6) Hileman, R. E., G. Holleschak, L. A. Turner, R. S. Thoma, C. R. Brown and J. D. Astwood (2001). Characterization and equivalence of the Cry3Bb1 protein produced by E. coli fermentation and corn event MON 863. MSL-17274, an unpublished study conducted by Monsanto Company.

Study Title

Characterization and Equivalence of the Cry3Bb1 Protein Produced by *E. coli* Fermentation and Corn Event MON 863

Authors

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Study Completed On

July 6, 2001

Performing Laboratory

Monsanto Company
Product Safety Center
Biotechnology Regulatory Sciences
700 Chesterfield Parkway North
St. Louis, MO 63198

Laboratory Project ID

MSL Number: 17274
Study Number: 01-01-39-30

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[redacted]

Information claimed confidential on the basis of its falling within the scope of FIFRA section 10(d)(1)(A), (B) or (C) has been removed to a confidential appendix, and is cited by cross reference number in the body of the report.

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Statement of Compliance

This study meets the GLP requirements for 40 CFR Part 160 (EPA) except for the following:

The MALDI-TOF mass spectrometer equipment records were not GLP compliant, in that no maintenance or calibration history was recorded and no SOP was available. The analytical reference standard characterization records were not GLP compliant; an expected mass for the reference standard was derived from its history of use as a calibration standard for this instrument. In addition, residual trypsin was present at the expected mass in each sample. Collected data were well documented according to the Monsanto Company Guidelines for Keeping Research Records (GRR 10/1/99).

The densitometer and microplate scanning spectrophotmeter equipment records were not GLP compliant, in that no equipment SOP was available. Nevertheless, these instruments were calibrated at the time of use and records for calibration and maintenance have been retained. Collected data were well documented according to the Monsanto Company Guidelines for Keeping Research Records (GRR 10/1/99).

_____ Date: _____
Submitter

Ravinder S Sidhu Date: July 6, 2001
Sponsor

R W E-Thorn Date: 6 JULY 2001
Study Director

Quality Assurance Unit Statement

Study Title: Characterization and Equivalence of the Cry3Bb1 Protein Produced by *E. coli* Fermentation and Corn Event MON 863

Study Number: 01-01-39-30

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
05/10/2001	Glycosylation	05/17/2001	05/17/2001
05/11/2001	Insect Bioassay	05/25/2001	05/25/2001
05/16/2001	Amino Acid Analysis	05/29/2001	05/29/2001
07/03/2001	Draft Report Review	07/06/2001	07/06/2001
07/03/2001	Raw Data Audit	07/06/2001	07/06/2001
07/03/2001	Raw Data Audit	07/06/2001	07/06/2001

Barbara A. Foy

Date: July 6, 2001

Barbara A. Foy
Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

MSL Number: 17274

Title: Characterization and Equivalence of the Cry3Bb1 Protein Produced by *E. coli* Fermentation and Corn Event MON 863

Primary Testing Facility:
Monsanto Company
Product Safety Center
700 Chesterfield Parkway North
Chesterfield, Missouri 63198

Additional Testing Facility:
Monsanto Company
Ecological Safety Center
800 North Lindbergh Blvd
St. Louis, MO 63167

Sponsor Representative: Ravinder S. Sidhu

Authors:
Ronald E. Hileman
Gyula Holleschak
Larry A. Turner
Richard S. Thoma
Christopher R. Brown
James D. Astwood

Contributors:
Chris M. Dalton
Jian J. Duan
Changjian Jiang
Fred Moshiri
Janet C. Obert
James E. Peister
Brian Shipley
Jamie J. Thorp

Study Start Date: May 3, 2001

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Records Retention: All study specific raw data, electronically stored files and final report will be retained at Monsanto-St. Louis.

Signatures of Final Report Approval:

Ravinder S Sidhu
Sponsor
Date: July 6, 2001

Director, Product Safety Center
Date: July 6, 2001

Study Director
Date: 6 JULY 2001

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Abbreviations and Definitions

aa	amino acid
α-cyano	alpha-cyano-4-hydroxy cinnamic acid
BSA	Bovine serum albumin
B.t.	*Bacillus thuringiensis*
CHAPS	(3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate
CI	Confidence interval
CPB	Colorado potato beetle, *Leptinotarsa decemlineata*
DMP	Dimethylpimelimidate
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
GenBank	A public genetic database maintained by the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-thiogalactopyranoside
IUPAC-IUB	International Union of Pure and Applied Chemistry - International Union of Biochemistry
kDa	Kilodalton
LB	Lennox L broth
LC_{50}	Concentration required to kill 50% of the test larvae relative to control
MALDI	Matrix Assisted Laser Desorption and Ionization
NIST	National Institutes of Standards and Technology
NFDM	Non-fat dried milk
OD	Optical density
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing Tween-20
PCR	Polymerase chain reaction
psi	pounds per square inch
PTH	Phenylthiohydantoin
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecylsulfate
SOP	Standard operating procedure
TB	Terrific broth
TFA	Trifluoroacetic acid
TOF	Time of Flight
Tris	Tris (hydroxymethyl)aminomethane

1.0 Summary

Genetically modified corn, corn event MON 863, produces a variant of the *Bacillus thuringiensis* (*B.t.*) Cry3Bb1 protein. Plants producing this modified Cry3Bb1 protein are resistant to larval feeding damage from the coleopteran insect, corn rootworm.

The purpose of this study was twofold: first, to comprehensively characterize the modified Cry3Bb1 proteins produced in *E. coli* and corn event MON 863 and second, to evaluate the physicochemical and functional similarity of these proteins. Demonstration of the physicochemical and functional equivalence supports the use of the *E. coli*-produced protein in studies designed to evaluate the safety or other aspects of this protein.

Equivalence of these proteins was judged by comparison of the results obtained from a variety of analytical methods including MALDI-TOF mass spectrometry, N-terminal sequencing, immunoblotting, insect bioassay, SDS-PAGE, glycosylation analyses and amino acid compositional analyses.

The identity of each protein was confirmed using a combination of matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, N-terminal sequence analyses and immunoblot analyses. Masses were identified that indicated that the N-terminal methionine residue (position 1) was post-translationally processed from each protein. Furthermore, the C-termini of each protein were confirmed to be intact. N-terminal sequence analyses again confirmed the identity of these proteins. Each protein displayed comparable electrophoretic mobility and immunoreactivity on blots probed with either polyclonal or monoclonal antibodies that identify the Cry3Bb1 protein.

The purity of the *E. coli*- and corn-produced proteins was estimated to be 92.6% and 53.9%, respectively. The proteins displayed comparable molecular weights on SDS-PAGE analysis and were consistent with the predicted molecular weight (~74 kDa) for the full length protein. While the purity of the corn-produced protein was too low to obtain useful amino acid composition values, it was suitable for use in all other methods designed to assess physicochemical and functional equivalence. The amino acid composition of the *E. coli*-produced protein was consistent with the expected composition. Analysis for covalently bound N- or O-linked carbohydrate was used to assess glycosylation. Neither the *E. coli*- nor corn-produced Cry3Bb1 protein was glycosylated. Functional activity was assessed using Colorado potato beetle larvae fed diets that incorporated either the *E. coli*- or corn-produced proteins. The estimated LC_{50} values (and corresponding 95% confidence intervals) of the *E. coli*- and corn-produced proteins were 0.76 µg/mL (0.57-0.92 µg/mL) and 0.63 µg/mL (0.48-0.77 µg/mL), respectively. Because there was considerable overlap between the 95% confidence intervals, these proteins were considered functionally equivalent.

The stability of each protein was assessed using SDS-PAGE analysis for samples stored at common storage temperatures (4, −20 and −80 °C) for the duration of the experimental phase (29 days). Each protein was observed to degrade to ~66 kDa when stored at 4 °C during this period. No significant degradation was observed for solutions stored at −20 and −80 °C. With the exception of samples designated for storage stability, all samples used in other analyses were stored at −80 °C until used.

