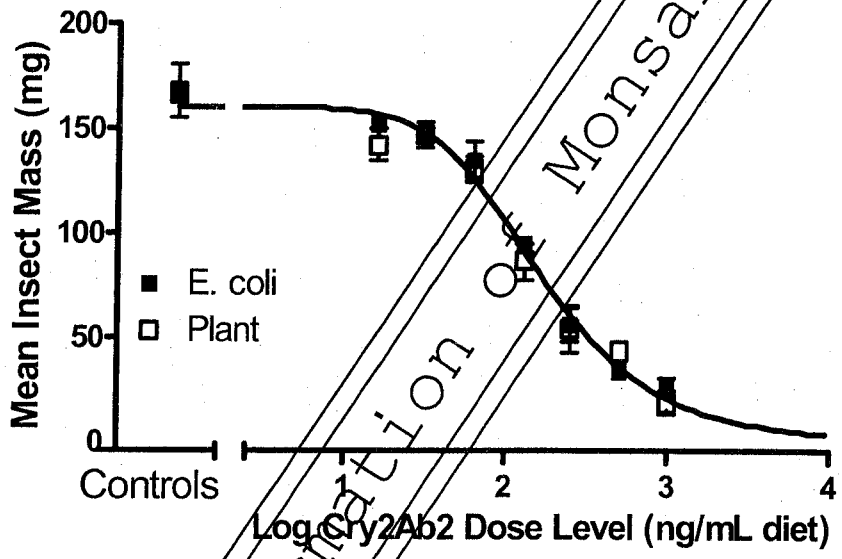
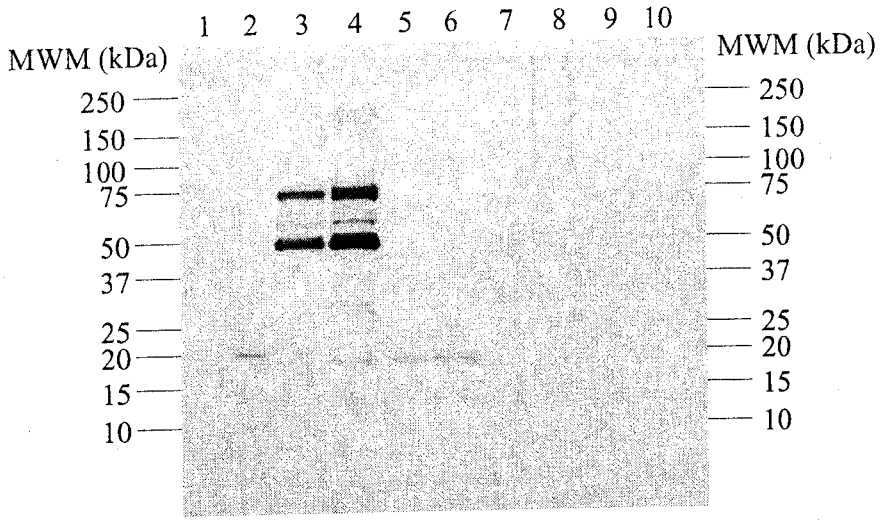


Figure 5. Functional Assay: Dose-Response Relationships for *E. coli*- and Plant-Produced Cry2Ab2 Proteins in a CEW Diet-Incorporation Bioassay.

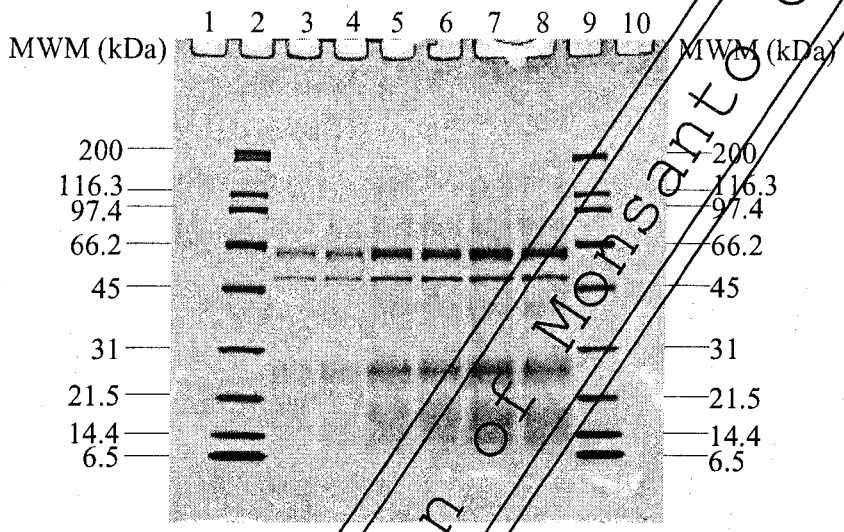
Dose-response relationships for the *E. coli*-produced Cry2Ab2 reference standard and plant-produced Cry2Ab2 protein in the CEW bioassay. Each data point represents the mean of the bioassay replicates (n = 3) at each dose level along with the standard error of the mean. Dose units on the *x*-axis are expressed in log nanograms Cry2Ab2/mL diet. Dose-response curves were prepared using GraphPad Prism software (v. 4.02).



Lane	Sample	Amount (µg)
1	Empty Lane	N/A
2	MW Markers (Precision Plus Dual Color)	N/A
3	Transferrin (positive control)	0.5
4	Transferrin (positive control)	1
5	*E. coli*-produced Cry2Ab2 protein (negative control)	0.5
6	*E. coli*-produced Cry2Ab2 protein (negative control)	1
7	Plant-produced Cry2Ab2 protein	0.5
8	Plant-produced Cry2Ab2 protein	1
9	Empty Lane	N/A
10	Empty Lane	N/A

Figure 6. Glycosylation Analysis of the Plant-Produced Cry2Ab2 Protein Isolated from Grain of MON 89034.

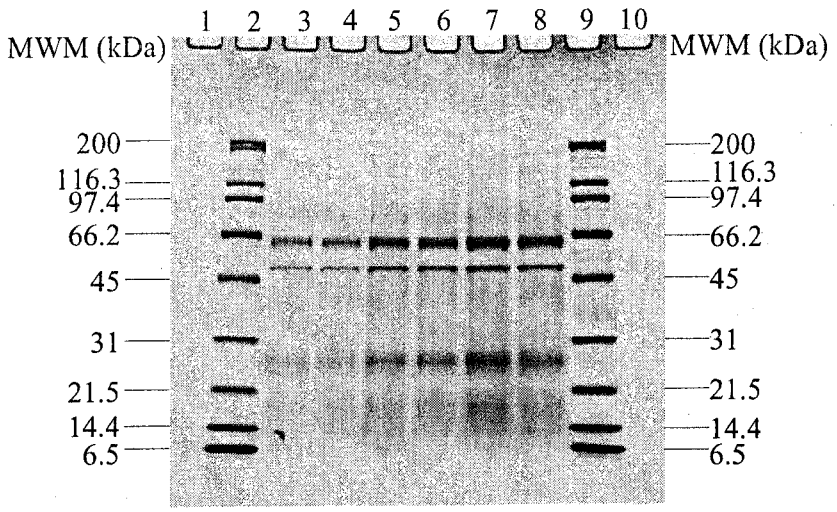
Samples of the plant-produced Cry2Ab2 protein, *E. coli*-produced Cry2Ab2 reference standard (negative control) and transferrin (positive control) were separated by a tris-glycine 4→20% SDS polyacrylamide gel and electrotransferred to PVDF membrane. Where present, protein-bound carbohydrate moieties were labeled with biotin, and detected with streptavidin-horseradish peroxidase and enhanced chemiluminescence (3 min exposure shown). Amount refers to total protein loaded per lane, except for the *E. coli* and the plant-produced proteins whose concentrations were corrected for purity.



