Study Title

Characterization of the Cry1Ac Protein Purified from the Harvested Seed of MON 87701 Soybean and Comparison of the Physicochemical and Functional Properties of the MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins

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

Submitter	Date
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Quality Assurance Unit Statement

Study Title: Characterization of the Cry1Ac Protein Purified from the Harvested Seed of MON 87701 Soybean and Comparison of the Physicochemical and Functional Properties of the MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins

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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 characterization protocol.

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

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
11/28/07	Western Blot	2/18/08	2/18/08
6/13/08	Draft Report Review	6/13/08	6/13/08
4/4/08, 4/7/08	Raw Data Audit	4/7/08	6/13/08

Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

12/08/08
Date

Study Certification

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

Signatures of Final Report Approval:

E Bell — 12/10/08

Erin Bell, Ph.D.
Study Director — Date

Kathleen S Crowley — 12/8/08

Kathleen S. Crowley, Ph.D.
Principal Investigator — Date

E A Rice — 12/8/08

Elena A. Rice, Ph.D.
Protein Team Lead — Date

Study Information

Study Number:	REG-07-270
Title:	Characterization of the Cry1Ac Protein Purified from the Harvested Seed of MON 87701 Soybean and Comparison of the Physicochemical and Functional Properties of the MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins
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Table of Contents

List of Tables

List of Figures

List of Appendices

Abbreviations and Definitions[1]

AA	Amino acid
AEX	Anion Exchange Chromatography
APS	Analytical Protein Standard
B.t.	*Bacillus thuringiensis*
CEW	Corn Earworm
COA	Certificate of Analysis
Da	Dalton
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	Ethylenediamine tetraacetic acid
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
GLP	Good Laboratory Practice
HRP	Horseradish peroxidase
MALDI-TOF MS	Matrix assisted laser desorption ionization - time of flight mass spectrometry
MH+	Protonated mass ion
MS	Mass spectrometry
MSL	Monsanto Technical Report
NIST	National Institute of Standards and Technology
NFDM	Non-fat dried milk
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline - Tween® 20
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene difluoride
PVPP	Polyvinylpolypyrrolidone
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard operating procedure
TFA	Trifluoroacetic acid
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 Company has developed insect-protected soybean MON 87701 that produces the Cry1Ac insecticidal crystal (Cry) protein (δ-endotoxin) derived from *Bacillus thuringiensis* (*B.t.)* var. *kurstaki*. The Cry1Ac protein provides protection from feeding damage caused by targeted lepidopteran pests. MON 87701-produced Cry1Ac protein is identical to the Cry1Ac protein produced in Bollgard cotton MON 531, but contains four additional amino acids at its N-terminus that are derived from a chloroplast targeting peptide, CTP1.

The level of Cry1Ac protein in the harvested seed of MON 87701 is relatively low. Therefore, it was necessary to produce the protein in recombinant bacteria in order to purify sufficient quantities of the protein for safety testing. An *E. coli*-produced Cry1Ac protein was engineered to match the Cry1Ac protein detected in MON 87701. This report describes the physicochemical and functional properties of the Cry1Ac protein isolated from MON 87701 seed and demonstrates the equivalence of this MON 87701-produced Cry1Ac protein to the previously characterized *E. coli*-produced Cry1Ac protein.

A panel of analytical techniques was used to characterize the MON 87701-produced Cry1Ac protein and to assess its equivalence to the *E. coli*-produced Cry1Ac protein. These analytical techniques included: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, densitometry, matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry, N-terminal sequence analysis, amino acid quantitation, glycosylation analysis, and Cry1Ac biological activity assay. The short-term stability of the MON 87701-produced Cry1Ac protein was assessed using SDS-PAGE by estimating the purity and molecular weight of the protein after storage for 48 days in a -80 °C freezer.

Identity of the purified, MON 87701-produced Cry1Ac protein was confirmed using western blot analysis with previously characterized anti-Cry1Ac antibody, as well as by N-terminal sequencing. MALDI-TOF mass spectrometry analysis of the full-length MON 87701-produced Cry1Ac protein, after trypsin digestion, yielded peptide masses consistent with the peptide masses expected for the Cry1Ac protein. The identified peptide masses yielded about 66% overall coverage of the expected amino acid sequence (787 out of 1182 amino acids) of the MON 87701-produced Cry1Ac protein. Purity and apparent molecular weight of the MON 87701-produced Cry1Ac protein were determined using densitometric analysis of a silver stained SDS-PAGE gel. Total purity, calculated as the sum of the percent optical density of Cry1Ac stained bands, was 77%. The apparent molecular weight of the full-length MON 87701-produced Cry1Ac protein was estimated to be 133.4 kDa. The MON 87701-produced Cry1Ac protein was determined to not be glycosylated. The functional activity of the MON 87701-produced Cry1Ac protein was determined using an insect bioassay with the larvae of a sensitive insect species, corn earworm (CEW). The MON 87701-produced Cry1Ac protein was

shown to be biologically active against CEW with a mean seven-day EC_{50} value of 0.0039 μg/ml of diet. The MON 87701-produced Cry1Ac protein was stable for at least 48 days when stored in a -80 °C freezer.

The equivalence of the MON 87701-produced and *E. coli*-produced Cry1Ac proteins was evaluated by comparing their full-length molecular weights, immunoreactivity with anti-Cry1Ac antibodies, glycosylation status, and functional activity. Based on results obtained, the MON 87701-produced Cry1Ac protein is equivalent to the *E. coli*-produced Cry1Ac protein.

These data provide a detailed characterization of the Cry1Ac protein isolated from MON 87701 and establish the equivalence of the MON 87701-produced Cry1Ac protein to the *E. coli*-produced Cry1Ac protein.

2.0 Introduction

Monsanto Company has developed insect-protected soybean MON 87701 that produces the Cry1Ac insecticidal crystal (Cry) protein (δ-endotoxin) derived from *Bacillus thuringiensis* (*B.t.*) var. *kurstaki*. The Cry1Ac protein provides protection from feeding damage caused by targeted lepidopteran pests. The nucleotide sequence of the *Cry1Ac* gene in MON 87701 was confirmed by DNA sequence analysis (Arackal et al., 2008).

The level of Cry1Ac protein in harvested seed of MON 87701 is relatively low. For that reason, the protein was expressed in bacteria (*E. coli*) in order to produce sufficient quantities for safety testing. Consequently, it was necessary to demonstrate equivalence between the MON 87701-produced and *E. coli*-produced proteins.

The plant transformation construct for MON 87701 was engineered to include a chloroplast transit peptide (CTP1) at the N-terminus to direct the Cry1Ac protein into chloroplasts. During translocation through chloroplast membranes CTP1 is usually rapidly cleaved, producing mature protein. To confirm the N-terminal sequence of the mature Cry1Ac protein, a small amount of Cry1Ac was isolated from MON 87701 and analyzed. The analysis indicated that four amino acids derived from the C-terminal end of CTP1 preceded the N-terminus of the Cry1Ac protein. For Cry1Ac, cleavage of both N-terminal and C-terminal regions occurs in the insect gut environment to generate a functionally active core protein (Lightwood et al., 2000). Thus, the CTP1-derived four additional amino acids present at the N-terminus of Cry1Ac in MON 87701, designated by the sequence CMQA, are not expected to change the functional properties of the Cry1Ac protein.

The construct for the *E. coli*-produced Cry1Ac was engineered to encode the same Cry1Ac protein as that found in MON 87701.

3.0 Purpose

The purpose of this study was to characterize the physicochemical and functional properties of the Cry1Ac protein produced in harvested seed of MON 87701 soybean, and to compare the physicochemical and functional properties of the MON 87701-

produced protein with the previously characterized *E. coli*-produced Cry1Ac protein (Orion lot 10000804).

4.0 Materials

4.1 MON 87701-Produced Cry1Ac Protein

The MON 87701-produced Cry1Ac protein (Orion lot 10000801) was purified as described below from harvested seed of MON 87701 prior to the initiation of this study. The seed used for the isolation of Cry1Ac protein, lot GLP-0612-17898-S, was produced under protocol IP036 by the Monsanto Trait Development group. The identity of the harvested seed containing MON 87701 was confirmed by event-specific PCR; a copy of the Certificate of Analysis for this seed lot is archived in the Monsanto Regulatory archives with the records documenting protein isolation. The purified MON 87701-produced protein was stored in a -80 ºC freezer in a buffer solution containing 50 mM CAPS, pH 10.8, 1 mM EDTA, 2 mM DTT, 1 mM benzamidine HCl, ~30 mM NaCl, ~1% ethylene glycol, and a trace amount of PMSF. The records describing the purification of this MON 87701-produced protein are archived under the Orion lot 10000801 in the Monsanto Regulatory archives.

4.2 *E. coli*-produced Cry1Ac Reference Protein

E. coli-produced Cry1Ac reference protein (Orion lot 10000804) was purified from the fermentation of *E. coli* transformed with plasmid pMON107800. The DNA sequence encoding this Cry1Ac reference protein was confirmed both prior to and following fermentation of *E. coli*. Records pertaining to the purification of this *E. coli*-produced reference protein are archived under Orion lot 10000804. The *E. coli*-produced Cry1Ac reference standard was previously characterized (APS Characterization Plan 20-100133) and a copy of the Certificate of Analysis (COA) is included as Appendix 1 of this report. The *E. coli*-produced Cry1Ac protein was stored in a -80 °C freezer in a buffer solution (50 mM CAPS, 1 mM benzamidine-HCl, 1 mM EDTA, and 2.5 mM DTT, pH 10.25) at a total protein concentration of 1.4 mg/ml.

The *E. coli*-produced Cry1Ac protein was used as a reference protein for the immunoblot assay, the functional activity assay, and the purity and molecular weight evaluation, and as a negative control in the glycosylation analysis.

4.3 Assay Controls

Protein molecular weight standards (BioRad, Hercules, CA) were used to calibrate SDS-PAGE gels and verify protein transfer to PVDF membranes. A peptide mixture (CalMix2 from the Sequazyme Peptide Mass Standards kit, Applied Biosystems, Foster City, CA) was used to calibrate the MALDI-TOF mass spectrometer for tryptic mass analysis. A PTH-amino acid standard mixture (Applied Biosystems) was used to calibrate the sequencer for N-terminal sequence analysis. Dilutions of an amino acid standard (NIST) were used to generate a standard curve for determining protein

concentration. Transferrin and horseradish peroxidase (both from Sigma, St. Louis, MO) were used as positive controls for glycosylation analysis. CandyCane Glycoprotein Molecular Weight Standards (Molecular Probes, Eugene, OR) were used as molecular weight markers and positive and negative controls for glycosylation analysis.

5.0 Methods

5.1 Protein Purification

The MON 87701-produced Cry1Ac protein was purified from harvested seed of MON 87701 soybean prior to initiation of this study. The purification procedure was not performed under a GLP protocol or plan; however, all procedures were documented on worksheets and, where applicable, SOPs were followed. The Cry1Ac protein was purified at 4 °C from an extract of ground seed using a combination of ammonium sulfate fractionation, anion exchange chromatography, and immunoaffinity chromatography.

Approximately one kilogram of MON 87701 seed was ground to powder using a Perten Laboratory Mill. Ground material was stored in a -80 °C freezer until use. To de-fat the seed powder, two ~500 g batches were extracted four times with warm hexane (~50 °C) added at a ratio of ~3 ml of hexane per gram of ground seed, and then air dried. The final weight of the de-fatted seed powder was ~760 g.

The Cry1Ac protein was purified from a total of four ~100 g aliquots of the ground, de-fatted MON 87701 seed in four separate runs that were pooled to generate the final MON 87701-produced Cry1Ac protein sample.

Each run included the following series of extraction and chromatography steps:

PBS wash – To promote extraction of neutral pH-soluble proteins, seed powder was stirred in cold PBS pH 7.0, 1 mM benzamidine HCl, 0.5 mM PMSF, 1% PVPP at 7.5 ml/g powder for about 1 h. The Cry1Ac-containing washed ground seed pellets were collected by centrifugation.

CAPS solubilization – Cry1Ac protein was extracted from the washed ground seed pellet with CAPS solubilization buffer (100 mM CAPS, pH 10.8, 1 mM benzamidine HCl, 0.5 mM PMSF, 1 mM EDTA, 10 mM DTT) added at 5 ml/g of starting powder. The suspension was stirred for 1-2 h, and solubilized proteins, including Cry1Ac, were separated from insoluble material by centrifugation.