These data established that the *E. coli*- and corn-produced Cry3Bb1 proteins were physicochemically and functionally equivalent. Physicochemical and functional equivalence supports the use of the *E. coli*-produced protein in studies designed to evaluate the food, feed or environmental safety of the modified Cry3Bb1 protein produced in corn event MON 863.

2.0 Introduction

Genetically modified corn, corn event MON 863, produces a variant of the *Bacillus thuringiensis* (*B.t.*) Cry3Bb1 protein. Corn plants producing this Cry3Bb1 protein variant are resistant to larval feeding damage from the coleopteran insect, corn rootworm (Coleoptera, Chrysomelidae, *Diabrotica* sp). The physical and functional equivalence of the modified Cry3Bb1 produced in corn event MON 863 to the Cry3Bb1.11098 protein produced by *B.t.* strain EG11098 was previously demonstrated (Holleschak *et al.*, 2001a; Holleschak *et al.*, 2001b). Recent DNA sequencing of the *cry3Bb1* coding region in corn event MON 863 has shown that it encodes a Q349R substitution in the expected Cry3Bb1.11098 protein (Cavato and Lirette, 2001). This was subsequently verified using MALDI-TOF mass spectrometry of the Cry3Bb1 protein produced in corn event MON 863 (Thoma *et al.*, 2001).

The wild type *cry3Bb1* gene (GenBank Accession No. M89794) was initially modified utilizing a *B.t.* plasmid and expression system (English *et al.*, 2000) in order to design a Cry3Bb1 protein with increased activity. The *cry3Bb1.11098* gene, which produces the Cry3Bb1.11098 protein, was observed to have increased bioactivity towards corn rootworm when expressed in *B.t.* Specific modifications present in the Cry3Bb1.11098 protein produced in *B.t.* strain EG11098 have been previously described (Hileman and Astwood, 2001; Hileman *et al.*, 2001). This *B.t.* protein sequence differs from the wild type Cry3Bb1 protein by 5 amino acid substitutions.

DNA encoding the Cry3Bb1.11098 protein was then modified to optimize cloning and expression in plants prior to transforming corn. Three nucleotides (GCC at positions 4, 5 and 6) were inserted at the 5' end of the coding sequence to create an *Nco* I restriction endonuclease site and facilitate cloning of the *cry3Bb1* gene into a plant transformation

vector. This manipulation resulted in the insertion of an alanine (A) residue at position 2 of the predicted plant amino acid sequence. When the coding region of this *B.t.* gene was modified for expression in corn, it was predicted to encode a separate additional change relative to the *B.t.* strain EG11098 Cry3Bb1.11098 protein sequence (Hileman and Astwood, 2001). This additional change corresponded to a Q349R substitution. Thus, the Cry3Bb1 protein produced in corn event MON 863 differs from the *B.t.* strain EG11098 Cry3Bb1.11098 protein by 2 amino acids and differs by 7 amino acids (1 aa insertion and 6 aa substitutions) from the wild type Cry3Bb1 protein.

Food, feed and environmental safety evaluations that utilize purified protein require gram quantities. Because of the relatively low level of the Cry3Bb1 protein variant in tissues from corn event MON 863, it was not feasible to isolate protein directly from plants. Therefore, an *E. coli* heterologous protein production system was designed using the same *cry3Bb1* DNA sequence present in corn event MON 863. The DNA sequence and deduced amino acid sequence of the *E. coli*- and corn-produced proteins were identical (Figure 1).

This report describes the physicochemical and functional characterization of the *E. coli*-produced Cry3Bb1 protein and of the Cry3Bb1 protein produced in corn event MON 863. Physicochemical and functional equivalence of these proteins was demonstrated by comparison of the results obtained from each of the analytical methods employed. Demonstration of physicochemical and functional equivalence supports the use of the *E. coli*-produced protein in studies designed to evaluate the safety or other aspects of the Cry3Bb1 protein produced in corn event MON 863.

3.0 Purpose

The purpose of this study was to characterize the physicochemical and functional properties of the *E. coli*- and corn-produced Cry3Bb1 proteins and to evaluate the physicochemical and functional equivalence of these proteins. Assessment of the equivalence of these proteins was judged by comparison of the results obtained from the following analytical methods: (1) mass spectrometric analyses, (2) N-terminal sequence analyses, (3) immunoblot analyses, (4) insect bioassay analyses, (5) sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses, (6) glycosylation analyses and (7) amino acid compositional analyses.

4.0 Materials

4.1 *Test substances.* There were two test substances in this characterization study. Both test substances represent the same variant of the wild type Cry3Bb1 protein and designed to be identical in amino acid sequence (Figure 1) and differ only by the host organism that produced each protein sample. Methods used for purification of the test substances are described in Appendix 1.

4.1.1 *E. coli*-produced Cry3Bb1 protein. The first test substance was the bacterially produced Cry3Bb1.11098(Q349R) protein (lot 6962478), isolated using chromatographic methods from a large-scale fermentation of *E. coli* containing the pET24d(+)/25097 expression plasmid. For this report, this test substance (lot 6962478), is referred to as the *E. coli*-produced protein.

4.1.2 Corn-produced Cry3Bb1 protein. The second test substance was the plant produced Cry3Bb1.11098 protein (lot 6957088) isolated from corn hybrid RX 670 containing event MON 863. This protein was purified using chromatographic methods from the grain grown under Production Plans (in progress) 00-01-39-16 and 00-01-39-18. For this report, this test substance (lot 6957088), is referred to as the corn-produced protein.

4.2 *Test substance characterization.* DNA sequence that encompassed the *cry3Bb1* coding region of the pET24d(+)/25097 expression plasmid was confirmed prior to fermentation of *E. coli*. Records pertaining to the identity and isolation of the *E. coli*-produced protein were archived with this study.

The identity of corn event MON 863 grain from which the test substance was isolated was confirmed by event specific polymerase chain reaction (PCR). Test substance characterization was archived under Production Plans (in progress) 00-01-39-16 and 00-01-39-18. Records pertaining to the isolation of the corn-produced protein from grain of corn event MON 863 were archived with this study.

5.0 Methods

5.1 *Protein purification.* Pre-study protein purification is described in Appendix 1.

5.2 *MALDI-TOF analysis.* MALDI-TOF mass spectrometry was used to assess the identity of the *E. coli*- and corn-produced CryBb1 proteins.

5.2.1 *SDS-PAGE separation of proteins.* Aliquots of *E. coli*- and corn-produced protein were diluted with 2× Laemmli (Laemmli, 1970) sample buffer [62.5 mM Tris-HCl, pH 6.8, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 25 % (v/v) glycerol and 0.01% (w/v) Bromophenol Blue]. Markers (~0.75 µg/band, Amersham Life Science, Buckinghamshire, England) were used to estimate molecular weight. All samples were heated at approximately 100 °C for 3 min and applied to separate pre-cast Novex® 4→20% polyacrylamide gradient mini-gels (Invitrogen™, Carlsbad, CA). Test protein samples (~20 µg total protein) were loaded into 4 separate lanes. Electrophoresis was performed under reducing conditions according to SOP PB-EQP-005-01 at constant voltage (150 V) for approximately 1.3 h (until the dye front reached the bottom of the gel). Proteins were fixed in the gels by gentle shaking with 40% (v/v) methanol and 7% (v/v) glacial acetic acid, stained with Colloidal Brilliant Blue G stain (Sigma Chemical Co., St. Louis, MO) and visualized after destaining with 25% (v/v) methanol.