Lane	Sample	Amount (µg)
1	Empty Lane	N/A
2	MW Markers (Broad Range)	4.5
3	Plant-produced Cry2Ab2 protein	1
4	Plant-produced Cry2Ab2 protein	1
5	Plant-produced Cry2Ab2 protein	2
6	Plant-produced Cry2Ab2 protein	2
7	Plant-produced Cry2Ab2 protein	3
8	Plant-produced Cry2Ab2 protein	3
9	MW Markers (Broad Range)	4.5
10	Empty Lane	N/A

Figure 7. Storage Stability (Day 32) of the Plant-Produced Cry2Ab2 Protein Stored in a 4 °C Refrigerator.

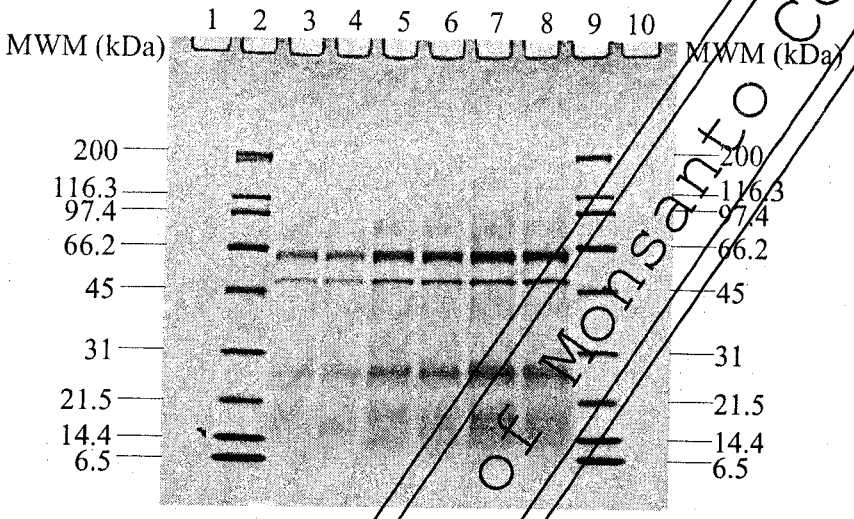
Purity and molecular weight of the plant-produced Cry2Ab2 protein stored in a 4 °C refrigerator for 32 days were re-evaluated to assess the protein storage stability. Samples were separated by a tris-glycine 4→20% SDS polyacrylamide gel and stained with Brilliant Blue G-Colloidal stain. Amounts loaded correspond to total protein loaded per lane.



Lane	Sample	Amount (µg)
1	Empty Lane	N/A
2	MW Markers (Broad Range)	4.5
3	Plant-produced Cry2Ab2 protein	1
4	Plant-produced Cry2Ab2 protein	1
5	Plant-produced Cry2Ab2 protein	2
6	Plant-produced Cry2Ab2 protein	2
7	Plant-produced Cry2Ab2 protein	3
8	Plant-produced Cry2Ab2 protein	3
9	MW Markers (Broad Range)	4.5
10	Empty Lane	N/A

Figure 8. Storage Stability (Day 32) of the Plant-Produced Cry2Ab2 Protein Stored in a –20 °C Freezer.

Purity and molecular weight of the plant-produced Cry2Ab2 protein stored in a – 20 °C freezer for 32 days were re-evaluated to assess the protein storage stability. Samples were separated by a tris-glycine 4→20% SDS polyacrylamide gel and stained with Brilliant Blue G-Colloidal stain. Amounts loaded correspond to total protein loaded per lane.



Lane	Sample	Amount (µg)
1	Empty Lane	N/A
2	MW Markers (Broad Range)	4.5
3	Plant-produced Cry2Ab2 protein	1
4	Plant-produced Cry2Ab2 protein	1
5	Plant-produced Cry2Ab2 protein	2
6	Plant-produced Cry2Ab2 protein	2
7	Plant-produced Cry2Ab2 protein	3
8	Plant-produced Cry2Ab2 protein	3
9	MW Markers (Broad Range)	4.5
10	Empty Lane	N/A

Figure 9. Storage Stability (Day 32) of the Plant-Produced Cry2Ab2 Protein Stored in a –80 °C Freezer.

Purity and molecular weight of the plant-produced Cry2Ab2 protein stored in a – 80 °C freezer for 32 days were re-evaluated to assess the protein storage stability. Samples were separated by a tris-glycine 4→20% SDS polyacrylamide gel and stained with Brilliant Blue G-Colloidal stain. Amounts loaded correspond to total protein loaded per lane.

Appendix 1. List of Applicable SOPs.

SOP Number	SOP Title
BR-ME-0044-03	Diet Incorporation Insect Bioassay for Use in Determining Biological Activity
BR-EQ-0265-02	Applied Biosystems 494 Procise™ Protein Sequencing System
BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-ME-0525-01	Bio-Rad Protein Assay
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-PO-0537-02	Characterization of Proteins for Use as TCR Substances
BR-EQ-0599-02	Bio-Rad GS-710 and GS-800 Densitometers
BR-EQ-0600-03	PowerWave Xi Microplate Reader
BR-ME-0602-01	Staining of Carbohydrate Moieties Using Commercially Available Kits
BR-PO-0655-02	Biotechnology Analytical Protein Standards Program
BR-PO-0722-01	Production and Isolation of Protein Standards
BR-EQ-0783-01	Applied Biosystems Voyager DE Pro Biospectrometry™ Workstation
BR-ME-0802-01	Protein Fragmentation via In-Gel Trypsin Digestion
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-ME-0926-01	Staining of Proteins on Blot Membranes
BR-EQ-0935-01	Konica SRX X-Ray Film Processors
BR-ME-0956-02	Protein Percent Purity and Apparent Molecular Weight Determination
BR-ME-0985-01	Zip-Tip Fractionation of Proteins and Peptides
BR-ME-0986-01	Protein Drop Dialysis
BR-ME-0994-01	Coomassie Blue Staining of Polyacrylamide Gels
AG-EQ-1051-02	Atlas Chromatography Data System

Appendix 2. Certificate of Analysis APS Lot number 20-100071.