$(NH_4)_2 SO_4$ precipitation and re-solubilization – An ammonium sulfate precipitate was prepared by the addition of ammonium sulfate salt to the CAPS solubilization supernatant to a final saturation of 40%. After mixing for 2-4 h, precipitated proteins were collected by centrifugation, and were re-solubilized in 50 mM Bis-Tris propane, pH 9.0, 150 mM NaCl, 1 mM EDTA, 2 mM benzamidine HCl, 1 mM PMSF, 5 mM DTT at 0.75 ml per starting ml of CAPS supernatant. Insoluble material was removed by centrifugation at 37000×g for 1 h, and the supernatant was diluted with

50 mM Bis-Tris propane, pH 9.0, 1 mM EDTA, 2 mM benzamidine HCl, 1 mM DTT to bring the NaCl concentration to 50 mM.

Anion exchange chromatography – The diluted Cry1Ac-containing protein solution (18-26 column volumes, depending on the run) was loaded at a flow rate of 1.4-2.4 ml/min onto a CaptoQ (GE Healthcare, Piscataway, NJ) anion exchange column (100 ml, 50 x 50 mm) equilibrated with 50 mM Bis-Tris propane, pH 9.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM benzamidine HCl buffer. After loading, the column was washed with 2.7-3.5 column volumes of the equilibration buffer. Proteins were then eluted in two steps, the first consisting of 4-7 column volumes of 50 mM Bis-Tris propane, pH 9.0, 300 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM benzamidine HCl buffer, and the second consisting of 3-4 column volumes of 50 mM Bis-Tris propane, pH 9.0, 600 mM NaCl, 1 mM EDTA, 1 mM DTT, 2 mM benzamidine HCl buffer. Cry1Ac protein was predominantly present in the second elution step, which was collected as a single fraction. All wash and elution steps were carried out at a flow rate of 6 ml/min.

Immunoaffinity chromatography – For immunoaffinity chromatography, resin was prepared by binding and then chemically cross-linking a monoclonal anti-Cry1Ac antibody to protein A agarose (Sigma, St. Louis, MO). The Cry1Ac-containing fraction from the anion exchange column (~300-400 ml, depending on the run) was loaded on to the immunoaffinity column (6 ml; 20 x 15 mm, h x d) equilibrated with 5-10 column volumes of 50 mM Bis-Tris propane, pH 9.0, 600 mM NaCl, 1 mM EDTA, 5 mM DTT, 2 mM benzamidine HCl, 1 mM PMSF. To maximize Cry1Ac binding to the immunoaffinity column, the load solution was recirculated through the column overnight. Following the load, the column was washed with 4-6 column volumes of the equilibration buffer. Proteins were then eluted in two elution steps, the first consisting of 4-7 column volumes of 50 mM Bis-Tris propane, pH 9.0, 800 mM NaCl, 1 mM EDTA, 5 mM DTT, 2 mM benzamidine HCl, 1 mM PMSF buffer, and the second consisting of ~ 7 column volumes of 50 mM Bis-Tris propane, pH 9.0, 800 mM NaCl, 30% (v/v) ethylene glycol, 1 mM EDTA, 5 mM DTT, 2 mM benzamidine HCl, 1 mM PMSF. Cry1Ac proteinwas predominantly present in the second elution step, which was collected as several ~ 4 ml fractions. Equilibration, load, wash and first elution step were carried out at a flow rate of ~2 ml/min; the flow rate for the final elution step was ~0.7 ml/min. Fractions collected from the final elution step were evaluated for the presence and amount of Cry1Ac by quantitative immunoblot, and fractions with the highest amounts of Cry1Ac protein were pooled for each run.

All operations described above were carried out at 4 °C. Following the final immunoaffinity chromatography run, the four batches of purified Cry1Ac protein were pooled. The pooled sample (~140 ml) was concentrated ~ 9-fold by diafiltration using a polysulfone hollow fiber cartridge with a 30 kDa molecular weight cut-off, diluted ~10-fold with a buffer containing 50 mM CAPS, pH 10.8, 1 mM EDTA, 2

mM DTT, 1 mM benzamidine HCl, and re-concentrated by diafiltration using the same cartridge. The final concentrated solution of the Cry1Ac protein (~ 1 ml) was diluted with the same buffer to a final volume of ~ 4 ml. This material was submitted to the APS program and assigned lot number 22-100135. The lot number was later reassigned as Orion lot 10000801 due to adoption of a new tracking database. The physical appearance of the protein solution was a clear liquid.

5.2 Protein Concentration

The total protein concentration of the MON 87701-produced Cry1Ac protein was determined using AccQ-Tag® (Waters Corp, Milford, MA) derivatized amino acid analysis, which allows for high sensitivity fluorescent detection of amino acids. Along with five replicates of the test sample, a hydrolysis blank sample, four dilutions of a calibration standard (NIST), and a BSA protein control were also analyzed. An internal calibrant, α-aminobutyric acid, was included in all non-blank samples. All samples were evaporated to dryness in hydrolysis tubes using a Speed-Vac concentrator. A 500 µl volume of hydrolysis solution (6N HCl, 1% phenol) was added and the tubes were transferred to a vacuum chamber. Samples were hydrolyzed for 90 min at 150±2 °C under vacuum. After cooling, the vacuum was released and the tube contents were evaporated to dryness using a Speed-Vac concentrator. A 20 µl volume of reconstitution solution (20 mM HCl) was added and tubes were vortexed to resuspend the sample. A 60 µl volume of AccQ-Fluor Borate Buffer and a 20 µl volume of AccQ-Fluor reagent were added sequentially to each vial with vortexing after each addition. The samples were transferred individually to autosampler vials, capped, and heated to 55 °C for 10 min. Samples were analyzed using a 2695 Separation Module (Waters Corp., Milford, MA) in conjunction with reverse-phase HPLC for detection of AccQ-Tag derivatized amino acids. Chromatographic data were collected using Atlas software (Thermo Electron Corp.).

5.3 Molecular Weight and Purity Analysis – SDS-PAGE

Aliquots of the *E. coli*-produced and MON 87701-produced Cry1Ac proteins were mixed with 5× sample buffer (0.31 M Tris-HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, 25% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue) to a final total protein concentration of 22 ng/µl and 17 ng/µl, respectively. The MON 87701-produced protein was analyzed in duplicate at 95, 189, and 284 ng of total protein per lane. The *E. coli*-produced Cry1Ac reference standard was loaded at 198 ng total protein, in a single lane. The Broad Range Molecular Weight marker (Bio-Rad, Hercules, CA) was loaded at 360 ng total protein. All samples were heated in a thermo-block at 95.8 °C for 5 min and applied to a pre-cast tris-glycine 4-20% gradient polyacrylamide gel. Electrophoresis was performed at a constant voltage of 125 V for 105 min.

The gel was stained using a Silver Staining Kit from Owl Separation Systems (Portsmouth, NH) according to the manufacturer's protocol. The gel was fixed for 15

min in 100 ml of Fixing Solution I. This was followed by incubation for 15 min in 100 ml of Fixing Solution II. Next, the gel was incubated in 100 ml of Pretreatment Solution for 10 min, followed by a 5 min wash in 100 ml of deionized water. The gel was stained using 100 ml of Silver Staining Solution for 12 min, followed by three 3-5 min washes, each in 100 ml of deionized water. Next the gel was incubated in 100 ml of Developer for 5 min, followed by addition of 5 ml of Stopper Solution to the Developer and incubation for 15 min. Finally, the gel was washed twice for 10 min each in 100 ml of deionized water. All incubations occurred at room temperature with gentle shaking.

Analysis of the gel was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software (version 4.4.0, Hercules, CA). For the purity evaluation, all visible bands within each lane were quantified. Stained bands corresponding to immunoreactive bands identified by anti-Cry1Ac antibody and migrating between ~133 kDa, the size of the full-length Cry1Ac protein and ~64 kDa, the lowest size of an immunoreactive band on a western (see Appendix 2), were included in the calculation of total Cry1Ac protein purity. Purity of the full-length Cry1Ac protein was also calculated. The purity values and full-length molecular weight of the MON 87701-produced Cry1Ac protein were calculated as the average of the six values obtained by densitometric analysis.

5.4 Protein Identity

5.4.1 Immunoblot Analysis

Immunoblot analysis was performed to confirm the identity of the MON 87701-produced Cry1Ac protein and compare immunoreactivity of the MON 87701-produced and *E. coli*-produced proteins. The MON 87701-produced Cry1Ac protein and the *E. coli*-produced Cry1Ac reference protein (each corrected for the purity of the full-length protein), were loaded on gels at 10, 20, or 30 ng per lane. Each protein was mixed with 5× sample buffer, heated at 96.2 °C for 5 min, and applied to a pre-cast tris-glycine 4-20% gradient polyacrylamide gel. Electrophoresis was performed at a constant voltage of 200 V for 60 min. Precision Plus Dual Color molecular weight marker (Bio-Rad, Hercules, CA) was used to verify electrotransfer of protein to the membrane and estimate the size of the immunoreactive bands. Electrotransfer to a PVDF membrane was performed at a constant voltage of 100 V for 44 min.

The membrane was blocked overnight with 5% (w/v) NFDM in 1× PBST. The membrane was probed with a 1:500 dilution of goat affinity-purified anti-Cry1Ac antibody (Orion lot 10000963) in PBST containing 1% (w/v) NFDM for 1 h. Excess antibody was removed using three 5 min washes with PBST. Finally, the membrane was probed with HRP-conjugated anti-goat IgG (Pierce, Rockford, IL) at a dilution of 1:10000 in PBST containing 1% (w/v) NFDM for 2 h. Excess HRP-conjugate was removed using three washes, each at least 5 min, with PBST.

The blocking step was performed at 4 °C. All other incubations were performed at room temperature. Immunoreactive bands were visualized using the ECL detection system (GE Healthcare, Piscataway, NJ) and exposed (1, 2, and 5 min) to Hyperfilm ECL high performance chemiluminescence film (GE Healthcare, Piscataway, NJ). Films were developed using a Konica SRX-101A automated film processor.

Analysis of the film was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One software.

5.4.2 MALDI-TOF Tryptic Mass Map Analysis

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry was used to confirm the identity of the MON 87701-produced Cry1Ac protein. The MON 87701-produced Cry1Ac protein was subjected to electrophoresis on an SDS-polyacrylamide gel. The protein sample was mixed with 5× DTT-containing sample buffer (250 mM Tris-HCl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, 0.25% (w/v) bromophenol blue, 0.5 M DTT) heated at 98.6°C for 5 min, and loaded across eight lanes of a pre-cast tris-glycine 4-20% gradient polyacrylamide gel. Precision Plus Dual Color molecular weight marker was loaded to enable estimation of molecular weight. Electrophoresis was performed at a constant voltage of 125 V for 105 min. Proteins were stained with Brilliant Blue G Colloidal stain (Sigma, St. Louis, MO) for 1 h, and destained according to manufacturer's protocol with 3 h of destaining in Destain Solution B prior to gel scanning.

The band representing full-length MON 87701-produced Cry1Ac protein was excised from several lanes of the gel, destained, reduced, and alkylated. Briefly, each excised gel band was destained for 30 min by incubation in 100 µl of destain solution in a microfuge tube. Following destaining, each excised gel band was incubated in 100 µl of 100 mM ammonium bicarbonate buffer for 40 min at room temperature. Each gel band was reduced in 100 µl of 10 mM DTT solution for 2 h at 37 °C. Each band was alkylated by the addition of 100 µl of 20 mM iodoacetic acid. The alkylation reaction was allowed to proceed at room temperature for 25 min in the dark. Each gel band was subsequently washed in 200 µl of 25 mM ammonium bicarbonate buffer for 15-45 min at room temperature. This step was repeated two additional times, following which each gel band was dried using a Speed Vac concentrator. Three gel bands were combined and rehydrated with 60 µl of 0.02 µg/µl trypsin in 25 mM ammonium bicarbonate, 10% acetonitrile, and the sample was incubated for about 1 h at room temperature. Next, excess liquid was removed and the sample was incubated overnight at 37 °C in 120 µl of 25 mM ammonium bicarbonate, 10% acetonitrile. The following day, the sample was sonicated for 5 min, and the supernatant was transferred to a new tube and dried using a Speed Vac concentrator (Extract 1). The gel material was resuspended in 90 µl 60% acetonitrile, 0.1% TFA, 0.1%

octyl-β-D-glucopyranoside solution, and sonicated for 5-10 min. After transfer of the supernatant to a new tube, this step was repeated one time, and the combined supernatants were dried using a Speed Vac concentrator (Extract 2). Extracts 1 and 2 were each resuspended in 20 µl 0.1% TFA and then dried using a Speed Vac concentrator. Extract 1 was resuspended in 5 µl of 50% acetonitrile, 0.1% TFA, while Extract 2 was resuspended in 10 µl of the same solution. Each extract was sonicated for 5 min. The extracts were then ready for loading onto the MALDI-TOF sample plate.