5.2.2 *In-gel protein digestion.* From each gel, bands that migrated to ~74 kDa were excised, destained, reduced, alkylated and subjected to an in-gel trypsin (Promega, Madison, WI) digest. Briefly, each gel band was destained by incubation in 100 µL of destaining buffer [40% (v/v) methanol, 10% (v/v) glacial acetic acid] three times, for 30 min each. A blank region of the same gel was cut and similarly processed to serve as a negative control sample. Following destaining, gel fragments were incubated in 100 µL buffer A (100 mM ammonium bicarbonate, J.T. Baker) solution for 30 min. Proteins were reduced in 100 µL buffer A containing 10 mM dithiothreitol (Calbiochem®, LaJolla, CA) for 2 h at 37 °C. Proteins were alkylated by the addition of 100 µL of buffer A containing 200 mM iodoacetic acid (Sigma Chemical Co.). The alkylation reaction was allowed to proceed at room temperature for 20 min in the dark. Gel fragments were incubated in 100 µL buffer A for 30 min, washed twice at room temperature for 30 min each with 100 µL of 50% (v/v) acetonitrile (Perkin Elmer, Norwalk, CT) and dried in a Speed Vac concentrator (Savant, Holbrook, NY) after each wash. Dried gel bands were rehydrated with 40 µL 25 mM ammonium bicarbonate solution containing 33 µg/mL trypsin and incubated for 16 h at 37 °C. After digestion, peptides were extracted 3 times for 1 h each at room temperature with 50 µL 70% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA, Perkin Elmer). Extraction supernatants from each sample were then combined into a single tube and dried in a Speed Vac concentrator to a final volume of ~10 µL (Williams *et al.*, 1997).

5.2.3 *Sample preparation.* Approximately half of each digested sample was desalted using Millipore (Bedford, MA) $ZipTip_{C18}$ pipette tips. Prior to desalting,

the tips were washed with methanol and equilibrated with 0.1% (v/v) TFA. Five µL of each sample was applied to separate tips and washed with 5 µL of Wash 1 [0.1% (v/v) TFA], 5 µL of Wash 2 [20% (v/v) acetonitrile containing 0.1% (v/v) TFA], 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].

5.2.4 *MALDI-TOF instrumentation and mass analysis.* Mass spectral analyses were performed using a Perseptive Voyager DE™-Pro (Perkin Elmer, Foster City, CA) MALDI-TOF instrument with the supplied Data Explorer™ software (version 4.0). Mass calibration of the instrument was performed using a trypsin digested porcine somatotropin mixture (lot 70003PX). From each desalted sample (described in Section 5.2.3), 0.3 µL was co-crystallized with 0.75 µL α-cyano-4-hydroxy cinnamic acid (Ciphergen Biosystems, Palo Alto, CA). Samples were analyzed in the 500 to 5000 Dalton range in reflector mode using 100 laser shots per spectrum at a laser intensity setting of 1939 (a unitless MALDI-TOF instrument specific value). Protonated (MH+) peptide masses were observed monoisotypically in reflector mode (Aebersold, 1993; Billeci and Stults, 1993). MacBioSpec™ software (PE Sciex Instruments, version 1.0.1 1992, Thornhill, Ontario, Canada) was used to generate a theoretical trypsin digest of the expected protein sequence. Masses were calculated for each theoretical peptide and compared to the raw mass data. Experimental masses (MH+) were assigned to peaks when three (or more) isotopically resolved ion peaks were observed in the raw mass data. Peaks were ignored if there were less than 3 isotopically resolved peaks in the spectra, when 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. Masses obtained from the negative control sample (blank gel) were attributed to matrix and ignored if a corresponding mass was observed in mass spectra of the test substances.

5.3 *N-terminal sequence analysis.* Aliquots of *E. coli*- and corn-produced Cry3Bb1 protein were diluted with 2× Laemmli (Laemmli, 1970) sample buffer. Pre-stained molecular weight markers (~1 µg/band, Amersham Life Science, Buckinghamshire, England) were used to verify electrotransfer of protein to the membrane. Separation of proteins using SDS-PAGE was performed under reducing conditions according to SOP PB-EQP-005-01 and similar to that described in Section 5.2.1. Test protein samples (~10 µg total protein) were loaded into 4 separate lanes. Proteins were electrotransferred to a 0.2 µm PVDF membrane (Novex) for 1.1 h at a constant 200 mA. Protein bands were detected by briefly staining the membranes with Ponceau S reagent [Sigma Chemical Co., 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid] followed by 3 washes, 5 min each in deionized water.

Bands that migrated to ~74 kDa were excised from the membranes. An additional band (~220 kDa) was excised from membrane containing the corn-produced protein. N-terminal sequence analysis was performed for 15 cycles using automated cycles of Edman degradation (Hunkapillar *et al.*, 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient, 785A Programmable Absorbance Detector, Procise Control Software (version 1.1a) and Model 610A Data Analysis Software (version 2.1a) was used following SOP BR-EQ-0265-01. Chromatography calibration was used for each analysis using of PTH-amino acid standards (Applied Biosystems, Foster City, CA). This mixture also served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. Bracketing test proteins, a β-lactoglobulin control (10 pmole, Applied Biosystems) was analyzed for 15 cycles to verify that the sequencer met acceptable performance criteria for repetitive yield and sequence identity.

5.4 *Immunoblot analysis.* The study protocol was amended (Appendix 3, Amendment 01) to include the use of a monoclonal antibody for this analysis. Polyclonal antibody (lot 6199830B) was produced by repeated immunization of a rabbit with wild type Cry3Bb1 protein (EG 11037). Characterization of the polyclonal antibody has been previously described (Dudin *et al.*, 1999). Data regarding the production of the monoclonal antibody (lot 6199915) was archived with this study. Characterization of the immunogen used to generate the monoclonal antibody, *B.t.* strain EG 11231 Cry3Bb1.11231 protein (lot 6024274) was previously reported (Hileman *et al.*, 2001). Monoclonal antibody was produced by repeated immunization of a mouse and the specificity of this monoclonal antibody was previously demonstrated (Holleschak *et al.*, 2001b). The wild type DNA sequence (GenBank Accession No. M89794) and deduced protein sequence has been previously compared to the deduced protein sequence of the Cry3Bb1 variants produced in corn event MON 863 and *B.t.* strain EG 11231 (Hileman and Astwood, 2001). Because these modified Cry3Bb1 proteins were alleles of the wild type protein (Astwood *et al.*, 2001), antibodies raised against any one of the alleles was expected to cross-react with another allele. Therefore the use of these antibody preparations did not impact the results or interpretation of the immunoblot analyses.

Aliquots of *E. coli*- and corn-produced Cry3Bb1 protein were diluted with Laemmli (Laemmli, 1970) sample buffer for each of the polyclonal and monoclonal immunoblot analyses. Pre-stained molecular weight markers (~1 µg/band, Amersham Life Science, Buckinghamshire, England) were used to verify electrotransfer of protein to the membranes while ECL™ markers (Amersham Pharmacia) were used to assess the molecular weight of the immunoreactive bands. Electrophoresis was performed under reducing conditions according to SOP PB-EQP-005-01 and similar to that described in Section 5.2.1. Proteins were electrotransferred to 0.45 µm nitrocellulose membranes (Invitrogen) for 1 h (polyclonal immunoblot) or 1.5 h (monoclonal immunoblot) at

200 mA. Membranes were blocked by incubation in 10% (w/v) NFDM in PBST for at least 45 min. The polyclonal immunoblot was probed with a 1:5000 dilution of polyclonal antibody (lot 6199830B) for approximately 45 min. Polyclonal IgG bound to membrane was detected by incubation with a 1:7500 dilution of a goat anti-rabbit IgG HRP conjugate (Sigma Chemical Co., St. Louis, MO) in PBST containing 5% (w/v) NFDM for 45 min. The monoclonal immunoblot was probed with a 1:300 dilution of monoclonal antibody (lot 6199915) overnight at 4 °C. Monoclonal IgG and biotinylated markers bound to membrane were detected by incubation with a mixture of a 1:5000 dilution of goat anti-mouse IgG HRP conjugate (Sigma Chemical Co.) and Neutravidin HRP conjugate (Amersham Pharmacia) in PBST containing 5% (w/v) NFDM for 60 min. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL™) detection system (Amersham Pharmacia) and exposed to Hyperfilm™ ECL™ high performance chemiluminescence film (Amersham Pharmacia). Films were developed using a Konica SRX101 automated film processor (Tokyo, Japan).