Analytical Protein Standard Certificate of Analysis

MONSANTO — ANALYTICAL PROTEIN STANDARDS

Re-characterization No. 1

Sample Information:

Name of APS	APS Lot Number	Expiration Date
E. coli-produced Cry2Ab2.820 protein	20-100071	February 28, 2006
Common or Alias Name(s)	**Historical APS Lot Number(s)**	**Storage Requirements** (until use)
—	—	–80 °C
Source: Fermentation of *Escherichia coli* containing the pMON70528 expression plasmid		**Comment(s)** None
Additional Background Information: —		

Re-characterization Information		
Characteristic	**Method**	**Assay Date**
Concentration	Amino Acid Analysis	7/11/05
Purity/Molecular weight	SDS-PAGE Densitometry	8/6/05
Activity	Insect Bioassay	8/10/05

Characteristic	Method	Analysis Date	Result
Concentration	Amino Acid analysis	21 January 2005	0.5 mg/mL (total protein)
Purity	SDS-PAGE Densitometry	27 January 2005	87 %
Molecular weight	SDS-PAGE Densitometry	27 January 2005	61.1 kDa
Molecular weight	MALDI-TOF MS	7 February 2005	Results inconclusive
Identity	Immunoblot	14 March 2005	Confirmed
Identity	N-terminal sequence	4 February 2005	Confirmed MQAMDN
Identity	MALDI-TOF MS	7 February 2005	Confirmed sequence 54.9 % coverage of expected sequence
Activity	Insect Bioassay	18 February 2005	EC_{50} = 0.25 µg Cry2Ab2.820/mL diet

Buffer composition: 50 mM CAPS, pH 11, and 2 mM DTT
Physical description: Clear colorless solution
Short-term storage stability (25 days (4C, -20°C, and -80°C)) was evaluated during the certification process. Based upon the criteria provided in Characterization Plan 20-100071, no significant degradation was observed for samples stored at 4 °C, –20 °C and –80 °C.
Purity corrected concentration is 0.4 mg/mL (0.5 mg/mL × 0.87 = 0.4 mg/mL)

Quality Assurance Specialist — Date 8/30/05

Testing Facility Management — Date 8/30/05

Analytical Protein Standards Officer — Date 8/30/05

Appendix 3. Certificate of Analysis APS Lot number 60-100075.

Analytical Protein Standard
Certificate of Analysis

MONSANTO ANALYTICAL PROTEIN STANDARDS

Sample Information:

Name of APS	**APS Lot Number**	**Recertification Date**
Plant–produced Cry2Ab Protein	60-100075	—
Common or Alias Name(s)	**Historical APS Lot Number**	**Storage Requirements (until use)**
YieldGard II Cry2Ab2	—	–80 °C
Source		**Comment(s)**
YieldGard II corn grain event MON 89034		None
Additional Background Information		
Historic lot Cry2Ab.820-040705		

Characteristic	Method	Assay Date	Result
Concentration	Bio-Rad protein assay	19 May 2005	0.25 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	23 May 2005	33%
Molecular weight	SDS-PAGE/Densitometry	23 May 2005	61.3 KDa
Molecular weight	MALDI-TOF MS	8 June 2005	Mass not observed
Identity	Immunoblot	6 June 2005	Confirmed*
Identity	N-terminal sequence	24 June 2005	Full length - not observed Internal Cry2Ab fragment starting at position 145 XVPL/SIIT
Identity	MALDI-TOF MS	7 June 2005	Confirmed sequence 47.7% coverage of expected sequence
Composition	Glycosylation	27 May 2005	None detected
Activity	Insect Bioassay	15 June 2005	EC_{50} = 160 ng of Cry2Ab protein/mL diet

* Denotes immunoreactive bands of expected size were observed.
Buffer composition: 50 mM CAPS, 2 mM DTT, pH 11
Physical description: Clear colorless solution
Short-term storage stability (32 days) was evaluated during the certification process. Based upon the criteria provided in Characterization Plan 60-100075, no significant degradation was observed for samples stored at 4, –20, and –80°C.

Purity corrected concentration is 0.1 mg/mL (0.25 mg/mL × 0.33 = 0.1 mg/mL)

Shawn M Ryder-Heath — September 7, 2005
Quality Assurance Specialist — Date

Testing Facility Management — 9-8-2005
Date

Analytical Protein Standards Officer — 9/8/2005
Date

Page 1 of 1

Appendix 4. Insect Bioassay Summary.

Insect Bioassay Summary for:

Characterization of the Cry2Ab2 Protein Purified from the Corn Grain of MON 89034 and Comparison of the Physicochemical and Functional Comparability of the Plant-Produced and *E. Coli*-Produced Cry2Ab2 Proteins

Purpose:

The purpose of this analysis was to compare the biological activity between plant-produced Cry2Ab2 protein recovered from the corn grain of MON 89034 and a reference *E. coli*-produced Cry2Ab2 protein by determining EC_{50} values in a corn earworm (CEW) diet-incorporation insect bioassay. The EC_{50} value is defined as the level of Cry2Ab2 protein in the diet that results in 50% growth inhibition.

Materials:

Plant-Produced Cry2Ab2 protein, *E. coli*-produced Cry2Ab2 Reference Standard Protein and Control Substance:

The reference standard, an *E. coli*-produced Cry2Ab2 protein (lot # 20-100071) and a plant-produced Cry2Ab2 protein (lot # 60-100075) recovered from corn grain of MON 89034, were received from the Monsanto Product Characterization Center (PCC). The total protein concentration of the *E. coli*-produced Cry2Ab2 protein aliquots was 0.50 mg/mL, with a purity of 87%, and a purity corrected concentration of 0.4 mg Cry2Ab2/mL. The total protein concentration of the plant-produced Cry2Ab2 protein aliquots was 0.25 mg/mL with a purity of 33%, and a purity corrected concentration of 0.1 mg Cry2Ab2/mL. Both proteins were suspended in 50 mM CAPS, 2 mM DTT, pH 11.0, buffer. The control substance used in the bioassays was buffer of the same composition used to store the *E. coli*-produced and plant-produced proteins, 50 mM CAPS, 2 mM DTT, pH 11, buffer (lot # 7622108-C). The plant-produced and *E. coli*-produced Cry2Ab2 proteins were stored in a -80° C freezer and the buffer was stored in a 4° C refrigerator.

Methods:

Insects. CEW were obtained from Benzon Research Inc. Insect eggs were incubated at temperatures ranging from 10° C to 27° C, to achieve the desired hatch time.

Bioassays. CEW were used to measure activity of the plant- and *E. coli*-produced Cry2Ab2 protein samples in accordance with the Monsanto SOP BR-ME-0044-03. The bioassay was replicated three times on separate days with separate batches of insects. The plant- and *E. coli*-produced proteins were run in parallel during each bioassay. Each bioassay replicate for the *E.*

coli-produced and plant-produced Cry2Ab2 proteins consisted of a series of seven dilutions and a buffer control yielding a dose series with a 2-fold separation factor ranging from 0.016 – 1.0 µg Cry2Ab2 protein/mL diet. The dose-response curves for each protein included a buffer control. Each buffer control contained an amount of their respective buffer equivalent to the amount of protein in the highest dose level. The Cry2Ab2 protein dosing solutions were prepared by diluting the protein with purified water and incorporating the dilution into an agar-based insect diet (Southland). This dose series in diet was chosen to adequately characterize the dose-effect relationship on CEW weight gain for the proteins from both sources. The diet mixture was then dispensed in 1 mL aliquots into a 128 well tray (#BIO-BA-128, CD International, Pitman, NJ). Insect larvae were placed on these diets using a fine paintbrush, with a target number of 16 insects per treatment. The infested wells were covered by a ventilated adhesive cover (#BIO-CV-16, CD International, Pitman, NJ) and the insects were allowed to feed for a period of seven days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14 h light:10 h dark. The combined weight of the surviving insects at each dose level for each source of protein was recorded at the end of the 7-day incubation period.