Mass spectral analyses were performed as follows. Mass calibration of the instrument was performed using an external peptide mixture (CalMix 2; Applied Biosystems, Foster City, CA). Extract 1 and Extract 2 samples (0.1-0.25 µl) were co-crystallized with 0.75 µl each of the following matrix solutions: α-cyano-4-hydroxy cinnamic acid (α-cyano), dihydroxybenzoic acid (DHB), and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) at separate locations on the analysis plate. The samples in α-cyano matrix were analyzed in the 500 to 6000 Da range using 200 shots at a laser intensity setting of 2511. The laser intensity setting is a unit-less MALDI-TOF instrument-specific value. The samples in DHB matrix were analyzed in the 550 to 6000 Da range using 200 shots at a laser intensity setting of 3101. The samples in sinapinic acid matrix were analyzed in the 900 to 8000 Da range using 200 shots at a laser intensity setting of 3247. Protonated (MH+) peptide masses were observed monoisotopically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993), except above 3000 Da, where mass-averaged values were observed. GPMAW32 software (Applied Biosystems, Foster City, CA) was used to generate a theoretical trypsin digest of the expected Cry1Ac protein sequence, which was based upon the nucleotide sequence of the inserted *cry1Ac* gene and the N-terminal sequence analysis that identified the amino terminus of the protein. Masses were calculated for each theoretical fragment and compared to the raw mass data. Experimental masses (MH+) were assigned to ion peaks in the 500 to 1000 Da range if there were two or more isotopically resolved ion peaks, and in the 1000 to 8000 Da range if there were three or more isotopically resolved ion peaks in the spectra. Ion peaks were not assessed if the ion 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 Da from the mass analyzed. Known autocatalytic fragments from trypsin digestion were identified in the raw data.

5.4.3 N-terminal Sequence Analysis

N-terminal sequence analysis was used to confirm the identity of the MON 87701-produced Cry1Ac protein. The MON 87701-produced Cry1Ac protein was subjected to electrophoresis on an SDS-polyacrylamide gel. MON 87701-produced Cry1Ac protein was mixed with 5× DTT-containing sample buffer, heated at 98.6 °C for 5 min and then loaded across eight lanes of a

pre-cast tris-glycine 4-20% gradient polyacrylamide gel. Precision Plus Dual Color molecular weight marker was used to estimate molecular weights and verify protein transfer to a PVDF membrane. Electrophoresis was performed at a constant voltage of 125 V for 105 min. Electrotransfer to a PVDF membrane was performed at a constant voltage of 25 V for 2 h. After transfer the blot was washed in deionized water three times for 2-5 min each, then briefly (≤ 2 min) stained in Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad, Hercules, CA). The blot was destained in Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad, Hercules, CA) for ~ 5 min, and the blot image was captured using a Bio-Rad GS-800 densitometer with the supplied Quantity One software.

Two bands were excised from the stained membrane: a band with a molecular weight of ~133 kDa, corresponding to full-length Cry1Ac protein, and a band with a molecular weight of ~75 kDa that, by purity analysis, represented ~10% of the total protein. N-terminal sequence analysis was performed on each of the excised bands for 15 cycles using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and 785A Programmable Absorbance Dectector and Procise Control Software (version 1.1a) was used. Chromatographic data were collected using Atlas software (Thermo Scientific, Woburn, MA) A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically 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 (β-lactoglobulin, Applied Biosystems, Foster City, CA) was analyzed before and after the test protein to verify that the sequencer met acceptable performance criteria for repetitive yield and sequence identity.

5.5 Glycosylation Analysis

Glycosylation analysis was used to determine whether the MON 87701-produced Cry1Ac protein was post-translationally modified with covalently bound carbohydrate moieties. Aliquots of the MON 87701-produced Cry1Ac protein, the *E. coli*-produced Cry1Ac reference protein, and the positive controls, transferrin and horseradish peroxidase (both from Sigma, St. Louis, MO), were each mixed with 5× sample buffer. These samples were heated at 96 °C for 4 min, cooled, and loaded on a tris-glycine 4-20% gradient polyacrylamide gel. Both *E. coli*- and MON 87701-produced Cry1Ac proteins were loaded at 50 and 100 ng purity-corrected for the full length protein. The Precision Plus Dual Color pre-stained protein molecular weight was loaded to verify electrotransfer of the proteins to the membrane and as markers for molecular weight, and the CandyCane Glycoprotein Molecular Weight Standard (Molecular Probes, Eugene, OR) was loaded as markers for molecular weight and to provide additional positive and negative controls for glycosylation. Electrophoresis was performed at a constant voltage of 150 V for 15 min, then 200 V for 55 min.

Electrotransfer to a PVDF membrane was performed at a constant voltage of 25 V for 80 min.

Carbohydrate detection was performed directly on the PVDF membrane using the Pro-Q® Emerald 488 Glycoprotein Gel and Blot Stain Kit (Molecular Probes, Eugene, OR). All steps were performed at room temperature. The PVDF membrane was fixed in two changes of 25 ml each of a solution containing 50% methanol and 5% glacial acetic acid, with the first fix step for 60 min and the second overnight. Two 15 min washes (50 ml each) of 3% (v/v) glacial acetic acid (wash solution), were followed by a 20 min oxidation in 25 ml of the kit-supplied oxidizing solution. After oxidation, three 15 min washes in wash solution prepared the membrane for staining. The blot was incubated in 25 ml of Pro-Q Emerald Staining Solution prepared as recommended for blot staining. After 75 min of staining in the dark, two 15 min washes were followed by one 20 min wash, all in 50 ml of wash solution. The final wash cycles included two 1 min deionized water washes followed by three 25 ml, 5 min washes in 100% methanol. Last, the blot was washed for 10 min in deionized water. The blot was then scanned using the BioRad Molecular Imager FX using the Alexa 488 illumination setting in order to visualize fluorescent signal from the glycosylated proteins.

After glycosylation analysis, the blot was stained to visualize the proteins present on the membrane. The blot was stained for 2 min in Coomassie Brilliant Blue R-250 Staining Solution (BioRad). The blot was destained in Coomassie Brilliant Blue R-250 Destaining Solution (BioRad) for ~ 15 min, and the blot image was captured using a BioRad GS-800 densitometer with the supplied Quantity One software.

5.6 Functional Activity Assay

The functional activities of the MON 87701-produced Cry1Ac protein and the *E. coli*-produced Cry1Ac reference protein were compared using an insect bioassay. Aliquots of MON 87701-produced Cry1Ac protein and *E. coli*-produced Cry1Ac reference protein were transferred to the Monsanto Ecological Technology Center for testing in an assay using corn earworm (CEW; *Helicoverpa zea)*, an insect species known to be susceptible to Cry1Ac protein (Sivasupramaniam et al., 1999). Dose-response assays were performed for Cry1Ac proteins from both sources in parallel and assays were repeated on three separate days to estimate the mean EC_{50} value, the effective concentration necessary to inhibit CEW growth by 50% relative to the control response. Bioassay methodology and data analysis are described in Appendix 3.

5.7 Storage Stability

The short-term stability of the MON 87701-produced Cry1Ac protein stored in a -80 °C freezer was evaluated by comparing the purity and molecular weight values obtained on Day 0 to the purity and molecular weight values obtained on Day 48 of storage. Aliquots of the MON 87701-produced Cry1Ac protein were stored in a

-80 °C freezer for the duration of the experimental phase of the study. Day 0 stability analysis corresponds to the molecular weight and purity determination described in Section 5.3. On Day 48, a sample was removed from storage, mixed with 5× sample buffer to a final total protein concentration of 17 ng/µl, heated at 96.5 °C for 5 min, and loaded in duplicate (95, 189, and 284 ng protein per lane) onto a tris-glycine 4-20% gradient gel. Staining and densitometric analysis were performed as described in Section 5.3 for Molecular Weight and Purity Estimation.

5.8 Equivalence Criteria

The equivalence of the MON 87701- and *E. coli*-produced Cry1Ac proteins was established by direct comparison of the proteins in terms of their apparent molecular weight, immunoreactivity with anti-Cry1Ac antibodies, functional activity and glycosylation state. The criteria for each of these tests were pre-established during developmental work taking into consideration the inherent variability of each analytical method. These equivalence criteria were as follows:

A. The apparent molecular weight of the full-length MON 87701-produced protein is within ± 5% of the *E. coli*-produced Cry1Ac protein.

B. The immunoreactivity of the MON 87701-produced Cry1Ac protein with Cry1Ac-specific antibodies is within ± 35% of the *E. coli*-produced Cry1Ac immunoreactivity.

C. The functional activity of the MON 87701-produced protein, expressed as an EC_{50} value, is less than 3-fold different than the EC_{50} value for the *E. coli* produced protein.

D. The MON 87701-produced and the *E. coli*-produced Cry1Ac are not glycosylated.

If these criteria are met then the proteins are considered equivalent to one another.

6.0 Data Rejected or Not Reported

Two runs of the protein concentration analysis were rejected. The first run was rejected because the sample was diluted inappropriately and gave an anomalous result. The second run was rejected because the concentration of the BSA control did not meet the acceptance criteria stated in the relevant SOP. One run of the glycosylation analysis was rejected because one of the blot fixation intervals substantially exceeded the recommended time. One run of the insect bioactivity assay was rejected because the visually estimated EC_{50} for the *E. coli*-produced reference protein was outside the typical range (e.g. ~10 times the typical value) observed for that reference protein. Additionally, the average mass of the surviving control insects was extremely low, which is likely indicative of poor quality insects. One run of the stability analysis was not reported because the storage stability period evaluated did not fully bracket all other experimental analyses in the study.

7.0 Study Amendments and Deviations

There were no amendments to the study. There were six deviations, none of which had an impact on the study. The deviations are detailed in Appendix 4.

8.0 Control of Bias and Quality Measures

Controls and standards were included with each analysis. Replicate analyses were used for the insect bioassay, and replicate samples were used for concentration determination, comparative immunoblot, purity and molecular weight determination, stability, and glycosylation analyses.

9.0 Results and Discussion

9.1 Protein Concentration

The concentration of total protein in the MON 87701-produced Cry1Ac sample was calculated by amino acid analysis to be 42.4 μg/ml.

9.2 Molecular Weight and Purity Analysis

For molecular weight and purity analysis, MON 87701-produced Cry1Ac protein was separated using SDS-PAGE and stained using a Silver Staining Kit (Owl Separation Systems) (Figure 1, lanes 3-8). The full-length MON 87701-produced Cry1Ac protein had an estimated molecular weight of 133.4 kDa (Table 1), and migrated to the same position on the SDS-PAGE gel as the *E. coli*-produced Cry1Ac reference standard (Figure 1, lane 9). The apparent molecular weight of the full-length *E. coli*-produced Cry1Ac reference protein, as reported on its COA (Appendix 1) is 131.7 kDa. The difference in the estimated molecular weights between the MON 87701-produced and *E. coli*-produced Cry1Ac full-length proteins was 1.3% (Table 2). Because the experimentally determined difference in apparent molecular weight met the pre-set acceptance criteria (≤ 5% difference), the MON 87701-produced and *E. coli*-produced Cry1Ac proteins are considered equivalent based on their molecular weights.