5.5 *Molecular weight and purity analysis.* *E. coli*- and corn-produced Cry3Bb1 proteins were diluted with 2× Laemmli (Laemmli, 1970) sample buffer to a final concentration of approximately 0.25 µg/µL. Multiple marker proteins (Life Technologies, Bio-Rad and Amersham Life Science) were used for molecular weight estimation. Electrophoresis was performed under reducing conditions according to SOP PB-EQP-005-01 and similar to that described in Section 5.2.1 using separate loads of 1, 2 and 3 µg of each test substance. The gel was incubated in 40% (v/v) methanol containing 7% (v/v) acetic acid for approximately 30 min to fix proteins, stained for approximately 1 h using Colloidal Brilliant Blue G (Sigma Chemical Co.), briefly destained in 25% methanol containing 10% (v/v) acetic acid for approximately 30 sec and fully destained in 25% methanol for approximately 1 h.

Methods and results are described for the second of two SDS-PAGE analyses used to assess molecular weight and purity. The first analysis was rejected. The molecular weight markers were distorted and the gel was considered unacceptable.

Analysis of the gel was performed using a Bio-Rad Laboratories GS-710 densitometer with the supplied Quantity One software (version 4.0.3, Hercules, CA). Molecular weight values supplied by the corresponding manufacturer were used to estimate the molecular weight of each band observed in the test substances. All visible bands within each lane were quantitated. Purity was estimated as the sum of bands (relative percent optical density) attributed to Cry3Bb1 protein.

5.6 *Glycosylation analysis.* Aliquots of *E. coli*- and corn-produced Cry3Bb1 protein were diluted with 2× Laemmli (Laemmli, 1970) sample buffer. The analytical positive

control sample, transferrin protein, was provided in the ECL™ glycoprotein detection system kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and prepared by mixing 1 mg lyophilized material with 1 mL water. Transferrin protein was further diluted with water and mixed with an equal volume of 2× Laemmli sample buffer to yield a concentration of 20 ng/µL. ECL protein (Amersham Life Science, Buckinghamshire, England) and broad range biotinylated markers (Bio-Rad Laboratories, Hercules, CA) were also diluted in 2× Laemmli sample buffer. Pre-stained molecular weight markers (~1 µg/band, Amersham Life Science) were used to verify electrotransfer of protein to the membrane. All samples were heated at approximately 100 °C for 4 min and applied to a pre-cast Novex® 4→20% polyacrylamide gradient mini-gel (Invitrogen™, Carlsbad, CA). Electrophoresis was performed under reducing conditions according to SOP PB-EQP-005-01 at constant voltage for approximately 1.5 h (until the dye front reached the bottom of the gel). Proteins were electrotransferred to a 0.45 µm PVDF membrane (Hybond-P, Amersham Pharmacia Biotech) for 1.5 h at 25 V (constant voltage).

Carbohydrate detection was performed directly on the PVDF membrane. Each incubation step was performed on an orbital shaker. The PVDF membrane was gently shaken for approximately 20 min in PBS and transferred to a solution of 100 mM sodium acetate buffer, pH 5.5, containing the oxidation reagent, 10 mM sodium metaperiodate. The membrane was incubated in the dark for 20 min. The oxidation reagent was removed from the membrane by two brief rinses in PBS followed by 3 × 10 min washes. The membrane was transferred to a solution of 100 mM sodium acetate buffer, pH 5.5, containing 25 nM biotin hydrazide and incubated for 40 min. Biotin hydrazide solution was removed by washing in PBS as previously described for the 10 mM sodium metaperiodate. Blocking was performed by incubating the membrane for 60 min in 5% (w/v) NFDM in PBS. Blocking solution was removed by washing in PBS as previously described. The membrane was incubated with streptavidin-HRP conjugate (diluted ~1:6000 in PBS) for approximately 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 enhanced chemiluminescence (ECL™) detection system (Amersham Pharmacia) and exposed to Hyperfilm™ ECL™ high performance chemiluminescence film (Amersham Pharmacia). Films were developed using a Konica SRX101 automated film processor (Tokyo, Japan).

5.7 *Protein assay.* Protein concentration was assessed using a Bio-Rad Laboratories (Hercules, CA) colorimetric dye-binding assay (Bradford, 1976) in a 96-well plate, performed according to SOP GEN-PRO-015-00. The concentration of the *E. coli*- and corn-produced Cry3Bb1 proteins were estimated relative to a standard curve of BSA as the mean of separate dilutions. Data was collected using a BioTek Instruments, Inc. Powerwave Xi microplate scanning spectrophotometer (Winooski, VT) at 595 nm.

5.8 *Amino acid composition analysis.* Aliquots of *E. coli*- and corn-produced Cry3Bb1 proteins were analyzed using a Hitachi L-8800 Amino Acid Analyzer with AAA System Manager Software according to SOP BR-EQ-0376-01. Test samples, NIST BSA (used as a system suitability standard) and NIST amino acid calibration control standard (Gaithersburg, MD) were spiked with an internal reference (norvaline, Sigma Chemical Co.) and dried in a Savant Speed Vac (Holbrook, NY). Vapor phase acid hydrolysis [6 N HCl containing 1% (v/v) phenol] was performed at ~150 °C for approximately 90 min. Cooled samples were again evaporated, reconstituted in protein hydrolyzate buffer-1 (Hitachi Instruments) and loaded onto the instrument. Amino acids were detected using post-column ninhydrin derivatization. Each protein sample was analyzed in triplicate. Due to an intermittent system error during the analysis, three of the results were rejected (see study specific SOP deviation, Appendix 3). Protein concentration was determined from acceptable runs and averaged.

5.9 *Storage stability.* The SDS-PAGE analysis used for purity and molecular weight estimation was performed at the beginning of the experimental phase of this study. The extent of degradation at storage temperatures of approximately 4, −20 and −80 °C was assessed by comparison of samples analyzed using SDS-PAGE at the end of the experimental phase. Aliquots of *E. coli*- and corn-produced Cry3Bb1 proteins were stored at the specified temperatures for 29 days. Test substances and markers (Bio-Rad Laboratories) were diluted with 2× Laemmli (Laemmli, 1970) sample buffer and electrophoresed as described in Section 5.2.1. Polyacrylamide gels were fixed by incubation with 40% (v/v) methanol containing 7% (v/v) acetic acid for approximately 30 min and stained using Coomassie Brilliant Blue R (see Protocol Deviation, Appendix 3). Bands were visualized after destaining (approximately 3 days) with 25% (v/v) methanol containing 10% (v/v) acetic acid. Stability was evaluated by comparison of molecular weight(s) of bands observed for each storage temperature, estimated using densitometric analysis (described in Section 5.5).

5.10 *Insect bioassay.* Aliquots of *E. coli*- and corn-produced Cry3Bb1 proteins were transferred to the Entomology Laboratory of the Ecological Safety Technology Center, Monsanto Company. Bioassays were conducted according to SOP BR-ME-0044-02. Each test substance was assayed in duplicate using first instar Colorado potato beetle (CPB, *Leptinotarsa decemlineata*) larvae. Test substances were incorporated into a defined diet and fed to larvae (16 larvae per dose). The purity corrected dose concentrations used were approximately 5.73, 2.86, 1.43, 0.72, 0.36 and 0.18 µg/mL (*E. coli*-produced protein) and approximately 9.62, 4.81, 2.40, 1.20, 0.60 and 0.30 µg/mL (corn-produced protein). Vehicle (water) served as the negative control dose in each replicate assay. Larvae were scored for survival after 7 days. Data were analyzed using SAS System software (Release 8.1) from SAS Institute, Inc, (Cary, NC). LC_{50} values and

corresponding 95% confidence intervals (CI) were estimated using the SAS Probit procedure (SAS®, 1999) with the following model:

$$p_{ijk} = C + (1 - C)F\left(Source_i + Dose_{ij} + \beta_k + e_{ijk}\right)$$

where p_{ijk} corresponds to the observed probability of lethality under Dose level *j* with protein Source *i* in replicate *k*, C corresponds to the natural response rate, F corresponds to the normal cumulative distribution function, $Dose_{ij}$ corresponds to Dose level *j* for protein source *i*, β_k corresponds to the effect of replicate *k* and e_{ijk} corresponds to the random residual effect of Source *i*, $Dose_{ij}$ in replicate *k*. Differences in biological activity (LC_{50} values) against CPB larvae were determined for the two test substances by comparison of the 95% confidence intervals.