Dose Response Modeling and Results:

Data were entered into an Excel spreadsheet and transferred to the Statistics Technology Center for analysis. Dose response modeling and EC_{50} determinations were performed using a 3-parameter logistic regression model (equation 1) under the PROC NLIN procedure in SAS.

Equation 1:
$$Wt = \frac{W_0}{1+\left(\frac{DietDose}{EC50}\right)^B} + e$$

where Wt is the average CEW larvae weight and *DietDose* is the Cry1Ab protein diet dose level. Three parameters that are included in the model; W_0 represents the weight at *DietDose* = 0.0, EC_{50} represents effective concentration to reduce the growth of the target insect by 50%, and B reflects the rate of the weight loss as *DietDose* increases, and e denotes the residual (error).

The EC_{50} values for each replicate bioassay are summarized in Table 1 and the dose response relationships for the two protein sources are illustrated in Figure 1. The mortality rate of the control substance groups was less than 20% in each insect bioassay, which met the acceptability criteria for the assay.

Table 1. EC_{50} values and standard errors for *E. coli*- and plant-produced Cry2Ab2 proteins in a CEW diet-incorporation bioassay

Bioassay ID	EC_{50} Estimates (µg Cry2Ab2/mL diet)	Standard Error (µg Cry2Ab2/mL diet)
E. coli-produced replicate 1	0.13	0.03
Plant-produced replicate 1	0.17	0.03
E. coli-produced replicate 2	0.16	0.02
Plant-produced replicate 2	0.16	0.03
E. coli-produced replicate 3	0.20	0.02
Plant-produced replicate 3	0.16	0.02

Mean EC_{50} for *E. coli*-produced Cry2Ab2 protein: 0.16 µg Cry2Ab2/mL diet
Mean EC_{50} for plant-produced Cry2Ab2 protein: 0.16 µg Cry2Ab2/mL diet
SD for *E. coli*-produced Cry2Ab2 protein: 0.04 µg Cry2Ab2/mL diet
SD for plant-produced Cry2Ab2 protein: 0.01 µg Cry2Ab2/mL diet

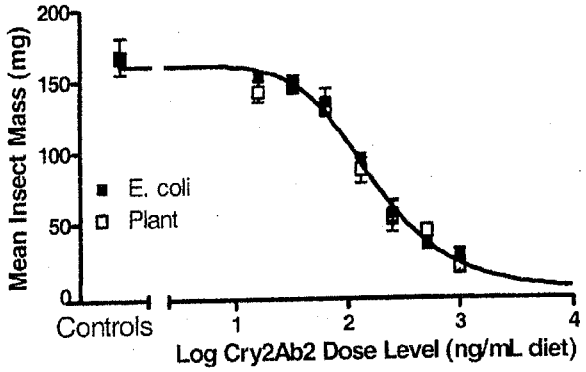


Figure 1. Corn earworm dose-response relationships for Cry2Ab2 from both protein sources in a diet-incorporation bioassay. Each data point represents the mean of the bioassay replicates (n = 3) at each dose level along with the standard error of the mean. Dose units on the *x*-axis are expressed in log nanograms Cry2Ab2/mL diet. Dose-response curves were prepared using GraphPad Prism software (v. 4.02).

Conclusions:

The mean EC_{50} values for the *E. coli*-produced protein and the plant-produced protein were identical and were determined to be 0.16 µg Cry2Ab2/mL diet, with standard deviations of 0.04 and 0.01 µg Cry2Ab2/mL diet, respectively.

Sources:
SAS Software Release 9.1 (TS1M3). Copyright 2002-2003 SAS Institute Inc., Cary, NC, USA.

Prepared by Joshua Uffman, Steven L. Levine Ph.D. and Changjian Jian Ph.D.:

Joshua Uffman, Ecological Technology Center author — Date 9/2/05

Steven Levine, Ecological Technology Center author — Date 9/2/05

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Study Title

Assessment of the Cry1A.105 and Cry2Ab2 Protein Levels in Tissues of Insect-Protected Corn MON 89034 Produced in 2005 U.S. Field Trials

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Study Completed On

August 25, 2006

Sponsor and Performing Laboratory

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Laboratory Project ID

Study 05-01-39-32
MSL 20285

The text below applies only to use of the data by the United States Environmental Protection Agency (U.S. EPA) in connection with the provisions of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

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No claim of data confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10(d)(1)(A), (B), or (C).

We submit this material to the U.S. EPA specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by the EPA strictly in accordance with FIFRA. By submitting this material to the EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to the EPA.

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Company

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Company Agent

_____

Title

_____

Signature Date

Statement of Compliance

This study meets the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160.

Submitter — Date

Sponsor Representative — 25-Aug-2006 — Date

Amelia J Hartmann — 8/25/06

Study Director — Date

Study Title: Assessment of the Cry1A.105 and Cry2Ab2 Protein Levels in Tissues of Insect-Protected Corn MON 89034 Produced in 2005 U.S. Field Trials

Study Number: 05-01-39-32

Quality Assurance Statement

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the study reported herein.

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
04/07/2006	ELISA	04/14/2006	04/14/2006
04/27/2006	Moisture Analysis	05/10/2006	05/10/2006
06/26/2006	Raw Data Audit	07/12/2006	07/12/2006
06/30/2006	Raw Data Audit	07/14/2006	07/14/2006
07/10/2006	Raw Data Audit	07/21/2006	07/21/2006
08/18/2006	Draft Report Review	08/25/2006	08/25/2006

Niki K Scanlon

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Quality Assurance Unit
Monsanto Regulatory, Monsanto Company

8/25/2006

Date

Study Information

Study Number:	05-01-39-32
Title:	Assessment of the Cry1A.105 and Cry2Ab2 Protein Levels in Tissues of Insect-Protected Corn MON 89034 Produced in 2005 U.S. Field Trials
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Study Initiation Date:	January 25, 2006
Study Completion Date:	August 25, 2006
Records Retention:	All study specific raw data (including rejected data and data not reported), protocols, final reports, and facility records will be retained at Monsanto, St. Louis.

Study Certification

This report is an accurate and complete representation of the study/project activities.

Signatures of Approval:

Amelia J Hartmann 8/25/06
Study Director Date

25-Aug-2006
Sponsor Representative Date

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Abbreviations[1] and Definitions

ANOVA	analysis of variance
B.t.	*Bacillus thuringiensis*
Cry1A.105	A modified *B.t.* Cry1A protein with 93.6% overall amino acid sequence identity to Cry1Ac
Cry2Ab2	A protein from *B.t.* subsp. *kurstaki*
CV	coefficient of variation
DTT	dithiothreitol
DWCF	dry weight conversion factor
dwt	dry weight of tissue
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
fwt	fresh weight of tissue
HRP	horseradish peroxidase
IgG	immunoglobulin G
LOD	limit of detection
LOQ	limit of quantitation
OSL	over season leaf
OSR	over season root
OSWP	over season whole plant
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
PCR	polymerase chain reaction
SEBA	Soil extraction buffer with L-ascorbic acid
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	standard operating procedure
TBA	Tris-borate buffer with L-ascorbic acid
TMB	3,3',5,5'-tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane
TSSP	tissue-specific site pool

[1] Standard abbreviations, e.g., units of measure, were used in this report according to format described in "Instructions to Authors" in the Journal of Biological Chemistry.