Purity of the MON 87701-produced Cry1Ac protein was evaluated using densitometric analysis. The data are summarized in Table 1. An immunoblot completed prior to the initiation of the study, which had been incubated with anti-Cry1Ac antibody and subjected to a long exposure to bring up minor bands, was used to determine which stained bands would be included in the total purity calculation for Cry1Ac within the study. Based on that immunoblot (Appendix 2) which showed numerous immunoreactive bands between ~133 kDa (the size of full-length Cry1Ac) and ~64 kDa, all stained bands on the purity gel between these molecular weights were included to determine total purity of the MON 87701-produced Cry1Ac protein. In insects, full-length Cry1Ac is processed following ingestion to its bioactive form (the trypsin-resistant core, which is ~ 55 kDa) by removal of an ~28 amino acid N-terminal peptide and removal of a C-terminal portion that represents approximately half the protein (Lightwood et al., 2000). The C-terminal portion is typically cleaved

during sequential proteolysis in the insect gut yielding intermediate fragments of different lengths (Choma et al., 1990; Dıaz-Mendoza et al. 2007). Proteolysis of the C-terminus has no effect on protein functionality and specificity toward target insects (de Maagd et al., 2003) because it has no biological activity. Furthermore, it has been shown for Cry1 proteins that their C-terminal regions are in general highly susceptible to the degradation with proteolytic enzymes present in the cells or released during protein purification (Clairmont et al. 1998; Gao et al., 2006). In the MON 87701-derived protein sample, N-terminal sequencing of the most abundant truncated immunoreactive protein (~75 kDa) was consistent with an intact Cry1Ac N-terminus and, hence, indicated that observed fragments resulted from degradation of the C-terminus as expected. Therefore, the bands included in the determination of Cry1Ac protein total purity, all of which are larger than the size of the Cry1Ac trypsin-resistant core, are expected to contribute to the insecticidal activity of the preparation.

Summing the stained bands between ~133 kDa and ~64 kDa yielded a total Cry1Ac protein purity of 77%. The analysis also determined the purity of the full-length Cry1Ac protein (~133 kDa), which was found to represent 43% of the total protein. Based upon the total protein concentration and the purity analyses, the total Cry1Ac protein concentration in the MON 87701-produced Cry1Ac protein sample is 32.6 µg/ml (42.4 µg/ml x 0.77), and full-length Cry1Ac protein concentration is 18.2 µg/ml (42.4 µg/ml x 0.43).

9.3 Protein Identity

The identity of the MON 87701-produced Cry1Ac protein was confirmed using three analytical methods: immunoblot analysis using anti-Cry1Ac antibody, MALDI-TOF MS tryptic mass map analysis, and N-terminal sequencing.

9.3.1 Immunoblot Analysis Using Anti-Cry1Ac Antibody

The MON 87701-produced Cry1Ac protein and corresponding *E. coli*-produced reference protein were loaded on the same gel, which was used for immunoblot analysis. The amount of each sample loaded was based on the concentration of the full-length Cry1Ac protein. The major immunoreactive band observed migrated with an apparent molecular weight of ~133 kDa (Figure 2), the expected molecular weight of the full-length Cry1Ac protein, and was present in both the MON 87701-produced and *E. coli*-produced Cry1Ac samples. As expected, the immunoreactive signal increased with increased loading levels of both the MON 87701-produced and *E. coli*-produced proteins. Faint immunoreactive bands with molecular weights below ~133 kDa represent degradation products of Cry1Ac. Faint immunoreactive bands with molecular weights around 250 kDa were also observed, and most likely represent aggregation of the Cry1Ac protein. Both protein degradation and protein aggregation are commonly observed during protein purification of Cry proteins. Cry proteins naturally aggregate into crystal

structures as has been observed for Cry1A proteins (Güereca and Bravo, 1998), while degradation occurs primarily due to the release of endogenous proteases during the purification procedure (Gao et al. 2006).

Densitometric analysis was conducted to compare the immunoreactivity of full-length MON 87701-produced and *E. coli*-produced Cry1Ac proteins. The relative immunoreactivity of each protein with Cry1Ac-specific antibody was determined by averaging intensity values of six protein bands corresponding to the full-length MON 87701-produced Cry1Ac protein and six bands corresponding to the full-length *E. coli*-produced Cry1Ac protein (Table 3). The averaged band intensity of the signal from the MON 87701-produced Cry1Ac lanes was 33.3% less than the averaged band intensity of the signal from the *E. coli*-produced Cry1Ac lanes. The observed difference was within the pre-set acceptance criteria for immunoreactivity (±35% difference). Thus, the immunoblot analysis established identity of the MON 87701-produced Cry1Ac protein and demonstrated that the MON 87701-produced and *E. coli*-produced Cry1Ac proteins are equivalent based on their immunoreactivity with Cry1Ac-specific antibody.

9.3.2 MALDI-TOF Tryptic Mass Map Analysis

The MON 87701-produced, full-length Cry1Ac protein was assessed by MALDI-TOF mass spectrometry. Prior to analysis, the protein sample was chemically reduced, alkylated, and digested with trypsin. The ability to identify a protein using this method is dependent on matching a sufficient number of observed mass fragments to expected (theoretical) mass fragments. In general, a protein identification made by peptide mapping is considered to be reliable if the measured coverage of the sequence is 15% or higher with a minimum of five matched peptides (Jensen *et al*., 1997). There were 70 peptides (out of 144 masses) identified that matched the expected masses of the Cry1Ac trypsin-digested peptides (Table 4). The identified masses were used to assemble a coverage map that indicates those matched peptide sequences within the protein sequence (Figure 3). The protein was confirmed as Cry1Ac based on the result that a significant portion of the protein, 787 of 1182 amino acids (66.6%), was contained in theoretical mass fragments that matched observed mass fragments.

9.3.3 N-terminal Sequence Analysis

N-terminal sequencing performed on MON 87701-produced Cry1Ac protein identified seven strong and two tenuous amino acids that matched the predicted N-terminal sequence for Cry1Ac containing four amino acids derived from CTP1 (Figure 4, panel A). The amino acid cysteine is shown in the predicted sequence at position one based on the coding sequence of the Cry1Ac construct in MON 87701. However, cysteine is unstable during the acid hydrolysis reaction used for N-terminal sequencing, and is usually not explicitly observed (Inglis and Liu, 1970). The clear identification of amino acids in subsequent cycles of the

sequencing analysis confirmed that an unidentified amino acid was present at position one. The N-terminal sequencing results for MON 87701-produced Cry1Ac protein were consistent with the sequencing results for the *E. coli*-produced Cry1Ac protein, which was engineered to contain a cysteine as the first amino acid but which also showed an unidentified amino acid at position one (see Appendix 1).

In addition to analysis of the full-length protein, a second band of approximately 75 kDa, which represented about 10% of total protein based on purity analysis, was also analyzed. Due to the reduced amount of this protein compared to the full-length protein, the signal intensity of the peaks in this analysis was low, and only three strong and three tenuous amino acids were identified. While this number of identified amino acids was insufficient to explicitly align the derived sequence to a known N-terminal sequence, the sequence obtained was consistent with the N-terminal sequence for Cry1Ac (Figure 4, panel B), suggesting that this protein is a truncated Cry1Ac protein.

9.4 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, strains of *E. coli* used for recombinant protein expression lack the necessary biochemical pathways required for protein glycosylation. To test whether post-translational glycosylation of the MON 87701-produced Cry1Ac protein occurred, it was analyzed for the presence of covalently bound carbohydrate moieties. The *E. coli*-produced Cry1Ac reference protein, horseradish peroxidase (positive control), and transferrin (positive control) were analyzed concurrently with the MON 87701-produced Cry1Ac protein. The results of this analysis are presented in Figure 5A. The positive controls were detected at the expected molecular weights, in a concentration-dependent manner (Figure 5A, lanes 2-5). No detectable signal was observed for either the MON 87701-produced or *E. coli*-produced Cry1Ac proteins (Figure 5A, lanes 6-9) at the expected molecular weight on the blot. Post-analysis staining of this blot with Coomassie stain to detect total protein confirmed that both MON 87701-produced and *E. coli*-produced Cry1Ac proteins were present on the blot at similar protein staining intensities as the positive controls (Figure 5B, lanes 6-9). Thus, the MON 87701-produced protein has been determined to not be glycosylated and is equivalent to the *E. coli*-produced Cry1Ac reference protein with respect to glycosylation.

9.5 Functional Activity

The functional activity of MON 87701-produced Cry1Ac protein was determined in an insect bioassay that assesses the impact of the Cry1Ac protein on growth of the test insect, corn earworm. The impact of Cry1Ac on insect growth is expressed as an

EC_{50} value, which represents the effective concentration of protein necessary to inhibit insect growth by 50% relative to a control population of insects not exposed to the insecticidal protein. The mean EC_{50} value determined for the MON 87701-produced Cry1Ac protein was 0.0039 µg Cry1Ac/ml diet. This EC_{50} value was very similar to the mean EC_{50} value of 0.0036 µg Cry1Ac/ml diet obtained for the *E. coli*-produced reference protein in the same assay. Because the difference between these values was within the pre-set acceptance criteria (<3 fold difference) for establishing equivalence, MON 87701-produced Cry1Ac is determined to have equivalent functional activity to that of *E. coli*-produced Cry1Ac.

A sub-report which summarizes the methods, results, and statistical evaluation of the functional activity assay for this study is attached as Appendix 3.

9.6 Storage Stability

Stability of the MON 87701-produced Cry1Ac protein stored in a -80 °C freezer was assessed after a 48 day storage period that encompasses the experimental phase of the study (Table 5, Figure 6). Stability was evaluated by comparison of the molecular weight and purity of the protein after storage to the molecular weight and purity values determined at the initiation of the study (Day 0). The molecular weight of the full-length Cry1Ac protein was estimated to be 130.6 kDa on Day 48. This value differed from the molecular weights obtained on Day 0 (133.4 kDa) by 2.1% (Table 6). The total purity value for the MON 87701-produced Cry1Ac protein on day 48 was 72%. This value differed from the total purity obtained on day 0 (77%) by 6.5% (Table 6). The differences in the purity and molecular weight were both within the pre-set acceptance criteria of ±10%; therefore, based on both molecular weight and purity, the MON 87701-produced Cry1Ac protein was established to be stable when stored in a -80 °C freezer for 48 days.

10.0 Conclusions

A panel of analytical techniques was used to characterize the purified MON 87701-produced Cry1Ac protein. Identity of the purified MON 87701-produced Cry1Ac protein was established by recognition with anti-Cry1Ac antibodies, identification of nine amino acids of the expected N-terminus by amino acid sequencing, and detection of tryptic peptide masses that yielded greater than 66% overall coverage of the expected Cry1Ac protein sequence. Purity of the MON 87701-produced Cry1Ac was 77%. The total Cry1Ac protein concentration in the MON 87701-derived sample was 32.6 µg/ml, providing sufficient levels to characterize the physiochemical and functional properties of the MON 87701-produced Cry1Ac protein. The apparent molecular weight of the full-length MON 87701-produced Cry1Ac protein was 133.4 kDa. The MON 87701-produced Cry1Ac protein was determined to not be glycosylated. Additionally, the functional activity of the MON 87701-produced Cry1Ac protein, expressed as a mean EC_{50} value, was 0.0039 µg Cry1Ac/ml. The MON 87701-produced Cry1Ac protein was

stable for at least 48 days when stored in a -80 °C freezer, which encompasses the duration of the experimental phase of this study.

The equivalence of the MON 87701-produced and *E. coli*-produced Cry1Ac proteins was evaluated by comparing their full-length molecular weights, immunoreactivity with anti-Cry1Ac antibodies, glycosylation status, and functional activity. The results obtained demonstrate that the MON 87701-produced Cry1Ac protein is equivalent to the *E. coli*-produced Cry1Ac protein. The established equivalence justifies the use of the *E. coli*-produced Cry1Ac protein in studies conducted to assess the safety of the Cry1Ac protein present in MON 87701 soybean.

11.0 References

Aebersold, R. (1993). Mass spectrometry of proteins and peptides in biotechnology. *Curr. Opin. Biotechnol.* **4**:412-419.

Arackal, S.M., Lawry, K.R., Song, Z., Groat, J.R., Rice, J.F., and Masucci, J.D. (2008). Molecular Analysis of Insect-Protected Soybean MON 87701. Monsanto Technical Report MSL-0021167. St. Louis, MO.

Bagshaw, R. D., Callahan, J. W., and Mahuran, D. J. (2000). Desalting of in-gel-digested protein sample with mini-C18 columns for matrix-assisted laser desorption ionization time of flight peptide mass fingerprinting. *Anal. Biochem.* **284**:432-435.