6.0 Results and Discussion

6.1 *Test substance identity.* Prior to initiation of this study, DNA sequence of corn event MON 863 (Cavato and Lirette, 2001) and the plasmid used to produce Cry3Bb1 protein in *E. coli* (Appendix 1) were translated *in silico*. The resulting deduced amino acid sequences were determined to be identical (Figure 1). This was expected because the *E. coli* protein production system was designed using DNA derived from the same vector used to create corn event MON 863. Three analytical methods were used to verify the identity of the test substances: MALDI-TOF, N-terminal sequencing and immunoblotting.

MALDI-TOF analysis. The identity of both the *E. coli*- and corn-produced Cry3Bb1 proteins was assessed using MALDI-TOF mass spectrometry. This is considered an indirect means of establishing protein identity because it is based on the number of experimentally derived mass fragments matched to computer generated expected mass fragments. The more mass fragments that match, the greater the likelihood the correct protein was identified. A protein can typically be identified from 10-15 mass fragments. In corn, 50 and in *E. coli*, 42 tryptic digest fragments matched expected fragments from the expected amino acid sequence (Table 1).

Less than 1 Dalton between the observed mass and its theoretical mass fragment was required to be designated as a match. In corn, 38 and in *E. coli*, 36 tryptic digest fragments corresponded directly to computer generated theoretical mass fragments. These matches were made without consideration of potential amino acid modifications. In *E. coli*, a fragment mass of 685.48 Daltons was observed. This corresponded to the N-terminal fragment of the *E. coli*-produced protein minus the methionine residue. The N-terminal methionine residue is often processed in proteins (Bradshaw *et al.*, 1998). This mass was not observed in the corn protein digest. Instead, a mass of 727.48 Daltons was observed. This was 42 Daltons greater than that observed in the *E. coli* digest, and

corresponds to an acetylation of the N-terminal alanine residue. Post-translational modifications such as N-terminal acetylation are commonly observed in eukaryotic organisms (Tsunasawa and Sakiyama, 1984). The MALDI-TOF mass spectrometry data strongly suggest that this has occurred in the corn-produced protein. This result was previously observed for the corn event MON 863 Cry3Bb1 protein (Thoma *et al.*, 2001) and was therefore expected. The remaining experimental masses corresponding to *E. coli*- and corn-produced proteins were identified after accounting for mass differences due to amino acid modifications during the MALDI-TOF mass spectrometry analysis. Reduction and alkylation with iodoacetic acid modifies cysteine residues to carboxymethyl cysteine with a corresponding mass increase of 58 Daltons (Mitchelhill, 2001). Oxidation of methionine and tryptophan residues increases a peptide's mass by 16 and 32 Daltons, respectively (Mitchelhill, 2001). Buffer salts can also increase a peptide mass. Several fragments were identified with a +22 Dalton mass, corresponding to a sodium salt ion on those peptides (Mitchelhill, 2001). Peptides containing these amino acid modifications are indicated in Table 1.

Masses that were designated as matches were used to build a coverage map for the entire protein. Figure 2 shows the regions of amino acid sequence identified using MALDI-TOF mass spectrometry. Approximately 69% (*E. coli*) and 72% (corn) of the amino acids were identified. These data unambiguously identified peptides that included the Q349R amino acid substitution, as well as both the N- and C-termini. Typically, a coverage of > 50% is considered excellent and sufficient for identification of a protein.

N-terminal sequence analysis. N-terminal sequence analysis was used to assess the identity of the *E. coli*- and corn-produced proteins. Confirmation of identity required that observed sequence(s) match the expected sequence. In these analyses, multiple residues were observed in most cycles, making direct determination of a designated sequence impossible. N-terminal sequence determination, however, was possible after comparison of the observed results to the expected sequence. Two sequences, starting at positions 2 and 32, were determined for the *E. coli*-produced protein. Three sequences, starting at positions 19, 25 and 36, were determined for the corn-produced protein (Figure 3). These data confirmed the identity of both the *E. coli*- and corn-produced proteins and also indicated that these proteins had truncated N-termini. This observation indicated that these proteins were partially degraded, probably due to exposure to proteases during the purification process. Regardless, these results demonstrated that the aggregate of bands that migrated to ~74 kDa each contained sequence that matched the expected sequence for this Cry3Bb1 variant.

From these sequencing data, it was observed that the *E. coli*-produced protein was missing the N-terminal methionine. This corroborates the results of MALDI-TOF mass spectrometry where a peptide mass was observed that corresponded to an N-terminal

peptide minus methionine (residues 2-7, Table 1). No corresponding N-terminal sequence was observed from the corn-produced protein. The earliest observed sequence started at position 19. Again, the results MALDI-TOF mass spectrometry strongly suggested that the N-terminus of the corn-produced protein was acetylated and would therefore be refractory to N-terminal sequencing. The N-terminal sequencing data was consistent with the MALDI-TOF mass spectrometry data.

N-terminal sequence analysis was also performed on a large molecular weight band (~220 kDa) observed only in the corn-produced protein sample. Similar to that observed for the *E. coli*- and corn produced Cry3Bb1 N-terminal sequence determinations, multiple residues were observed for the ~220 kDa band in most cycles making the direct determination of a designated sequence impossible. However, no similarity was found between the data for the ~220 kDa protein and any portion of the expected Cry3Bb1 variant protein sequence (data not shown) indicating that these high molecular weight bands were impurities found only in the corn-produced protein preparation.

Immunoblot analysis. Immunoblot analyses were performed for both the *E. coli*- and corn-produced Cry3Bb1 proteins using polyclonal and monoclonal antibodies. A molecular weight of ~74 kDa was expected for the intact 653 amino acid protein calculated from the expected Cry3Bb1 amino acid sequence. Multiple immunoreactive bands were observed at the apparent electrophoretic mobility ranging from ~66 kDa to ~74 kDa in lanes containing the test substances (Figure 4A, lanes 3-8; Figure 4B, lanes 4-9). Corroborating the results of N-terminal sequence analysis, these data again indicated that both the *E. coli*- and corn-produced proteins were partially degraded. The aggregate of immunoreactive bands ranging from ~66-74 kDa were thus identified as Cry3Bb1 protein. For bands observed in this region of the blots, both the *E. coli*- and corn-produced Cry3Bb1 proteins had visually indistinguishable electrophoretic mobility and comparable immunological responses when detected using immunospecific antibodies.

The higher molecular weight bands (>75 kDa) observed for the corn-produced protein probed with polyclonal antibody (Figure 4A, Lanes 6-8) were not observed when probed with monoclonal antibody (Figure 4B, Lanes 7-9), suggesting that these were due to non-specific binding. These higher molecular weight bands were not observed where approximately 10-fold more protein was loaded (Figure 4B), further demonstrated that the higher molecular weight bands observed in Figure 4A were due to non-specific binding to contaminant corn protein(s), and did not represent Cry3Bb1 protein(s).

6.2 *Molecular weight and purity estimation.* Densitometric analysis of *E. coli*- and corn-produced proteins separated using SDS-PAGE was performed to estimate molecular weight and purity (Table 2). The average molecular weight and purity of each test substance was obtained from three separate load levels (estimated using total protein

concentration) on a polyacrylamide gel run at the initiation of this study (Figure 5). Analysis of the *E. coli*- and corn-produced proteins showed that multiple bands were present at an apparent electrophoretic mobility of 66-75 kDa. Because peptide fragments were identified using MALDI-TOF mass spectrometry analysis that matched both intact N-termini (minus the methionine residue) and C-termini, differences in the estimated molecular weight value for the uppermost band in the region expected (74 kDa) for the *E. coli* (72.0 kDa) and corn (74.6 kDa) Cry3Bb1 protein was apparently an artifact and likely due to the relative purity of these proteins. That is, nearly twice the level of protein attributed to Cry3Bb1 protein was present in the *E. coli*-produced protein preparation (when corrected for purity) relative to the corn-produced protein (Figure 5). The increased amount of may have affected the migration pattern.