1.0 Summary

Monsanto has developed corn MON 89034, which produces the Cry1A.105 and Cry2Ab2 insecticidal proteins and is protected from feeding damage caused by European corn borer (*Ostrinia nubilalis*) and other lepidopteran insect pests. Cry1A.105 is a modified *B.t.* Cry1A protein. Cry2Ab2 is a *B.t.* (subsp. *kurstaki*) protein. The combination of the Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant provides better insect control and offers an additional insect-resistance management tool.

The purpose of this study was to determine the levels of Cry1A.105 and Cry2Ab2 proteins by validated enzyme-linked immunosorbent assays (ELISA) in corn tissues collected from MON 89034 produced in U.S. field trials. Tissue samples were collected from plants grown in the U.S. at five field sites in 2005 under Production Plan 05-01-50-02. In this study, over season leaf (OSL-1-4), over season root (OSR-1-4), over season whole plant (OSWP-1-4), forage, stover, forage root, senescent root, silk, pollen, and grain tissues were used for ELISA analysis. The over season samples (leaf, root, and whole plant) were collected four times at different growth stages: (1) V2 – V4 stage, (2) V6 – V8 stage, (3) V10 – V12 stage, and (4) pre-VT stage. All protein levels for all tissue types were calculated on a microgram (µg) per gram (g) fresh weight (fwt) basis. Moisture content was then measured for all tissue types and all protein levels were converted and reported on a dry weight (dwt) basis.

The means for Cry1A.105 protein levels across all sites were 5.9 µg/g dwt in grain, 42 µg/g dwt in forage, 12 µg/g dwt in pollen, 520 µg/g dwt in OSL-1, 120 µg/g dwt in leaves OSL-4, 12 µg/g dwt in forage root, and 50 µg/g dwt in stover. In tissues harvested throughout the growing season, mean Cry1A.105 protein levels across all sites ranged from 72-520 µg/g dwt in leaf, 42-79 µg/g dwt in root, and 100-380 µg/g dwt in whole plant.

The means for Cry2Ab2 protein levels across all sites were 1.3 µg/g dwt in grain, 38 µg/g dwt in forage, 0.64 µg/g dwt in pollen, 180 µg/g dwt in OSL-1, 160 µg/g dwt in OSL-4, 21 µg/g dwt in forage root, and 62 µg/g dwt in stover. In tissues harvested throughout the growing season, mean Cry2Ab2 protein levels across all sites ranged from 130-180 µg/g dwt in leaf, 26-58 µg/g dwt in root, and 39-130 µg/g dwt in whole plant.

2.0 Introduction

2.1 Background

Monsanto has developed corn MON 89034, which produces the Cry1A.105 and Cry2Ab2 insecticidal proteins and is protected from feeding damage caused by European corn borer (*Ostrinia nubilalis*) and other lepidopteran insect pests. Cry1A.105 is a modified *B.t.* Cry1A protein. Cry2Ab2 is a *B.t.* (subsp. *kurstaki*) protein. The combination of the

Cry1A.105 and Cry2Ab2 insecticidal proteins in a single plant provides better insect control and offers an additional insect-resistance management tool.

Cry1A.105 and Cry2Ab2 protein levels were determined in corn plants produced at five U.S. field sites in 2005. Field sites were selected to represent geographical regions representing commercial corn production. The corn was planted in a three replicate, randomized, complete block field design at each test site.

2.2 Purpose

The purpose of this study was to determine the levels of Cry1A.105 and Cry2Ab2 proteins in corn tissues collected from MON 89034. Tissue samples were collected from plants grown in the U.S. at five field sites in 2005 under Production Plan 05-01-50-02.

3.0 Materials

3.1 Test, Control, and Reference Substances

3.1.1 Test Substance. The test substance was MON 89034 grown in 2005 U.S. field trials. Tissue samples were collected as outlined in Production Plan 05-01-50-02 from plants grown from starting seed lot GLP-0411-15624-S.

3.1.2 Control Substance. The negative control substance was a conventional corn with a similar genetic background to the test plants grown in 2005 U.S. field trials. Tissue samples were collected as outlined in Production Plan 05-01-50-02 from plants grown from starting seed lot GLP-0411-15630-S.

3.1.3 Characterization of Test and Control Substances. The identities of the test and control substances were confirmed by verifying the chain-of-custody documentation prior to analysis. To further confirm the identities of the test and control substances, event-specific polymerase chain reaction (PCR) analyses were conducted on seed and grain samples. The PCR analyses of the planted seed were archived by the Sponsor under the seed lot numbers described in sections 3.1.1 and 3.1.2. The identities of grain samples harvested from the field were verified by PCR and the verification of identity was referenced back to the starting seed lot numbers.

3.1.4 Reference Substances. Two *E. coli*-produced protein standards were used in this study and the certificates of analysis were archived with the study data.

A Cry1A.105 protein standard (lot 20-100086) was used as the reference substance for the analysis of Cry1A.105 protein levels. The purity-corrected protein concentration of the purified standard was 1.0 mg/ml by amino acid composition

analysis. The purity was 80% as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric analysis.

A Cry2Ab2 protein standard (lot 20-100071) was used as the reference substance for the analysis of Cry2Ab2 protein levels. The purity-corrected protein concentration of the purified standard was 0.4 mg/ml by amino acid composition analysis. The purity was 87% as determined by SDS-PAGE and densitometric analysis.

4.0 Methods

4.1 Generation of Plant Samples

4.1.1 Summary of Field Design. Production Plan 05-01-50-02 (Hull, 2006) was initiated during the 2005 planting season to generate test and control substances at various corn-growing locations in the U.S. The field sites were as follows: Jefferson County, IA; Warren County, IL; Clinton County, IL; York County, NE; and Fayette County, OH. These field sites were located within the major corn-growing regions of the U.S. and provided a variety of environmental conditions. At each site, three replicated plots of MON 89034, as well as the conventional control, were planted using a randomized complete block field design. Over season leaf (OSL 1-4), over season root (OSR 1-4), over season whole plant (OSWP 1-4), forage, stover, forage-root, senescent root, pollen, silk, and grain tissues were collected from each replicated plot at all field sites. The over season samples (leaf, root, and whole plant) were collected four times at different growth stages: (1) V2 – V4 stage, (2) V6 – V8 stage, (3) V10 – V12 stage, and (4) pre-VT stage. The identification of corn growth and development stages were based on the descriptions in "How a Corn Plant Develops" (Ritchie et al., 1997). Throughout the field production, sample identity was maintained by using unique sample identifiers and proper chain-of-custody documentation. All tissue samples, except grain, were stored and shipped on dry ice to the Monsanto Sample Processing facility in Saint Louis, Missouri. Grain samples were stored and shipped at ambient temperature.

4.2 Tissue Processing and Protein Extraction Methods

4.2.1 Processing Method. All tissue samples produced at the field sites were shipped to the Monsanto Sample Processing facility. Processed tissue samples were stored in a -80°C freezer until shipped on dry ice to Monsanto's analytical facility. All processed tissue samples were stored in a -80°C freezer during the study.

4.2.2 Extraction Methods. The Cry1A.105 and Cry2Ab2 proteins were extracted from corn tissues as described in standard operating procedures (SOPs) BR-ME-1027-01 and BR-ME-1026-01, respectively. Extraction parameters for each protein and

tissue type are described in Appendices 2 and 3. All processed tissues were kept on dry ice during extract preparation. All tissues were extracted using a Harbil mixer. Insoluble material was removed from the extracts by using a Serum Filter System (Fisher Scientific, Pittsburgh, PA), or by centrifugation. The extracts were aliquoted and stored in a -80°C freezer until ELISA analyses.