Billeci, T.M. and Stults, J.T. (1993). Tryptic mapping of recombinant proteins by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* **65**:1709-1716.

Choma, C.T., Surewicz, W.K., Carey, P. R., Pozsgay, M., Raynor, T., and Kaplan, H. (1990). Unusual proteolysis of the protoxin and toxin from Bacillus thuringiensis. *European Journal of Biochemistry* 189, 523-527.

Clarimont, F.R., Milnet, R.E., Pham, V.T., Carriw, M.B., and Kaplan, H. (1998). Role of DNA in the Activation of the Cry1A Insecticidal Crystal Protein from *Bacillus thuringiensis. J. Biol. Chem*. 273, 9292-9296.

De Maagd, R.A., Bravo, A., Berry, C., Crickmore, N., and Schnepf, H.E. (2003). Structure, diversity and evolution of protein toxins from spore-forming entomopathogenic bacteria. *Ann. Rev. Genet*. 37, 409-443.

Dıaz-Mendoza, M., Perez-Farinos, G., Hernandez-Crespo, P., Castanera, P., and Ortego, F. (2007). Proteolytic processing of native Cry1Ab toxin by midgut extracts and purified trypsins from the Mediterranean corn borer Sesamia nonagrioides. *J. Insect. Physiol.* 53, 428–435.

Gao, Y., Fencil, K. J., Xu, X., Schwedler, D. A., Gilbert, J. R., and Herman, R. A. (2006). Purification and characterization of a chimeric Cry1F ä-endotoxin expressed in transgenic cotton plant. *J. Agric. Food Chem.* **54**:829-835.

Güereca, L. and Bravo, A. (1999). The oligomeric state of Bacillus thuringiensis Cry toxins in solution. *Biochimica et Biophysica Acta* **1429**: 342-350.

Hunkapillar, M.W., Hewick, R.M., Dreyer, W. J., and Hood, L.E. (1983). High-sensitivity sequencing with gas-phase sequenator. *Methods Enzymol.* **91**:399-413.

Inglis, A.S. and Liu, T. (1970). The stability of cysteine and cystine during acid hydrolysis of proteins and peptides. *J. Biol. Chem.* **245**:112-116.

Jensen, O.L., A.V. Podtelejnikov, and Mann, M. (1997). Identification of the components of simple protein mixtures by high-accuracy peptide mass mapping and database searching. *Anal. Chem.* **69**:4741-4750.

Lightwood, D.J., Ellar, D.J., and Jarrett, P. (2000). Role of Proteolysis in Determining the Potency of Bacillus thruingiensis Cry1Ac δ-Endotoxin. *Appl. Environ. Micro*. **66**:5174-5181.

Rademacher, T. W., Parekh, R. B., and Dwek, R. A. (1988). Glycobiology. *Annu. Rev. Biochem*. **57**:785-838.

Sivasupramaniam, S., Kabuye, V., Holden, L., Roberts, J.,Gouzov, V., and Shappley, Z. (1999). Independent and Combined Activity of Cry1Ac and Cry2Ab2 in *in vitro* and Plant Tissue Studies. Monsanto Technical Report MSL-16204. St. Louis, MO.

Williams, K., LoPresti, M., and Stone, K. (1997). Internal protein sequencing of SDS-PAGE-separated proteins: Optimization of an in-gel digest protocol. *Tech. in Protein Chem.* **8**:79-98.

Table 1. Molecular Weight and Purity of MON 87701-Produced Cry1Ac Protein

Cry1Ac Sample Loaded	Apparent Mol. Wt. (kDa) of Full-Length Cry1Ac	Total Purity[1] (%)	Full-length Cry1Ac Purity[2] (%)
95 ng Load (Figure 1, lane 3)	133.2	80.9	49.3
95 ng Load (Figure 1, lane 4)	132.4	79.3	48.4
189 ng Load (Figure 1, lane 5)	133.0	74.9	40.0
189 ng Load (Figure 1, lane 6)	133.3	76.5	40.4
284 ng Load (Figure 1, lane 7)	133.9	75.1	40.6
284 ng Load (Figure 1, lane 8)	134.5	75.4	41.0
Average Values (Total for each lane / 6)	**133.4**	**77**[3]	**43**[3]

[1] Total purity as determined by summing purity of stained bands from ~133 kDa to ~64 kDa.
[2] Purity of major stained band, at ~133 kDa.
[3] Average purity is rounded to the nearest whole number.

Table 2. Molecular Weight Difference Between Full-Length MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins.

Molecular Weight of Full-Length MON 87701-Produced Cry1Ac Protein[1]	Molecular Weight of *E. coli*-Produced Cry1Ac Protein[2]	Percent Difference from *E. coli*-Produced Cry1Ac Protein[3]
133.4 kDa	131.7 kDa	1.3 %

[1] Reference Table 1 for the molecular weight of the full-length MON 87701-produced protein.
[2] Reference the Orion 10000804 COA (Appendix 1) for the molecular weight of the full-length *E. coli*-produced reference protein.
[3] Percent difference was calculated as follows: $\frac{133.4 - 131.7}{133.4} \times 100\% = 1.3\%$

Table 3. Comparison of Immunoreactive Signal Between Full-Length MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins.

Load Amount (ng)	MON 87701-produced Protein Signal Density[1]	*E. coli*-produced Protein Signal Density[1]
10	1.288	1.419
10	1.955	1.798
20	2.908	4.559
20	2.987	3.706
30	4.214	6.547
30	4.140	8.199
Sum	**17.492**	**26.228**
Average Density	**2.915**	**4.371**
Percent difference[2]	**33.3%**	

[1] The density of each band was determined by image analysis of the quantitative western blot shown in Figure 2. Values shown for signal density are contour quantity, i.e. average OD x contour area in mm^2.

[2] Percent difference is calculated using the equation:

$$\frac{\left| AverageDensityEcoli - AverageDensityPlant \right|}{AverageDensityEcoli} \times 100 = PercentDifference$$

Table 4. Summary of the Tryptic Masses Identified for the Full-Length MON 87701-Produced Cry1Ac Protein Using MALDI-TOF Mass Spectrometry.[1]

1	2	3	4	5	6				
AC-1	AC-2	DHB-1	DHB-2	SA-1	SA-2	Expected	Diff[2]	Fragment	Sequence[3]
579.40		579.38				579.33	0.07 (1)	754-757	YQLR
589.39		589.37				589.28 589.31	0.11 (1) 0.08 (1)	1028-1032 229-232	VCPGR DWIR
611.42						611.36	0.06 (1)	941-945	VHSIR
621.46		621.45				621.37	0.09 (1)	1033-1037	GYILR
649.46		649.44				649.37	0.09 (1)	258-262	TYPIR
688.10						688.37	0.27 (1)	98-103	NQAISR
727.46		727.45				727.35	0.11 (1)	233-237	YNQFR
731.46		731.46				731.36	0.10 (1)	428-433	QGFSHR
764.51		764.49				764.39	0.12 (1)	92-97	IEEFAR
		781.51				781.38	0.13 (3)	197-202	YNDLTR
804.41		804.58				804.46	0.05 (1)	263-269	TVSQLTR
816.52		816.52				816.40	0.12 (1)	222-228	VWGPDSR
832.44		832.44	832.62			832.48	0.04 (1)	671-677 743-749	ELSEKVK IDESKLK
854.43	854.54	854.55				854.41	0.02 (1)	1119-1125	SYTDGRR
907.60	907.74	907.61				907.46	0.14 (1)	178-185	DVSVFGQR
940.66		940.67				940.51	0.15 (1)	365-372	TLSSTLYR
976.66	976.80	976.67				976.50	0.16 (1)	434-441	LSHVSMFR
1027.69						1027.53	0.16 (1)	696-703	DINRQPER
1038.66	1038.82	1038.68		1038.49	1038.65	1038.50	0.16 (1)	214-221	WYNTGLER
1066.62		1066.66		1066.55		1066.43	0.19 (1)	1126-1133	ENPCEFNR
1074.72		1074.75				1074.55	0.17 (1)	286-296	GSAQGIEGSIR
		1078.73				1078.55	0.18 (3)	687-695	NLLQDSNFK
1144.74	1144.92	1144.78	1145.00	1144.73	1144.84	1144.57	0.17 (1)	454-462	APMFSWIHR

Table 4, continued

1	2	3	4	5	6				
AC-1	AC-2	DHB-1	DHB-2	SA-1	SA-2	Expected	Diff[2]	Fragment	Sequence[3]
1203.87	1204.05	1203.90		1203.83		1203.68	0.19 (1)	354-364	IVAQLGQGVYR
1216.77	1216.96	1216.82				1216.60	0.17 (1)	505-515	LNSSGNNIQNR
1237.78	1237.97	1237.83	1238.08	1237.76		1237.60	0.18 (1)	186-196	WGFDAATINSR
1249.85		1249.88				1249.65	0.20 (1)	595-606	NFSGTAGVIIDR
1253.84	1254.04	1253.87		1253.83		1253.65	0.19 (1)	442-453	SGFSNSSVSIIR
1258.84	1259.04	1258.87				1258.65	0.19 (1)	203-213	LIGNYTDHAVR
1303.85	1304.05	1303.90	1304.23	1303.85	1304.04	1303.67	0.18 (1)	970-980	IFTAFSLYDAR
		1352.96				1352.71	0.25 (3)	1137-1148	DYTPLPVGYVTK
1398.90		1398.95				1398.67	0.23 (1)	120-131	EWEADPTNPALR
1424.88	1425.07	1424.90				1424.65	0.23 (1)	999-1010	GHVDVEEQNNQR
		1552.08		1552.01		1551.81	0.27 (3)	896-907	EKLEWETNIVYK
	1577.36	1577.19		1577.10		1576.81	0.55 (2)	687-699	NLLQDSNFKDINR
						1576.87	0.49 (2)	628-642	AVNALFTSTNQLGLK
1599.06						1598.71	0.35 (1)	1125-1136	RENPCEFNRGYR
1704.09	1704.37	1704.21	1704.57	1704.14		1703.88	0.21 (1)	516-530	GYIEVPIHFPSTSTR
	1795.38	1795.16	1795.59	1795.10		1794.87	0.51 (2)	704-721	GWGGSTGITIQGGDDVFK
1801.13	1801.40	1801.26	1801.64	1801.15		1800.87	0.26 (1)	758-772	GYIEDSQDLEIYSIR
1901.15	1901.47	1901.27	1901.67	1901.21	1901.50	1900.91	0.24 (1)	270-285	EIYTNPVLENFDGSFR
1902.15	1902.49	1902.28		1902.21	1902.47	1901.82	0.33 (1)	1119-1133	SYTDGRRENPCEFNR
						1902.96	0.81 (1)	104-119	LEGLSNLYQIYAESFR
1904.22	1904.49	1904.24	1903.77	1904.24	1904.48	1904.06	0.16 (1)	625-642	AQKAVNALFTSTNQLGLK
1956.29	1956.59	1956.39	1956.82	1956.33	1956.58	1956.01	0.28 (1)	1011-1027	SVLVVPEWEAEVSQEVR
		2088.38				2088.94	0.56 (1)	1100-1118	GYNEAPSVPADYASVYEEK
2098.42	2098.82	2098.55	2098.94	2098.50	2098.81	2098.15	0.27 (1)	865-883	LGNLEFLEEKPLVGEALAR
		2118.44		2118.41		2118.11	0.33 (3)	463-482	SAEFNNIIASDSITQIPAVK
	2142.76	2142.42	2143.20	2142.41	2142.83	2142.08	0.68 (2)	607-624	FEFIPVTATLEAEYNLER
2149.32	2149.71	2149.43				2149.05	0.27 (1)	408-427	SGTVDSLDEIPPQNNNVPPR
2195.47	2195.84	2195.63	2196.12	2195.51	2195.82	2195.16	0.31 (1)	239-257	ELTLTVLDIVSLFPNYDSR