The aggregate of bands observed between ~66-75 kDa were identified as Cry3Bb1 protein using immunoblot analysis (Figure 4) Furthet, multiple N-termini were observed for both the *E. coli*- and corn-produced proteins (Figure 3) that corresponded to progressive truncation at the N-termini. Therefore, all molecular weight species in the range of ~66-75 kDa were thus considered to be derived from Cry3Bb1 protein (Figure 5 and Table 2). Purity was calculated as the sum of individual relative percent optical density of bands falling within the range of ~66-75 kDa for each test substance. The purity of the *E. coli*- and corn-produced Cry3Bb1 proteins were estimated to be 92.6% and 53.9%, respectively.

An accurate estimation of purity was required primarily for the insect bioassay (Section 6.7). Including the aggregate of bands ranging from ~66 to ~75 kDa was justified and necessary. Previous studies established that the N-terminal portion of Cry3Bb1 protein variants was not required for activity against target insect larvae. For example, the Cry3Bb1 variant proteins isolated and from *B.t.* strains EG11098 and EG11231 consisted of two N-terminally truncated polypeptides of ~66 and ~59 kDa (Hileman *et al.*, 2001). These proteins were shown to be highly active against the larvae of Western corn rootworm (*Diabrotica virgifera vergifera*) (Hileman *et al.*, 2001) and Colorado potato beetle (*Leptinotarsa decemlineata*) (Holleschak *et al.*, 2001b). In another study, these Cry3Bb1 protein variants were shown to retain activity towards Colorado potato beetle even when proteolytically degraded to the ~59 kDa form (Leach *et al.*, 2001).

A diffuse band corresponding to ~220 kDa in the corn-produced Cry3Bb1 protein preparation represented 25.5% of the total protein (Table 2). Although the identity of this protein could not be determined using N-terminal sequence analysis, it was determined not to be derived from the expected Cry3Bb1 protein sequence.

6.3 *Protein concentration.* Total protein concentration was assessed using a colorimetric dye-binding assay and amino acid compositional analysis. The differences

in estimated total protein concentration (Table 3) were considered to be within the limits of error for each analytical method. The results of the colorimetric dye-binding assay (measured relative to a standard curve of BSA) were used throughout this study where the amount of protein has been reported. The relatively larger amount of contaminant proteins present in the corn-produced protein preparation apparently had no effect on the ability to estimate the concentration of this sample, because similar values were obtained using each method. Where necessary, such as the insect bioassay, concentration values were adjusted for purity.

6.4 *Amino acid composition.* Mole percent values of each amino acid for both the *E. coli*- and corn-produced proteins are described in Table 4. The observed amino acid composition of the *E. coli*-produced protein was comparable to the theoretical amino acid composition and consistent with the identity of the test substance. The observed purity for the corn-produced protein was too low (53.9%) to make a meaningful comparison.

6.5 *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. The modified Cry3Bb1 protein sequence (shown in Figure 1) deduced from the coding region of corn event MON 863 contains 5 potential N-glycosylation consensus sites [defined as (Asp-X-Thr/Ser) where X = any amino acid]. A consensus sequence for O-glycosylation has not been defined. However, multiple serine and threonine residues are present in the deduced sequence of this modified Cry3Bb1 protein. Because the potential for post-translational glycosylation exists, the *E. coli*- and corn-produced proteins were analyzed for covalently N- or O-linked carbohydrate moieties.

Proteins were electroblotted to PVDF membrane and detection of protein bound carbohydrates was performed after periodate oxidation. This method is sensitive for the detection of any carbohydrate modification. The results of the glycosylation assay are shown in Figure 6A. A single band was clearly visible for transferrin protein, the positive control (Lanes 3-5). Prokaryotic organisms rarely glycosylate proteins (Moens and Vanderleyden, 1997) and are generally thought to lack the cellular machinery to glycosylate proteins. No bands were observed for the *E. coli*-produced protein (Lanes 6,7), as expected. The corn-produced protein yielded multiple bands (Lanes 8,9), none of which appeared in the region expected for this Cry3Bb1 protein preparation (~66-75 kDa). Indeed, the region corresponding to ~66-75 kDa appeared blank (i.e. negative signal) in Lanes 8 and 9 (Figure 6A). The banding pattern (i.e. positive signal) observed in Lanes 8 and 9 indicated the presence of impurities. An immunoaffinity purification step using antibody covalently bound to protein A agarose (Appendix 1), could have been a source of carbohydrate impurities. This protein preparation may have been

contaminated with traces of agarose, which would not be detected using protein specific methods.

To further verify the absence of N- or O-linked carbohydrate moieties, a replicate gel was analyzed using SDS-PAGE and stained with Colloidal Brilliant Blue G (Figure 6B). As observed earlier (Figure 4), multiple bands were observed the both the *E. coli*- and corn-produced proteins (Figure 6B, Lanes 6-9). The banding pattern for the aggregate of bands designated as Cry3Bb1 protein clearly migrated to a position below the transferrin positive control protein (Lanes 3-5) and to a position approximately equal to the 66.2 kDa marker (Lane 10). The same region in Figure 6A corresponded to the blank region, demonstrating the absence of glycosylated protein in that region of the blot. Combined, these results demonstrated that neither the *E. coli*- nor corn-produced Cry3Bb1 protein were glycosylated.

6.6 *Storage stability.* The stability of each protein was assessed at common storage temperatures (4, –20 and –80 °C) for the duration of the experimental phase (29 days). SDS-PAGE analysis performed at the initiation of this study (Figure 5) was used to represent day 0. Protein samples, stored as solutions, were stored at the initiation and analyzed at the termination of the experimental phase of this study. Visual comparison of samples separated using SDS-PAGE (Figure 7) clearly showed that the banding pattern of both the *E. coli*- and corn-produced proteins had changed when stored at 4 °C. A single dominant band was observed at ~65 kDa and ~66 kDa for the *E. coli*- and corn-produced proteins, respectively, rather than the aggregate of bands described earlier (Figure 5). Relative to samples analyzed on day 0, no significant degradation was observed for solutions stored at –20 and –80 °C.

Methods were employed to reduce the effect of proteases during the pre-study purification of the test substances and most steps were performed at 4 °C (Appendix 1). Samples of *E. coli*- and corn-produced Cry3Bb1 protein that were designated for use in this study were transferred to a –80 °C freezer prior to initiation of this study. With the exception of samples designated for storage stability analyses, the test substances used in this study were stored in a –80 °C freezer until analyzed. Therefore, degradation that was observed for samples stored at 4 °C would not impact the results of other analyses used in this study.

Apparently, the N-terminal portion of this protein, regardless of the source, is relatively sensitive to proteases. N-terminal truncation of closely related Cry3Bb1 protein variants isolated from *B.t.* has been observed (Hileman *et al.*, 2001). Similarly, several wild type Cry3 proteins have been shown to undergo degradation (Carroll *et al.*, 1997; Hori *et al.*, 1994; Rupar *et al.*, 1991; Von Tersch *et al.*, 1994). The closely related protein Cry3Ba1

(GenBank Accession No. X17123) and wild-type Cry3Bb1 (Cry3Bb1, GenBank Accession No. M89794) are also observed to produce proteolytic products in solution (Rupar *et al.*, 1991).

6.7 *Insect bioassay.* Functional activity was assessed using CPB larvae fed artificial diets containing varying amounts of the *E. coli*- and corn-produced proteins. A dose response was observed for each replicate assay, indicating that both proteins were functional and highly active. The LC_{50} estimates and corresponding 95% CI for each of the *E. coli*- and corn-produced proteins are shown in Table 5. These results demonstrated that there was considerable overlap between the 95% CI of each of the estimated LC_{50} values across all replicates. Therefore, these proteins were considered biologically equivalent.