4.3 ELISA Reagents and Methods

4.3.1 Cry1A.105 Antibodies. Goat polyclonal antibodies (lot 7509175) specific for the Cry1A.105 protein were purified using Protein-G Agarose affinity chromatography. The concentration of the purified IgG was determined to be 0.93 mg/ml by spectrophotometric methods. The purified antibody was stored in a phosphate buffered saline (1X PBS) buffer (pH 7.4) containing 0.001 M KH_2PO_4, 0.01 M $Na_2HPO_4 \cdot 7H_2O$, 0.137 M NaCl, and 0.0027 M KCl.

The purified Cry1A.105 antibodies were coupled with biotin (Sigma, St. Louis, MO) according to the manufacturer's instructions and assigned lot 7509180. The detection reagent was NeutrAvidin (Pierce, Rockford, IL) conjugated to horseradish peroxidase (HRP).

4.3.2 Cry2Ab2 Antibodies. Mouse monoclonal antibody (lot G-800601) specific for the Cry2Ab2 protein was purified using Protein-A Agarose affinity chromatography. The concentration of the purified IgG was determined to be 1.0 mg/ml by spectrophotometric methods. Production of the Cry2Ab2 monoclonal antibody was performed by Strategic Biosolutions (Newark, DE). The purified antibody was stored in a buffer (pH 7.2) containing 0.02 M $Na_2HPO_4 \cdot 7H_2O$ and 0.15 M NaCl with 0.05% NaN_3 added as a preservative.

Purified Cry2Ab2 antibodies (lot 7381862) were coupled with biotin (Sigma) according to the manufacturer's instructions and assigned lot 7381898. The detection reagent was NeutrAvidin-HRP.

4.3.3 Cry1A.105 ELISA Method. Goat anti-Cry1A.105 capture antibodies were diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM NaCl, pH 9.6) and immobilized onto 96-well microtiter plates at 5.0 µg/ml followed by incubation in a 4°C refrigerator for $\geq$8 h. Prior to each step in the assay, plates were washed with 1X PBS containing 0.05% (w/v) Tween-20 (1X PBST). For grain tissue only, plates were blocked with the addition of 100 to 150 µl per well of 1X PBST with 9% non-fat dried milk (NFDM) for 30 to 90 minutes at 37°C. Cry1A.105 protein standard or sample extract was added at 100 µl per well and incubated for 1 h at 37°C. The captured Cry1A.105 protein was detected by the addition of 100 µl per well of biotinylated goat anti-Cry1A.105 antibodies and NeutrAvidin-HRP (Pierce). Plates

were developed by adding 100 µl per well of HRP substrate, 3,3',5,5'-tetramethylbenzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4. Quantitation of the Cry1A.105 protein was accomplished by interpolation from a Cry1A.105 protein standard curve that ranged from 0.438 – 14 ng/ml.

4.3.4 Cry2Ab2 ELISA Method. Mouse anti-Cry2Ab2 capture antibody was diluted in coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) and immobilized onto 96-well microtiter plates at 5.0 µg/ml followed by incubation in a 4°C refrigerator for ≥8 h. Prior to each step in the assay, plates were washed with 1X PBST. Cry2Ab2 protein standard or sample extract was added at 100 µl per well and incubated for 1 h at 37°C. The captured Cry2Ab2 protein was detected by the addition of 100 µl per well of biotinylated goat anti-Cry2Ab2 antibodies and NeutrAvidin-HRP. Plates were developed by adding 100 µl per well of TMB. The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H_3PO_4. Quantitation of the Cry2Ab2 protein was accomplished by interpolation from a Cry2Ab2 protein standard curve that ranged from 0.219 – 7 ng/ml.

4.3.5 Cry1A.105 and Cry2Ab2 ELISA Validations. Appendices 2 and 3 summarize the results of validation of the ELISA methods used to assess the Cry1A.105 and Cry2Ab2 protein levels in corn tissues, respectively.

4.4 Control of Bias

The test and control substances were planted in a non-systematic manner at all field sites using a randomized complete block design as described in Production Plan 05-01-50-02. Representative tissues from each plot were collected as described in the production plan. All tissues, except pollen, were processed by thoroughly grinding before extraction to minimize sampling bias. All of the ELISA methods used were optimized to minimize method bias.

4.5 Moisture Analysis

All tissues were analyzed for moisture content using a IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO) according to SOP AG-EQ-1023-01. A homogeneous tissue-specific site pool (TSSP) was prepared consisting of a minimum of 4 samples of a given tissue type (including over season samples) grown at a given site. For stover tissue, only three samples were available and all three samples were used to prepare the TSSP. These pools were prepared for all tissues in this study. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$DWCF = 1 - [Mean\ TSSP\ Moisture / 100]$$

The DWCF were used to convert protein levels assessed on a ug/g fresh weight (fwt) basis into levels reported on a ug/g dry weight (dwt) basis using the following calculation:

$$Protein\ Level\ in\ Dry\ Weight = \frac{(Protein\ Level\ Fresh\ Weight)}{(DWCF)}$$

The protein levels that were reported to be less than or equal to the limit of detection (LOD) or the limit of quantitation (LOQ) on a fresh weight basis were not reported on a dry weight basis.

4.6 Data Analyses

All Cry1A.105 and Cry2Ab2 ELISA plates were analyzed on a SPECTRAmax Plus (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer using a dual wavelength detection method. All protein concentrations were determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 620-655 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO version 4.7.1 software. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve fit. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was converted to a "µg/g fwt" basis. For all proteins, this conversion utilized a sample dilution factor and a tissue-to-buffer ratio. The protein values in "µg/g fwt" were also converted to "µg/g dwt" by applying the DWCF. Microsoft Excel 2002 (Version 10.6730.6735 SP3, Microsoft, Redmond, WA) was used to calculate the Cry1A.105 and Cry2Ab2 protein levels in corn tissues.

4.7 Protocol Amendment and Deviations

The study protocol was amended to correct the lot number of the Cry1A.105 reference standard. This amendment had no impact on the data generated in the study.

A protocol deviation was written to address OSL-1, OSR-3, and forage samples that were analyzed more than one time. All data from the additional analyses were reported, resulting in minimal impact on the study data. A second protocol deviation was written because samples from site IA for MON 89034 stover were not included in the moisture or ELISA analyses. These samples were stored improperly and never entered the study. The absence of these samples had no impact on the moisture data generated and had minimal impact on the ELISA data generated. A third deviation was written for an OSWP-1 sample that was extracted for Cry1A.105 protein using an incorrect tissue-to-buffer ratio as a result of a miscalculation. The actual tissue-to-buffer ratio was used in all calculations for that sample, so there was no impact to the data generated.

5.0 Results

5.1 Cry1A.105 Protein Levels in Corn

Summaries of mean, standard deviation (SD), and range of the Cry1A.105 protein levels reported on a µg/g fwt and µg/g dwt basis in corn tissues collected from five U.S. field sites in 2005 for MON 89034 are presented in Tables 1 through 4. The levels of Cry1A.105 protein in tissue samples from the conventional control were below the Cry1A.105 assay LOQ or LOD for each tissue type, as expected.