Table 4, continued

1 AC-1	2 AC-2	3 DHB-1	4 DHB-2	5 SA-1	6 SA-2	Expected	Diff[2]	Fragment	Sequence[3]
2211.41	2211.76	2211.49	2212.03	2211.48	2211.80	2211.12	0.29 (1)	483-504	GNFLFNGSVISGPGFTGGDLVR
						2211.13	0.28 (1)	434-453	LSHVSMFRSGFSNSSVSIIR
		2278.60				2278.14	0.46 (3)	203-221	LIGNYTDHAVRWYNTGLER
						2278.21	0.39 (3)	516-534	GYIEVPIHFPSTSTRYRVR
			2376.24	2375.60		2375.24	1.00 (4)	777-799	HETVNVPGTGSLWPLSAQSPIGK
2616.65	2617.12	2616.80	2617.31	2616.79	2617.19	2616.36	0.29 (1)	946-969	EAYLPELSVIPGVNAAIFEELEGR
				3284.93		3284.61 Ma	0.32 (5)	104-131	LEGLSNLYQIYAESFREWEADPTNPALR
				3318.05		3318.71 Ma	0.66 (5)	258-285	TYPIRTVSQLTREIYTNPVLENFDGSFR
				3365.91		3365.71 Ma	0.20 (5)	911-940	ESVDALFVNSQYDQLQADTNIAMIHAADKR
			3374.20			3374.77 Ma	0.57 (4)	595-624	NFSGTAGVIIDRFEFIPVTATLEAEYNLER
	3731.09	3732.34		3731.25	3732.14	3731.12 Ma	0.03 (2)	373-406	RPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYR
					4371.09	4370.75 Ma	0.34 (6)	704-742	GWGGSTGITIQGGDDVFKENYVTLSGTFDECYPTYLYQK
			4676.21		4676.70	4675.45 Ma	0.76 (4)	136-177	IQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLR
	5564.48	5564.43	5563.75			5564.43 Ma	0.05 (2)	136-185	IQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVS VFGQR
					6142.52	6141.69 Ma	0.83 (6)	537-594	YASVTPIHLNVNWGNSSIFSNTVPATATSLDNLQSSDFGYFESA NAFTSSLGNIVGVR

[1] Only experimental masses that matched expected masses are listed in the table. All mass values shown were rounded to two decimal places. Columns 1-6 represent experimentally observed masses from Extract 1 or Extract 2 of trypsinized protein mixed with matices α-cyano-4-hydroxy cinnamic acid (AC), dihydroxybenzoic acid (DHB), or 3,5-dimethoxy-4-hydroxycinnamic acid (SA).
[2] Diff represents the difference between the experimental mass and the expected mass; the number in parenthesis indicates the column containing the experimental mass used to calculate the difference.
[3] Sixty-eight unique sequences are shown. Two of the 70 fragments identified were methionine-oxidized versions of two sequences shown.
[4] Ma indicates mass averaged value. Unless Ma is indicated, expected mass is monoisotopic mass.

Table 5. Molecular Weight and Purity Estimation for the Storage Stability (Day 48) Sample of MON 87701-Produced Cry1Ac Using SDS-PAGE Analysis.

Cry1Ac Sample Loaded	Apparent Mol. Wt. (kDa) of Full-Length Cry1Ac	Total Purity [1] (%)	Full-length Cry1Ac Purity [2] (%)
95 ng Load (Figure 6, lane 3)	131.8	72.4	40.7
95 ng Load (Figure 6, lane 4)	130.3	75.5	44.9
189 ng Load (Figure 6, lane 5)	129.7	71.5	45.4
189 ng Load (Figure 6, lane 6)	129.6	69.6	40.1
284 ng Load (Figure 6, lane 7)	130.3	72.1	39.2
284 ng Load (Figure 6, lane 8)	131.7	73.3	39.1
Average Values (Total for each lane / 6)	**130.6**	**72**[3]	**42**[3]

[1] Total purity as determined by summing purity of stained bands from ~133 kDa to ~64 kDa.
[2] Purity of major stained band, at ~133 kDa.
[3] Average purity is rounded to the nearest whole number.

Table 6. Storage Stability of the Cry1Ac MON 87701-Produced Protein Stored in a -80 °C Freezer for 48 Days.

	Day 0[1]	Day 48[2]	Percent Difference[3] (Day 48 vs. Day 0)
Apparent Molecular Weight (kDa)	133.4	130.6	2.1%
Total Cry1Ac Purity (%)	77	72	6.5%

[1] See Table 1 for the apparent molecular weight and total purity of the Day 0 sample.
[2] See Table 5 for the apparent molecular weight and total purity of the Day 48 sample.
[3] Percent difference for apparent molecular weight or purity was calculated as follows:

$$\frac{\left|Day0 - Day48\right|}{Day0} \times 100 = \%Difference$$

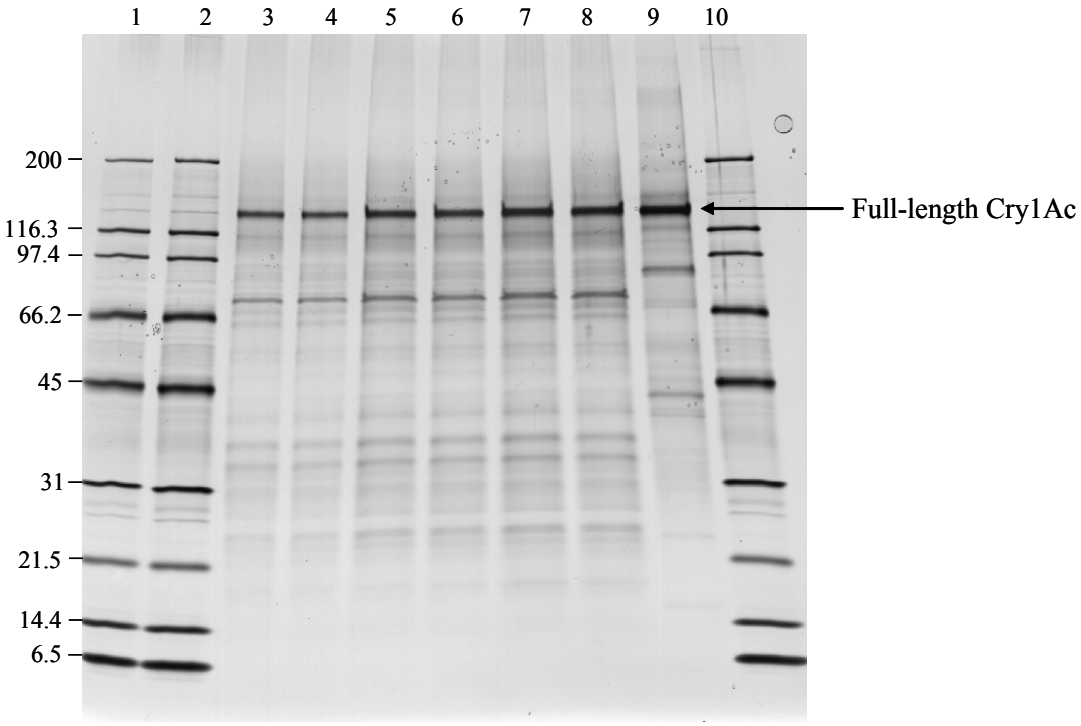


Figure 1. Purity and Molecular Weight Analysis of the MON 87701-Produced Cry1Ac Protein.

Aliquots of the MON 87701-produced Cry1Ac protein and *E. coli*-produced Cry1Ac reference protein were separated by SDS-PAGE, followed by silver staining. Approximate molecular weights (kDa) are shown on the left and correspond to the marker loaded in lanes 1, 2, and 10.

Lane	Sample	Amount loaded (ng)
1	BioRad Broad Range Marker	360
2	BioRad Broad Range Marker	360
3	MON 87701-produced Cry1Ac protein	95
4	MON 87701-produced Cry1Ac protein	95
5	MON 87701-produced Cry1Ac protein	189
6	MON 87701-produced Cry1Ac protein	189
7	MON 87701-produced Cry1Ac protein	284
8	MON 87701-produced Cry1Ac protein	284
9	*E. coli*-produced Cry1Ac protein	198
10	BioRad Broad Range Marker	360

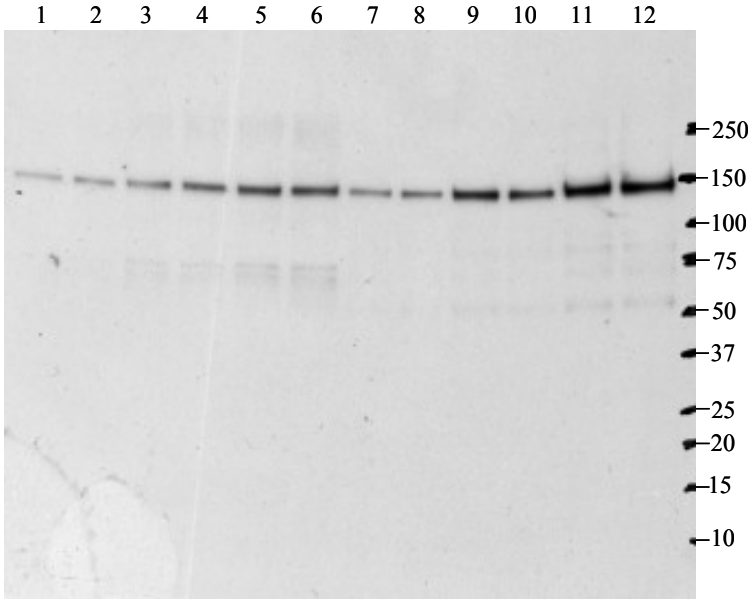


Figure 2. Western Blot Analysis and Immunoreactivity of MON 87701-Produced and *E. coli*-Produced Cry1Ac Proteins.

Aliquots of the plant-produced Cry1Ac protein and *E. coli*-produced Cry1Ac reference protein were separated by SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated with affinity-purified anti-Cry1Ac antibody, and immunoreactive bands were visualized using an ECL system. Approximate molecular weights (kDa) are shown on the right and correspond to the tick marks indicating the position of molecular weight markers loaded beyond lane 12. The 1 min exposure is shown. Amount loaded indicates full-length Cry1Ac amount.

Lane	Sample	Amount Loaded (ng)
1	MON 87701-produced Cry1Ac protein	10
2	MON 87701-produced Cry1Ac protein	10
3	MON 87701-produced Cry1Ac protein	20
4	MON 87701-produced Cry1Ac protein	20
5	MON 87701-produced Cry1Ac protein	30
6	MON 87701-produced Cry1Ac protein	30
7	*E. coli*-produced Cry1Ac protein	10
8	*E. coli*-produced Cry1Ac protein	10
9	*E. coli*-produced Cry1Ac protein	20
10	*E. coli*-produced Cry1Ac protein	20
11	*E. coli*-produced Cry1Ac protein	30
12	*E. coli*-produced Cry1Ac protein	30

```
1     CMQAMDNNPN  INECIPYNCL  SNPEVEVLGG  ERIETGYTPI  DISLSLTQFL
51    LSEFVPGAGF  VLGLVDIIWG  IFGPSQWDAF  LVQIEQLINQ  RIEEFARNQA
101   ISRLEGLSNL  YQIYAESFRE  WEADPTNPAL  REEMRIQFND  MNSALTTAIP
151   LFAVQNYQVP  LLSVYVQAAN  LHLSVLRDVS  VFGQRWGFDA  ATINSRYNDL
201   TRLIGNYTDH  AVRWYNTGLE  RVWGPDSRDW  IRYNQFRREL  TLTVLDIVSL
251   FPNYDSRTYP  IRTVSQLTRE  IYTNPVLENF  DGSFRGSAQG  IEGSIRSPHL
301   MDILNSITIY  TDAHRGEYYW  SGHQIMASPV  GFSGPEFTFP  LYGTMGNAAP
351   QQRIVAQLGQ  GVYRTLSSTL  YRRPFNIGIN  NQQLSVLDGT  EFAYGTSSNL
401   PSAVYRKSGT  VDSLDEIPPQ  NNNVPPRQGF  SHRLSHVSMF  RSGFSNSSVS
451   IIRAPMFSWI  HRSAEFNNII  ASDSITQIPA  VKGNFLFNGS  VISGPGFTGG
501   DLVRLNSSGN  NIQNRGYIEV  PIHFPSTSTR  YRVRVRYASV  TPIHLNVNWG
551   NSSIFSNTVP  ATATSLDNLQ  SSDFGYFESA  NAFTSSLGNI  VGVRNFSGTA
601   GVIIDRFEFI  PVTATLEAEY  NLERAQKAVN  ALFTSTNQLG  LKTNVTDYHI
651   DQVSNLVTYL  SDEFCLDEKR  ELSEKVKHAK  RLSDERNLLQ  DSNFKDINRQ
701   PERGWGGSTG  ITIQGGDDVF  KENYVTLSGT  FDECYPTYLY  QKIDESKLKA
751   FTRYQLRGYI  EDSQDLEIYS  IRYNAKHETV  NVPGTGSLWP  LSAQSPIGKC
801   GEPNRCAPHL  EWNPDLDCSC  RDGEKCAHHS  HHFSLDIDVG  CTDLNEDLGV
851   WVIFKIKTQD  GHARLGNLEF  LEEKPLVGEA  LARVKRAEKK  WRDKREKLEW
901   ETNIVYKEAK  ESVDALFVNS  QYDQLQADTN  IAMIHAADKR  VHSIREAYLP
951   ELSVIPGVNA  AIFEELEGRI  FTAFSLYDAR  NVIKNGDFNN  GLSCWNVKGH
1001  VDVEEQNNQR  SVLVVPEWEA  EVSQEVRVCP  GRGYILRVTA  YKEGYGEGCV
1051  TIHEIENNTD  ELKFSNCVEE  EIYPNNTVTC  NDYTVNQEEY  GGAYTSRNRG
1101  YNEAPSVPAD  YASVYEEKSY  TDGRRENPCE  FNRGYRDYTP  LPVGYVTKEL
1151  EYFPETDKVW  IEIGETEGTF  IVDSVELLLM  EE
```