7.0 Conclusions

The physicochemical and functional properties of the *E. coli*- and corn-produced proteins have been characterized and are summarized in Table 6. The deduced amino acid sequence assessed using MALDI-TOF mass spectrometric analyses yielded unambiguous data that confirmed the identity of the test substances. N-terminal sequence analyses further corroborated the identity of both test substances as the Cry3Bb1 protein. Immunoblot analyses demonstrated that these two proteins had equivalent electrophoretic mobility and immunoreactivity. Biological activity was assessed using the larvae of Colorado potato beetle. Estimation of the LC_{50} values and 95% confidence intervals demonstrated that the activity of these proteins was statistically indistinguishable. There was a minor difference in the observed molecular weights of the uppermost band, estimated using SDS-PAGE analysis. MALDI-TOF data confirmed that the N- and C-termini were identical. In combination, these data established the physicochemical and biological equivalence of the *E. coli*- and corn-produced Cry3Bb1 proteins. Equivalent physicochemical and functional properties support the use of the *E. coli*-produced protein in studies designed to evaluate the Cry3Bb1 protein variant produced in corn event MON 863.

8.0 References

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Table 1. Summary of masses observed using MALDI-TOF mass spectrometry. Identified amino acid modifications are shown in parentheses for relevant peptide sequences. The composition of each wash step used to desalt samples is described in Section 5.2.3

Expected mass (Daltons)	aa Position	Sequence	Corn				*E. coli*			
			Wash 1	Wash 2	Wash 3	Wash 4	Wash 1	Wash 2	Wash 3	Wash 4
564.31	45-48	EFLR trypsin autocatalytic fragment	564.41 568.23	564.48		564.55	564.42 568.26	564.48 568.31		564.57 568.39
619.34	299-303	TELTR	619.48							606.31
						644.30				628.30 644.31
685.34	_2-7	ANPNNR								650.32 666.30
686.42	155-160	TPLSLR	686.55	686.61		686.72	685.48			
727.35	Ac 2-7	(Ac-)ANPNNR	727.48					686.61		
752.38	123-128	IEEYAK	752.52				752.53	752.62		
779.47	418-424	VYLGVTK	779.63				779.70			
788.42	387-393	STEPVQK	788.55				788.57			
794.40	394-400	LSFDGQK trypsin autocatalytic fragment	794.54	794.64		794.74 842.86	794.57 842.69	794.63 847.67	794.68	795.77 855.43
880.48	122-128	KIEEYAK	880.63	880.74			880.65	880.74		861.44
897.58	513-520	ITQLPVVK	897.74			897.96	897.76			
925.47	573-580	YASTTNLR	925.66	925.75		925.87	925.67	925.75		
937.53	348-355	LRPGYFGK	937.69	937.81	937.85	937.92	937.73	937.80	937.87	937.95
1026.49	258-266	GSTYDAWVK	1026.68	1026.79	1026.92		1026.71	1026.80	1026.88	
1058.49	258-266	GSTYDA(W)VK		1058.78						
1128.56	377-386	TITSPFYGDK	1128.76	1128.90	1129.49	1129.01	1128.82	1128.88	1128.97	
1185.70	556-566	VTLNSAALLQR		1186.05	1186.14	1186.19	1185.93	1186.05	1186.12	1186.21
				1202.99						
1244.54	425-434	VDFSQYDDQK	1244.74					1244.93		
1350.64	170-180	ELFSQAESHFR	1350.93	1351.07	1351.13	1351.21	1350.92	1351.05	1351.13	1351.24
1362.70	490-500	GTIPFFTWTHR		1363.12	1363.19	1363.27	1362.98		1363.19	1363.30
1385.66	501-512	SVDFFNTIDAEK		1386.08	1386.18	1386.23	1385.94	1386.07	1386.14	
1394.70	490-500	GTIPFFT(W)THR		1395.10		1395.27				
						1408.22				
1457.74	404-417	TIANTDVAAWPNGK		1458.16				1458.16		
1460.86	554-566	FKVTLNSAALLQR			1461.38	1461.47				
1489.74	404-417	TIANTDVAA(W)PNGK		1490.20					1461.38	
1496.78	319-331	YGPTFLSIENSIR	1497.05	1497.25	1497.32	1497.41	1497.10	1497.22	1497.32	1497.41

Table 1 (continued)

Expected mass (Daltons)	aa Position	Sequence	Corn Wash 1	Corn Wash 2	Corn Wash 3	Corn Wash 4	*E. coli* Wash 1	*E. coli* Wash 2	*E. coli* Wash 3	*E. coli* Wash 4
1518.80	489-500	RGTIPFFTWTHR		1519.29	1519.35	1519.43		1519.30	1519.36	1519.43
						1535.40				1535.39
1590.92	641-653	IYIDKIEFIPVQL	1591.15				1591.25			
1619.83	168-180	IRELFSQAESHFR		1620.33	1620.43	1620.51			1620.43	1620.59
					1635.47					
					1680.45	1680.54				
				1738.30	1738.94				1738.40	1738.44
1755.80	49-64	MTEDSSTEVLDNSTVK		1755.29						
1764.98	304-318	DIFTDRIFLLTTLQK	1765.31		1765.60	1765.69	1765.35		1765.59	1765.70
1771.80	49-64	(M)TEDSSTEVLDNSTVK		1772.28						
						1787.69				1787.67
2001.04	332-347	KPFHLFDYLQGIEFHTR	2001.40	2001.65	2001.76	2001.87	2001.48		2001.75	2001.88
2023.04	332-347	KPFHLFDYLQGIEFHTR (+22 Na salt)				2023.90				2023.85
2041.08	581-597	LFVQNSNNDFLVIYINK	2041.40				2041.52			
2146.93	472-488	AYSHQLNYAE(C)FLMQDR	2147.73	2147.61	2147.72	2147.83	2147.43		2147.71	2147.80
2162.93	472-488	AYSHQLNYAE(C)FL(M)QDR	2163.14	2163.60	2163.69	2163.84	2163.43		2163.69	2163.85
		trypsin autocatalytic fragment		2211.78	2211.91				2211.86	2212.07
		trypsin autocatalytic fragment							2283.97	
										2300.04
2394.10	356-376	DSFNYWGSGNYVETRPSIGSSK	2394.57	2394.85	2394.96	2395.10	2314.75			
2402.35	191-211	REMTLTVLDLIVLFPFYDIR	2402.77				2394.71	2394.79	2394.95	2395.09
				2410.83			2402.90			
2426.10	356-376	DSFNY(W)GSGNYVETRPSIGSSK	2426.54	2426.85						
2484.06	212-232	DAQVFGEEWGYSSEDVAEFYR	2483.73		2484.19	2484.33	2426.62			
2499.08	425-445	VDFSQYDDQKNETSTQTYDSK		2499.83			2483.88		2484.19	2484.35
						2501.12		2499.79	2499.94	
2516.06	212-232	DAQVFGEE(W)GYSSEDVAEFYR	2516.54				2516.60			
2552.19	237-257	LTQQYTDH(C)VNWYNVGLNGLR	2552.69	2552.96	2553.12	2553.22	2552.65		2553.11	2553.29
2585.19	237-257	LTQQYTDH(C)VN(W)YNVGLNGLR	2585.63							
2637.29	131-153	ALAELQGLQNNFEDYVNALNSWK			2638.28	2638.34			2638.21	
2640.16	212-233	DAQVFGEEWGYSSEDVAEFYRR			2640.26					
2656.14	602-625	DDDLTYQTFDLATTNSNMGFSGDK		2656.01				2655.95		
							2670.88			
				2718.09						
2734.28	447-471	NNGHVSAQDSIDQLPPETTDEPLEK		2735.12				2736.08		
2890.38	446-471	RNNGHVSAQDSIDQLPPETTDEPLEK		2891.17				2891.14		
				3034.29						
3443.64	15-44	VTPNSELQTNHNQYPLADNPNSTLEELNYK		3444.65	3444.93	3445.11		3444.69	3444.81	

Table 2. Protein molecular weight and purity estimation. Average relative percent optical densities of each visible band and calculated average deviations are derived from densitometric analysis of the SDS polyacrylamide gel shown in Figure 5, Lanes 3-5 (*E. coli*) and Lanes 7-9 (corn). The average molecular weight and average deviation were calculated from the molecular weight markers (Figure 5, Lanes 1, 2, 6 and 10) using the manufacturer's supplied molecular weight values. Protein purity was calculated from values shown in bold.