5.2 Cry2Ab2 Protein Levels in Corn

Summaries of mean, SD, and range of the Cry2Ab2 protein levels reported on a µg/g fwt and µg/g dwt basis in corn tissues collected from five U.S. field sites in 2005 for MON 89034 are presented in Tables 1 through 4. The levels of Cry2Ab2 protein in tissue samples from the conventional control were below the Cry2Ab2 assay LOQ or LOD for each tissue type, as expected.

5.3 Stability of Test Materials

All of the test and control substances were extracted and analyzed by ELISA within the timeframe of verified tissue stability for the Cry1A.105 and Cry2Ab2 proteins.

6.0 Conclusions

MON 89034 was grown in U.S. field trials at five field sites in 2005. Tissue samples were collected at various growth stages throughout the growing season and assayed for Cry1A.105 and Cry2Ab2 protein levels using validated ELISA methods. These data estimate the protein levels of Cry1A.105 and Cry2Ab2 proteins on a fresh weight and dry weight basis in nineteen tissues throughout the growing season.

The means for Cry1A.105 protein levels across all sites were 5.9 µg/g dwt in grain, 42 µg/g dwt in forage, 72 µg/g dwt in pollen, 520 µg/g dwt in OSL-1, 120 µg/g dwt in leaves OSL-4, 12 µg/g dwt in forage root, and 50 µg/g dwt in stover. In tissues harvested throughout the growing season, mean Cry1A.105 protein levels across all sites ranged from 72-520 µg/g dwt in leaf, 42-79 µg/g dwt in root, and 100-380 µg/g dwt in whole plant.

The means for Cry2Ab2 protein levels across all sites were 1.3 µg/g dwt in grain, 38 µg/g dwt in forage, 0.64 µg/g dwt in pollen, 180 µg/g dwt in OSL-1, 160 µg/g dwt in OSL-4, 21 µg/g dwt in forage root, and 62 µg/g dwt in stover. In tissues harvested throughout the growing season, mean Cry2Ab2 protein levels across all sites ranged from 130-180 µg/g dwt in leaf, 26-58 µg/g dwt in root, and 39-130 µg/g dwt in whole plant.

7.0 Acknowledgments

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8.0 References

Hull, C.Y. 2006. Field Production of Tissue and Grain Samples from Lepidopteran Protected Corn MON 89034 and MON 89597, and their respective traditional crosses with MON 88017 Grown in the U.S. during 2005. Monsanto Technical Report, St. Louis, MO, MSL 20188.

Ritchie, S.W., Hanway, J.J., and Benson, G.O. January 1997. How a Corn Plant Develops: Special Report #48. Iowa State University of Science and Technology Cooperative Extension Service, Ames, IA.

Table 1. Summary of Cry1A.105 and Cry2Ab2 Protein Levels in Corn Leaf Tissues Collected from MON 89034 Produced in U.S. Field Trials Conducted in 2005

Tissue Type[1]	Cry1A.105 Protein Levels		Cry2Ab2 Protein Levels	
	Mean (SD)[2] Range[3] (µg/g fwt)[4]	Mean (SD) Range (µg/g dwt)[5]	Mean (SD) Range (µg/g fwt)	Mean (SD) Range (µg/g dwt)
OSL-1	85 (21) 56 – 130	520 (130) 380 – 850	29 (6.8) 19 – 43	180 (59) 94 – 270
OSL-2	28 (8.7) 12 – 45	140 (36) 80 – 200	32 (5.3) 23 – 44	170 (34) 110 – 230
OSL-3	16 (4.3) 9.4 – 24	72 (14) 47 – 89	29 (5.4) 23 – 41	130 (34) 85 – 200
OSL-4	30 (20) 6.3 – 59	130 (77) 27 – 240	37 (12) 11 – 56	160 (44) 48 – 210

1. Tissues were collected at the following growth stages (Ritchie et al., 1997):
   a. OSL-1: V2 – V4
   b. OSL-2: V6 – V8
   c. OSL-3: V10 – V12
   d. OSL-4: pre-VT
2. The mean and standard deviation were calculated across sites (n=15, except OSL-1 for Cry1A.105, n=16).
3. Minimum and maximum values were determined for each tissue type across sites.
4. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
5. Protein levels are expressed as µg/g on a dry weight (dwt) basis. The dry weight values were calculated by dividing the fwt by the dry weight conversion factors obtained from moisture analysis data.

Table 2. Summary of Cry1A.105 and Cry2Ab2 Protein Levels in Corn Root Tissues Collected from MON 89034 Produced in U.S. Field Trials Conducted in 2005

Tissue Type[1]	Cry1A.105 Protein Levels		Cry2Ab2 Protein Levels	
	Mean (SD)[2] Range[3] (µg/g fwt)[4]	Mean (SD) Range (µg/g dwt)[5]	Mean (SD) Range (µg/g fwt)	Mean (SD) Range (µg/g dwt)
OSR-1	8.9 (1.3) 7.3 – 12	79 (17) 52 – 110	6.4 (1.6) 4.4 – 10	56 (17) 33 – 100
OSR-2	5.8 (1.6) 3.0 – 8.5	48 (11) 30 – 63	7.6 (4.2) 2.5 – 15	58 (18) 25 – 86
OSR-3	6.4 (1.8) 4.4 – 10	45 (10) 26 – 64	5.0 (2.7) 2.2 – 12	35 (17) 15 – 74
OSR-4	6.7 (0.63) 5.6 – 8.1	42 (10) 30 – 63	4.2 (1.2) 3.2 – 7.6	26 (7.7) 15 – 45
Forage-Root	2.2 (0.35) 1.3 – 2.7	12 (3.1) 6.2 – 16	4.1 (1.4) 2.2 – 6.5	21 (5.9) 14 – 33
Senescent Root	2.2 (0.36) 1.7 – 3.1	11 (1.4) 9.4 – 15	5.3 (2.0) 2.4 – 9.1	26 (8.8) 13 – 43

1. Tissues were collected at the following growth stages (Ritchie et al., 1997):
   a. OSR-1: V2 – V4
   b. OSR-2: V6 – V8
   c. OSR-3: V10 – V12
   d. OSR-4: pre-VT
   e. Forage-Root: Early dent
   f. Senescent Root: After harvest
2. The mean and standard deviation were calculated across sites (n=15, except OSR-3 for Cry1A.105, n=16).
3. Minimum and maximum values were determined for each tissue type across sites.
4. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
5. Protein levels are expressed as µg/g on a dry weight (dwt) basis. The dry weight values were calculated by dividing the fwt by the dry weight conversion factors obtained from moisture analysis data.