Figure 3. **MALDI-TOF MS Coverage Map of the MON 87701-Produced Cry1Ac Protein.**

The amino acid sequence of the full-length MON 87701-produced Cry1Ac protein was deduced from the coding region of the full-length *Cry1Ac* gene present in MON 87701 (Arackal et al., 2008) and the observed N-terminal sequence of the protein. Boxed regions correspond to tryptic peptide masses that were identified from the ~131 kDa protein band using MALDI-TOF MS. In total, 70 fragments, covering 66.6% (787 of 1182 total amino acids) of the expected protein sequence, were matched to expected masses.

A.

Amino acid residue # from the N-terminus	→	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Predicted Cry1Ac Sequence[1]	→	C	M	Q	A	M	D	N	N	P	N	I	N	E	C	I
		|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Observed Sequence	→	X	M	Q	A	M	D	N	(N)	P	(N)	X	X	X	X	X

B.

Amino acid residue # from the N-terminus	→	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Predicted Cry1Ac Sequence[1]	→	C	M	Q	A	M	D	N	N	P	N	I	N	E	C	I
		|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Observed Sequence	→	X	M	Q	A	(M)	X	(N)	(N)	X	X	X	X	X	X	X

Figure 4. Summary of N-terminal Sequence Analysis.

The single letter amino acid codes are: A, Alanine; C, cysteine; D, Aspartic acid; E, Glutamic acid; I, Isoleucine; M, methionine; N, Asparagine, P, Proline; and Q, Glutamine. Amino acids in the experimentally-derived sequence that are in parentheses represent tenuous designations. X indicates an undesignated call in that cycle of the analysis
Panel A: N-terminal sequence determined from full-length (~133 kDa) Cry1Ac band.
Panel B: N-terminal sequence determined from ~ 75 kDa band.

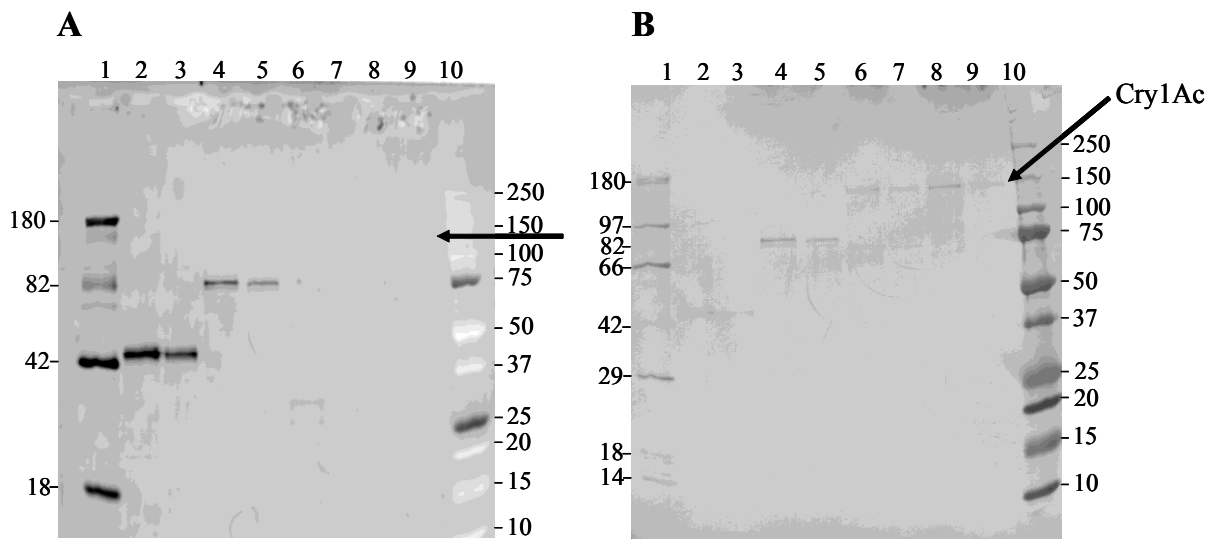


Figure 5. Glycosylation Analysis of the MON 87701-Produced Cry1Ac Protein.

Aliquots of horseradish peroxidase and transferrin (positive controls), MON 87701-produced Cry1Ac protein, and *E. coli*-produced Cry1Ac reference protein (negative control), were separated by SDS-PAGE and transferred to a PVDF membrane. For Cry1Ac samples, amount loaded indicates full-length protein amount. Approximate molecular weights indicated (in kDa) correspond to Candy Cane markers (lane 1) and dual color markers (lane 10). **Panel A**. Glycosylation analysis: Where present, periodate-oxidized protein-bound carbohydrate moieties reacted with Pro-Q Emerald 488 glycoprotein stain. The arrow indicates the approximate molecular weight for Cry1Ac. **Panel B.** Total protein staining: Following glycosylation analysis, the blot was stained for total protein.

Lane	Sample	Amount Loaded (ng)
1	Candy Cane MW Marker	-
2	Horseradish Peroxidase	100
3	Horseradish Peroxidase	50
4	Transferrin	100
5	Transferrin	50
6	MON 87701-produced Cry1Ac	100
7	MON 87701-produced Cry1Ac	50
8	*E. coli*-produced Cry1Ac	100
9	*E. coli*-produced Cry1Ac	50
10	Precision Plus Dual Color MW marker	-

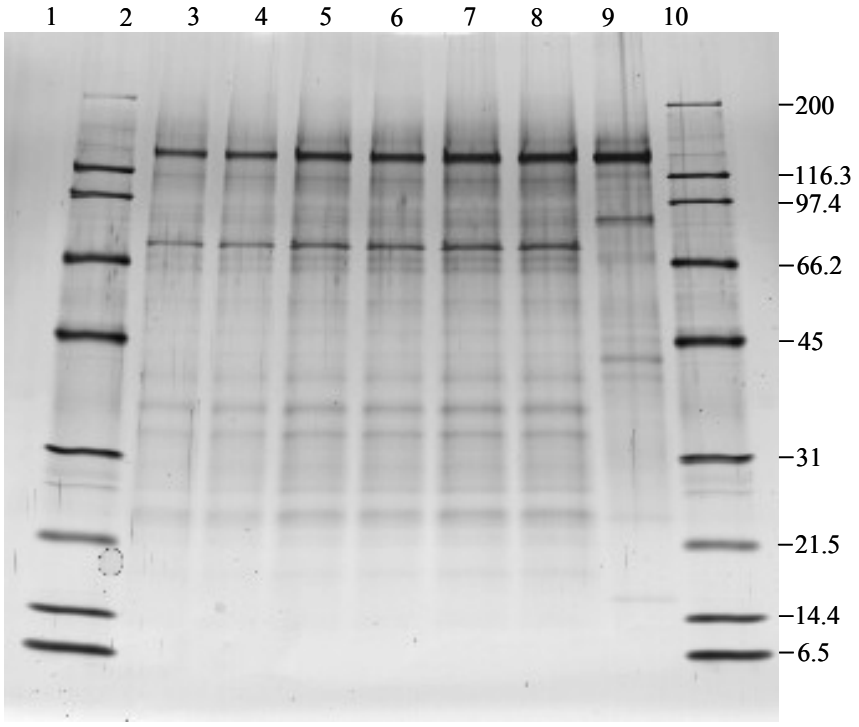


Figure 6. Storage Stability (Day 48) of the MON 87701-Produced Cry1Ac Protein Stored in a -80° C Freezer

SDS-PAGE analysis was performed on MON 87701-produced Cry1Ac samples stored in a -80° C freezer for 48 days. Aliquots of the MON 87701-produced Cry1Ac protein and *E. coli*-produced Cry1Ac reference protein were separated by SDS-PAGE, followed by silver staining. Approximate molecular weights (in kDa) are shown on the right and correspond to the markers loaded in lanes 2 and 10.

Lane	Sample	Amount loaded (ng)
1	empty	-
2	BioRad Broad Range Marker	360
3	MON 87701-produced Cry1Ac protein	95
4	MON 87701-produced Cry1Ac protein	95
5	MON 87701-produced Cry1Ac protein	189
6	MON 87701-produced Cry1Ac protein	189
7	MON 87701-produced Cry1Ac protein	284
8	MON 87701-produced Cry1Ac protein	284
9	*E. coli*-produced Cry1Ac protein	198
10	BioRad Broad Range Marker	360

Appendix 1. Certificate of Analysis for *E. coli*-produced Cry1Ac Reference Protein

Analytical Protein Standard
Certificate of Analysis

MONSANTO *ANALYTICAL PROTEIN STANDARDS*

Re-characterization No. 1

Sample Information:

Name of APS	Orion Lot Number	Expiration Date
E. coli-produced Cry1Ac [MON 87701]	10000804	May 31, 2008
Common or Alias Name(s)	**Historical APS Lot Number**	**Storage Requirements (until use)**
—	20-100133	–80 °C
Source Fermentation of *Escherichia coli* paste (APS lots 10-100116 through 10-100123, 10-100131 and 10-100132) containing the expression plasmid pMON107800.		**Comment(s)** None
Additional Background Information Historic Lot No. G-818529_26L		

Re-characterization Information		
Characteristic	**Method**	**Assay Date**
Total Protein Concentration	Amino Acid Analysis	4 October 2007
Purity/Molecular weight	SDS-PAGE/Densitometry	9 October 2007
Activity	Insect Bioassay	11 October 2007

Characteristic	Method	Assay Date	Result
Concentration	Amino Acid Composition	15 May 2007	1.4 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	17 May 2007	*80%
Molecular weight	SDS-PAGE/Densitometry	17 May 2007	131.7 KDa
Identity	Immunoblot	27 July 2007	Confirmed – immuno reactive band observed
Identity	N-terminal sequence	26 May 2007	Confirmed – XMQAMDNN(P)(N)(I)
Identity	MALDI-TOF MS (Trypsinized)	24 May 2007	Confirmed sequence 43.4 % coverage of expected sequence
Identity	MALDI-TOF MS (Native)	24 May 2007	Results inconclusive
Activity	Insect Bioassay	8 June 2007	EC_{50} = 2.1 ng of Cry1Ac [MON 87701] protein/mL diet

Buffer composition: 50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, and 1 mM benzamidine-HCl
Physical description: Clear solution
Short-term storage stability (41 days) was evaluated during the certification process. Based upon the criteria provided in Characterization Plan 20-100133, no significant degradation was observed for samples stored at 4°C, -20°C and –80°C.

* Purity is determined by summing all bands detected between the full-length protein (~130 KDa) and the tryptic core (~55 KDa) as well as a band immediately above the full-length POI.