Table 3. Summary of Cry1A.105 and Cry2Ab2 Protein Levels in Corn Whole Plant, Forage, and Stover Tissues Collected from MON 89034 Produced in U.S. Field Trials Conducted in 2005

Tissue Type[1]	Cry1A.105 Protein Levels		Cry2Ab2 Protein Levels	
	Mean (SD)[2] Range[3] (µg/g fwt)[4]	Mean (SD) Range (µg/g dwt)[5]	Mean (SD) Range (µg/g fwt)	Mean (SD) Range (µg/g dwt)
OSWP-1	40 (5.7) 30 – 52	380 (90) 230 – 570	13 (4.6) 5.2 – 21	130 (51) 52 – 230
OSWP-2	24 (3.7) 16 – 31	260 (52) 170 – 350	7.5 (1.8) 4.0 – 9.7	79 (18) 45 – 110
OSWP-3	11 (2.4) 7.0 – 15	100 (26) 58 – 160	4.2 (0.94) 2.4 – 5.8	40 (9.9) 22 – 61
OSWP-4	17 (3.7) 9.3 – 22	120 (29) 58 – 170	5.9 (2.6) 0.70 – 11	39 (16) 5.0 – 67
Forage	14 (3.6) 8.3 – 24	42 (9.4) 20 – 56	12 (4.0) 6.5 – 18	38 (14) 15 – 55
Stover	17 (4.4) 9.5 – 26	50 (17) 26 – 85	22 (3.6) 17 – 29	62 (15) 46 – 97

1. Tissues were collected at the following growth stages (Ritchie et al., 1997):
   a. OSWP-1: V2 – V4 c. OSWP-3: V10 – V12 e. Forage: Early dent
   b. OSWP-2: V6 – V8 d. OSWP-4: pre-VT f. Stover: After harvest
2. The mean and standard deviation were calculated across sites (n=15, except MON 89034 stover, n=12 and MON 89034 forage for Cry1A.105, n=30).
3. Minimum and maximum values were determined for each tissue type across sites.
4. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
5. Protein levels are expressed as µg/g on a dry weight (dwt) basis. The dry weight values were calculated by dividing the fwt by the dry weight conversion factors obtained from moisture analysis data.

Table 4. Summary of Cry1A.105 and Cry2Ab2 Protein Levels in Corn Silk, Pollen, and Grain Tissues Collected from MON 89034 Produced in U.S. Field Trials Conducted in 2005

Tissue Type[1]	Cry1A.105 Protein Levels		Cry2Ab2 Protein Levels	
	Mean (SD)[2] Range[3] (µg/g fwt)[4]	Mean (SD) Range (µg/g dwt)[5]	Mean (SD) Range (µg/g fwt)	Mean (SD) Range (µg/g dwt)
Silk	3.0 (0.57) 2.0 – 3.8	26 (3.9) 20 – 31	8.2 (3.6) 3.3 – 16	71 (35) 33 – 160
Pollen	6.4 (1.5) 3.8 – 8.8	12 (1.7) 8.5 – 16	0.34 (0.084) 0.21 – 0.47	0.64 (0.091) 0.49 – 0.79
Grain	5.1 (0.67) 4.1 – 6.0	5.9 (0.77) 4.7 – 7.0	1.1 (0.31) 0.67 – 1.8	1.3 (0.36) 0.77 – 2.1

1. Tissues were collected at the following growth stages:
   a. Silk: At pollination
   b. Pollen: At pollination
   c. Grain: Dried to approximately 12-15% moisture
2. The mean and standard deviation were calculated across sites (n=15).
3. Minimum and maximum values were determined for each tissue type across sites.
4. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
5. Protein levels are expressed as µg/g on a dry weight (dwt) basis. The dry weight values were calculated by dividing the fwt by the dry weight conversion factors obtained from moisture analysis data.

Appendix 1. Standard Operating Procedures

AG-EQ-1023-01	Denver Instrument IR-200 Moisture Analyzer
BR-ME-1026-01	Extraction and Indirect ELISA Analysis of Cry2Ab2 in Corn Tissues
BR-ME-1027-01	Extraction and Indirect ELISA Analysis of Cry1A.105 in Corn Tissues

Appendix 2. Summary of the Validation Results for the Cry1A.105 Protein ELISA in Corn Matrices

1.0 Accuracy

1.1 Extraction Efficiency and Spike and Recovery

Extraction Efficiency acceptance criteria = 70 – 100%.
Spike and Recovery acceptance criteria = 70 – 130%.
Spike and Recovery acceptance criteria (grain) = 60 – 140%.

Tissue	Tissue-to-Buffer Ratio	Extraction Efficiency[1]	Spike and Recovery[2]
Forage	1:100	100 %	72 – 78 %
Leaf	1:50	100 %	74 – 85%
Pollen	1:100	92 %	79 – 83%
Root	1:50	88 %	100 – 105 %
Silk	1:50	85 %	86 – 92%
Grain	1:50	100 %	60 – 67 %

1. Extraction efficiency for each tissue type was determined by comparing an aqueous extract to an extract in harsh buffer (e.g. 1X Laemmli buffer) on a western blot.
2. To evaluate the analytical accuracy of the ELISA, extracts prepared from each tissue type of conventional corn plants were spiked with known quantities of Cry1A.105 protein at three concentrations spanning the range of the standard curve.

1.2 Matrix Effects

No matrix interferences (non-specific binding) were noted when sample extracts were analyzed at matrix dilutions stated below. Matrix Effects acceptance criteria = 70 – 130%.

Tissue	Minimal Dilution to Avoid Matrix Effects	Average Recovery Range
Forage	1:10	92 – 109 %
Leaf	1:30	84 – 95 %
Pollen	1:25	87 – 100 %
Root	1:15	88 – 111 %
Silk	1:20	91 – 96 %
Grain	1:50	99 – 108 %

1.3 Parallelism

Parallelism is defined to mean that the plant-produced Cry1A.105 protein is immunologically equivalent to the *E. coli*-Cry1A.105 protein standard. Parallelism acceptance criteria = 70 – 130%.

Tissue	Parallelism between 70-130%
Forage	98 – 105 %
Leaf	91 – 101 %
Pollen	81 – 90 %
Root	96 – 99 %
Silk	95 – 102 %
Grain	92 – 113 %

2.0 Precision

Range of Quantitation: 0.438 – 14 ng/ml
Method for Curve Fit: 4-parameter

Intra-Assay Precision Acceptance Criteria: ≤ 15%
Inter-Assay Precision Acceptance Criteria: ≤ 25%
Precition Profile Acceptance Criteria: Standards 1-5 ≤ 15%
Standard 6 ≤ 25%

Intra-Assay Precision[3]: 3.8 %
Inter-Assay Precision[3]: 11.8 %
Intra-Assay Precision (Grain)[4]: 5.3 %
Inter-Assay Precision (Grain)[4]: 13.2 %

3. The inter- and intra-assay precision were assessed by determining the CV of the concentration of Cry1A.105 protein measured for the positive control sample from 57 independent ELISAs using one-way analysis of variance (ANOVA).
4. The inter- and intra-assay precision were assessed by determining the CV of the concentration of Cry1A.105 protein measured for the positive control sample from 21 independent ELISAs using one-way analysis of variance (ANOVA).

Precision Profile:

Standard Number	Concentration (ng/ml)	%CV (over 57 runs)
1	14	6.8 %
2	7	5.3 %
3	3.5	6.2 %
4	1.75	5.0 %
5	0.875	5.9 %
6	0.438	9.3 %

The total intra-assay precision based on the standard curve was calculated to be 6.4%.

Precision Profile (Grain):

Standard Number	Concentration (ng/ml)	%CV (over 21 runs)
1	14	6.4 %
2	7	6.7 %
3	3.5	6.8 %
4	1.75	7.2 %
5	0.875	8.3 %
6	0.438	12.0 %

The total intra-assay precision based on the standard curve was calculated to be 7.9%.