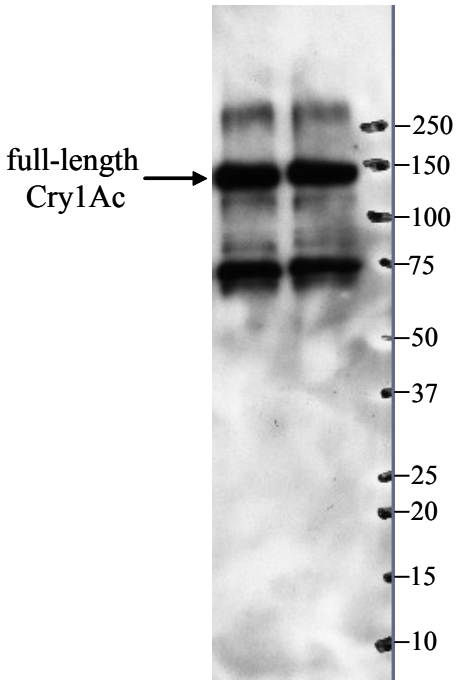
Purity corrected concentration is 1.1 mg/mL (1.4 mg/mL × 0.80 ≈ 1.1 mg/mL)

Quality Assurance Specialist — Date: 11/28/07

Analytical Protein Standards Officer — Date: 11/28/07

Page 1 of 1

Appendix 2. **Pre-study Immunoblot Used as a Reference for MON 87701 Cry1Ac Total Purity Determination**



In each lane 18.4 ng of MON 87701-produced Cry1Ac protein was loaded. Gel was blotted to PVDF and incubated with affinity purified anti Cry1Ac antibody. The 5 min exposure is shown.

Appendix 3. Insect Bioassay Summary

Insect Bioassay Summary for:

Characterization of the Cry1Ac Protein Purified from the Harvested Seed of MON 87701 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. Coli*-Produced Cry1Ac Proteins

Purpose:

The purpose of this analysis was to compare the biological activity of plant-produced Cry1Ac protein recovered from harvested seed of MON 87701 soybean and a reference *E. coli*-produced Cry1Ac protein by determining EC_{50} values in a corn earworm (CEW) diet-incorporation insect bioassay.

Materials:

Plant-Produced Cry1Ac protein, *E. coli*-produced Cry1Ac Reference Standard Protein and Control Substance:

The reference standard, an *E. coli*-produced Cry1Ac protein (Orion ID: 10000804) and a plant-produced Cry1Ac protein (Orion ID: 10000801) from the harvested seed of MON 87701 soybean, were received from the Monsanto Product Characterization Center (PCC). The total protein concentration of the *E. coli*-produced Cry1Ac protein aliquots was 1.4 mg/mL, with a purity of 80%, and a purity corrected concentration of 1.1 mg Cry1Ac/mL. The total protein concentration of the plant-produced Cry1Ac protein aliquots were 42 µg/mL with a purity of 77%, and a purity corrected concentration of 32 µg Cry1Ac/mL. The *E. coli*-produced Cry1Ac protein was suspended in 50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, 1 mM benzamidine-HCl buffer, while the plant-produced Cry1Ac protein was suspended in 50 mM CAPS, pH 10.8, 1 mM EDTA, 2 mM DTT, 1 mM benzamidine, and < 1% ethylene glycol buffer. Additionally, the buffers used to store the *E. coli*-produced and the plant-produced proteins, 50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mMDTT, and 1 mM benzamidine-HCl (lot # G-826331-A), and "Soy Cry1Ac Sample Buffer" (lot # G-824555B) were received from the PCC. The plant-produced and *E. coli*-produced Cry2Ab2 proteins were stored in a -80° C freezer and the buffers were stored in a 4° C refrigerator.

Methods:

Insects. CEW were obtained from Benzon Research Inc (Carlisle, PA). Insect eggs were incubated at temperatures ranging from 10^{o} C to 27^{o} C, to achieve the desired hatch time.

Bioassays. CEW were used to measure biological activity of the MON 87701-produced and *E. coli*-produced Cry1Ac protein samples in accordance with the Monsanto SOP BR-ME-1088-02. The bioassay was replicated three times on separate days with separate batches of insects. The MON 87701-produced and *E. coli*-produced substances were run in parallel during each bioassay. Each bioassay replicate for the *E. coli*-produced and MON 87701-produced Cry1Ac proteins consisted of a series of six dilutions yielding a dose series with a two-fold separation factor ranging from 0.00065 – 0.020 µg Cry1Ac protein/mL diet and two buffer controls. All dose levels, including the buffer controls, contained an equal volume and composition of buffer. The Cry1Ac protein dosing solutions were prepared by diluting the protein with purified water and incorporating the dilution into a Southland agar-based insect diet (Lake Village, AR). 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 (# BAW128, Bio-Serv, Frenchtown, 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 (# BACV16, Bio-Serv, Frenchtown, NJ) and the insects were allowed to feed for a period of approximately seven days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14 light:10 dark. The number of surviving insect and the combined weight of the surviving insects at each dose level was recorded at the end of the 7-day incubation period.

Statistical Analysis 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 NLMIXED procedure in SAS Software Release 9.1 (TS1M3).

Equation 1:
$$y_{ij} = \frac{w_0}{1 + \left(Dose_i / EC_{50}\right)^B} + e_{ij}$$

where y_{ij} is the average CEW larvae weight (mg) under dose level I; $Dose_i$ is the Cry1Ac protein diet dose level. Three parameters that are included in the model; W_0 represents the weight at $Dose_i = 0.0$, 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 $Dose_i$ increases, and e_{ij} 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.

Table 1. EC_{50} values and standard errors for *E. coli*- and MON 87701-produced Cry1Ac proteins in a CEW diet-incorporation bioassay

Bioassay Replicate	**EC_{50} Estimates (µg Cry1Ac/ml diet)**	**Standard Error (µg Cry1Ac/ml diet)**
E. coli-produced replicate 2	0.0031	0.00035
MON 87701-produced replicate 2	0.0050	0.00069
E. coli-produced replicate 3	0.0026	0.00022
MON 87701-produced replicate 3	0.0032	0.00021
E. coli-produced replicate 4	0.0050	0.00030
MON 87701-produced replicate 4	0.0034	0.00035

Mean EC_{50} for *E. coli*-produced Cry1Ac protein: 0.0036 µg Cry1Ac/mL diet
Mean EC_{50} for MON 87701-produced Cry1Ac protein: 0.0039 µg Cry1Ac/mL diet
SD for *E. coli*-produced Cry1Ac protein: 0.0013 µg Cry1Ac/mL diet
SD for MON 87701-produced Cry1Ac protein: 0.00098 µg Cry1Ac/mL diet

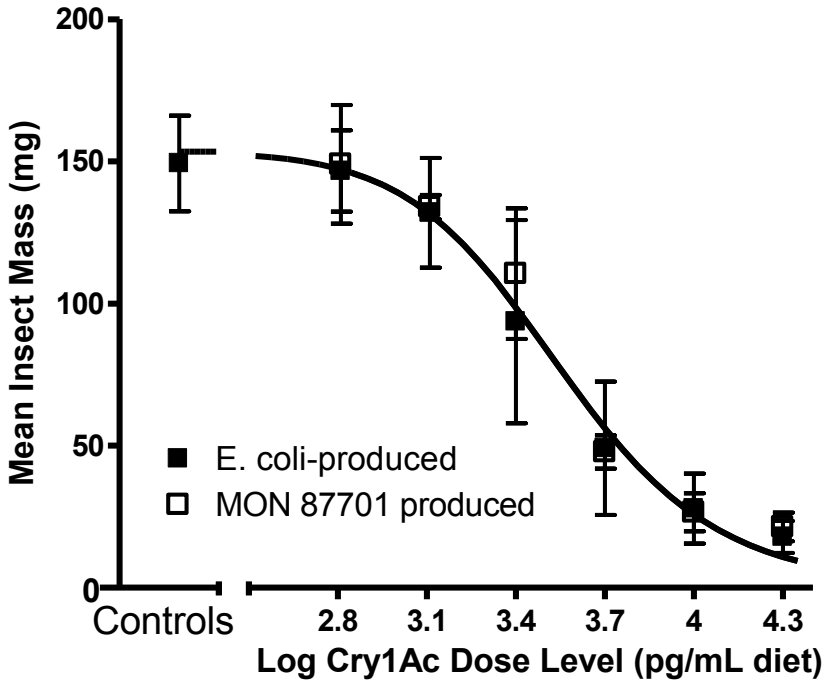


Figure 1. Dose-response curves for *E. coli*-produced and the MON 87701-produced Cry1Ac proteins in the corn earworm diet-incorporation bioassay. Each data point represents the mean of three bioassay replicates at each dose level along with the standard deviation. Dose units on the *x*-axis are expressed in log picograms Cry1Ac/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 MON 87701-produced protein were nearly identical, and therefore, were concluded to be functionally equivalent. The mean EC_{50} values for the *E. coli*-produced and the MON 87701-produced proteins were determined to be 0.0036 and 0.0039 µg Cry1Ac/mL diet, with standard deviations of 0.0013 and 0.00098 µg Cry1Ac/mL diet, respectively.

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

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Appendix 4. Study Deviations

There were four protocol deviations. These were:

1. The SDS-PAGE gel for purity and molecular weight was loaded based on an estimated concentration of the protein determined prior to the study, which differed slightly from the concentration determined within the study. Thus, rather than 100, 200, and 300 ng of protein being loaded as specified in the protocol, 95, 189, and 284 ng of the protein were loaded. In addition, the protocol stated that the reference protein would be loaded based on a purity-corrected value; in fact it was loaded based on total protein concentration. These changes did not have an impact on the study.
2. The gel band excised for MALDI-TOF tryptic mass analysis was alkylated in 20 mM iodo-acetic acid rather than 200 mM iodo-acetic acid as the protocol stated. The value listed in the protocol was a typographical error, and the solution used, 20 mM iodo-acetic acid, was the correct solution. This change did not have an impact on the study.
3. The actual time of two washes of the glycosylation blot following staining were shorter than specified in the manufacturer's protocol: 15 min and 20 min rather than 30 min for each. In addition, contrary to the manufacturer's protocol, the blot was scanned while wet rather than dry. These changes did not have an impact on the study.
4. A procedure not included in the protocol was performed. Following glycosylation, the blot was stained for total protein. This change did not have an impact on the study.

There were two additional deviations related to procedures stated in SOPs used in the study. These were:

1. The transfer buffer used in the electro-transfer of samples to PVDF membrane for glycosylation analysis was prepared incorrectly, from a transfer buffer stock of Bicine/BisTris/EDTA, rather than the standard transfer buffer stock of Tris/Glycine. There was no impact due to this deviation: despite the incorrect solution the prestained markers proteins transferred efficiently, as evaluated by visual examination of the blot following transfer and the signals observed from glycostaining of the blot as well as from total protein staining.
2. The system suitability criterion from SOP BR-EQ-0265-02 was not met for comparison of the PTH-alanine value to the value from the previous successful run. The difference observed was 15.6%; the criterion is < 15%. This slightly greater than normal variability did not have an impact on the

study, as chromatography amino acid peak elution time and peak resolution were unaffected by the varying amounts of PTH-standard analyzed.

Appendix 5. List of Applicable SOPs

SOP Number	Title
BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-ME-0234-01	Silver Staining of Polyacrylamide Gels
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-EQ-0599-03	Bio-Rad GS-800 Densitometer
BR-ME-0956-03	Protein Percent Purity and Apparent Molecular Weight Determination
BR-EQ-0935-01	Konica SRX X-Ray Film Processors
BR-EQ-0783-02	Applied Biosystems Voyager DE Pro Biospectrometry Workstation
BR-EQ-0265-02	Applied Biosystems 494 Procise™ Protein Sequencing System
BR-ME-0926-01	Staining of Proteins on Blot Membranes
BR-ME-0932-03	Assessment of Immunoreactive Bands from Western Blots Exposed to X-Ray Films Using Bio-Rad GS-800 Densitometer
BR-ME-1139-01	Vapor Phase Acid Hydrolysis Using 6 N HCl and Subsequent Amino Acid Analysis Using AccQ-Tag® Derivatization
BR-EQ-1138-01	Waters 2695 Separations Module for AccQ-Tag® Analysis
AG-EQ-1051-02	Atlas Chromatography Data System
BR-ME-1088-02	Determination of Cry1Ab/Cry1Ac/Cry2Ab/Cry1A.105 Biological Activity Using Corn Earworm (*Helicoverpa zea*) in a Diet Incorporation Bioassay
BR-EQ-1155-01	Bio-Rad PharosFX Plus Molecular Imager System