***E. coli*-produced Cry3Bb1 protein**		**Corn-produced Cry3Bb1 protein**	
Relative OD (%)	Mol. Wt. (kDa)	Relative OD (%)	Mol. Wt. (kDa)
1.0 ± 0.0	208.4 ± 9.7	6.3 ± 1.1	285.0 ± 13.8[a]
2.2 ± 0.1	153.0 ± 3.3	25.5 ± 1.6	219.5 ± 12.6
67.4 ± 1.4	**72.0 ± 0.4**	5.4 ± 0.9	104.5 ± 1.1
11.8 ± 1.4	**68.2 ± 0.6**	**10.5 ± 0.6**	**74.6 ± 0.1**
13.4 ± 1.3	**66.1 ± 0.4**	**17.9 ± 3.9**	**72.1 ± 0.4**
0.7 ± 0.0	29.2 ± 0.1	**8.0 ± 1.0**	**69.5 ± 0.7**
0.4 ± 0.0	28.6 ± 0.0	**7.8 ± 0.7**	**68.0 ± 0.6**
1.0 ± 0.1	25.7 ± 0.4	**9.7 ± 0.4**	**66.3 ± 0.4**
1.0 ± 0.1	11.2 ± 1.1	8.5 ± 0.7	53.5 ± 0.2
1.9 ± 0.2	7.8 ± 0.5	5.5 ± 0.0	28.9 ± 0.2

[a] Extrapolated from above the largest molecular weight marker used, Figure 5, Lanes 1, 2, 6 and 10.

Table 3. Estimation of total protein concentration. Errors correspond to the average deviation of all replicates used to calculate the mean.

	Protein Concentration (mg/mL)	
Assay Method	***E. coli***	**corn**
Colorimetric assay	0.58 ± 0.03[a]	0.46 ± 0.01[b]
Amino acid composition	0.59 ± 0.01[c]	0.48 ± 0.01[c]

[a] Calculated from 6 replicate determinations.
[b] Calculated from 4 replicate determinations.
[c] Calculated from 2 replicate determinations.

Table 4. Amino acid compositional analysis. Observed mole percent data was averaged from two separate analyses of the *E. coli*- and corn-produced Cry3Bb1 protein. The observed purity for the corn-produced protein was too low (53.9%) to make a meaningful comparison.

	Mole Percent		
	observed		
amino acid	*E. coli*	Corn	theoretical
ASX[a]	12.74	9.65	12.71
THR	7.50	10.13	7.81
SER	7.97	8.26	8.27
GLX[a]	11.08	6.39	10.26
PRO	4.13	6.92	3.98
GLY	5.90	11.92	5.21
ALA	6.48	5.94	5.51
CYS[b]	0.12	0.14	0.31
VAL	6.30	8.69	5.97
MET	1.14	0.96	1.22
ILE	5.01	4.67	5.05
LEU	9.77	8.60	9.34
TYR	4.57	4.08	4.75
PHE	5.97	5.40	6.28
LYS	6.30	4.70	5.97
HIS	1.26	0.00[c]	1.53
ARG	3.78	3.53	4.44
TRP[d]	n.d.	n.d.	1.38

[a] ASX and GLX correspond to the sum of aspartic acid and asparagine mole percents and the sum of glutamic acid and glutamine mole percents, respectively. These amino acid pairs are indistinguishable after amino acid hydrolysis. Asparagine and glutamine are deamidated and converted to the acid forms during the vapor phase acid hydrolysis step (Section 5.8).

[b] Recovery of cysteine is variable from sample to sample.

[c] No histidine was quantitated due to overlap with the free amine peak in that region of the chromatogram.

[d] Tryptophan is completely destroyed during acid hydrolysis.

Table 5. Insect bioassay results using the larvae of CPB. Doses were corrected for purity prior to analysis.

Protein Source	Assay Replicate[a]	LC_{50} (µg/mL)	95% CI
E. coli	1	0.86	0.51-1.10
	2	0.67	0.38-0.88
	Pooled[b]	0.76	0.57-0.92
Corn	1	0.56	0.39-0.72
	2	0.74	0.20-1.00
	Pooled[b]	0.63	0.48-0.77

[a] A total of 112 insects were tested for each replicate.
[b] Calculated using the all data from assay replicates 1 and 2.

Table 6. Summary of the Cry3Bb1 protein properties.

		Cry3Bb1 Protein Source	
Criteria	**Method**	***E. coli***	**Corn Event MON 863**
Identity	DNA sequence	confirmed[a]	confirmed[b]
Identity	MALDI-TOF	confirmed	confirmed
Identity	N-Terminal sequence analysis	confirmed	confirmed
Identity	Amino acid composition	consistent	N.D.[c]
Immunoreactivity	Immunoblot analysis	confirmed	confirmed
Bioactivity	CPB bioassay	LD_{50} of 0.76 µg/mL (0.57-0.92)	LD_{50} of 0.63 µg/mL (0.48-0.77)
Molecular weight	SDS-PAGE	66.0 - 72.0 kDa	66.3 - 74.6 kDa
Purity	SDS-PAGE	92.6%	53.9%
Concentration	Colorimetric assay	0.58 mg/mL	0.46 mg/mL

[a] Determined prior to initiation of this study (see Figure 1).
[b] Determined previously (Cavato and Lirette, 2001).
[c] Not determined.

This place holder page in place of the following pages and can be found in **Cross Reference Number 1** of the **Confidential Attachment.**

Page	MSL-17274 Pages 36-37 of 71
Page Title	**Figure 1.** Sequences of the *E. coli* and corn Cry3Bb1 protein variants
Reason for Deletion	Discloses commercial information
FIFRA Reference	10(d)(1)(A)

MSL-17274 Pages 36-37 of 71 are found in the **Confidential Attachment,** Pages 3-4 of 4.

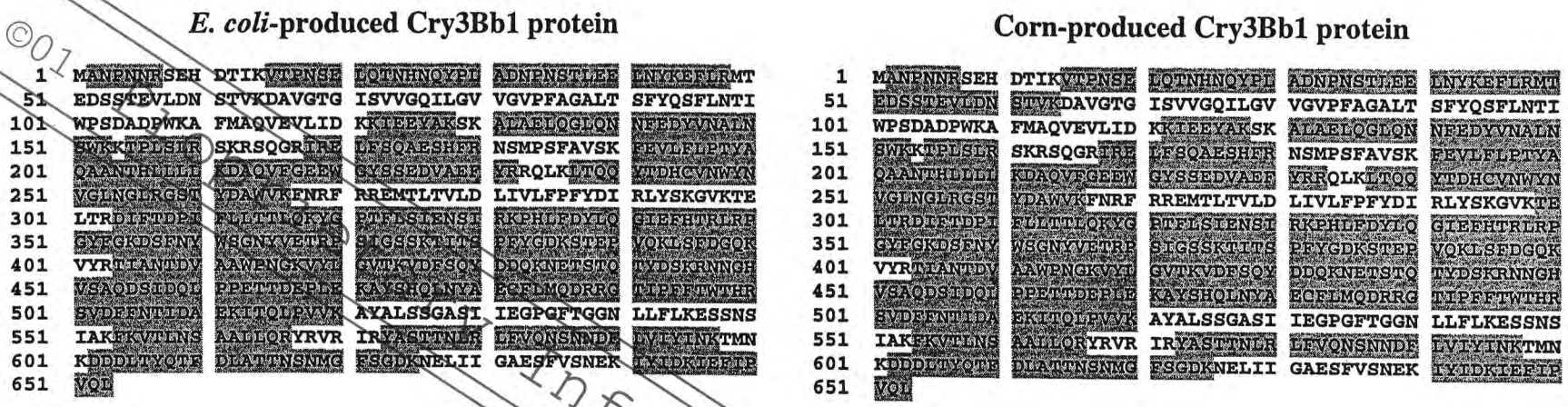
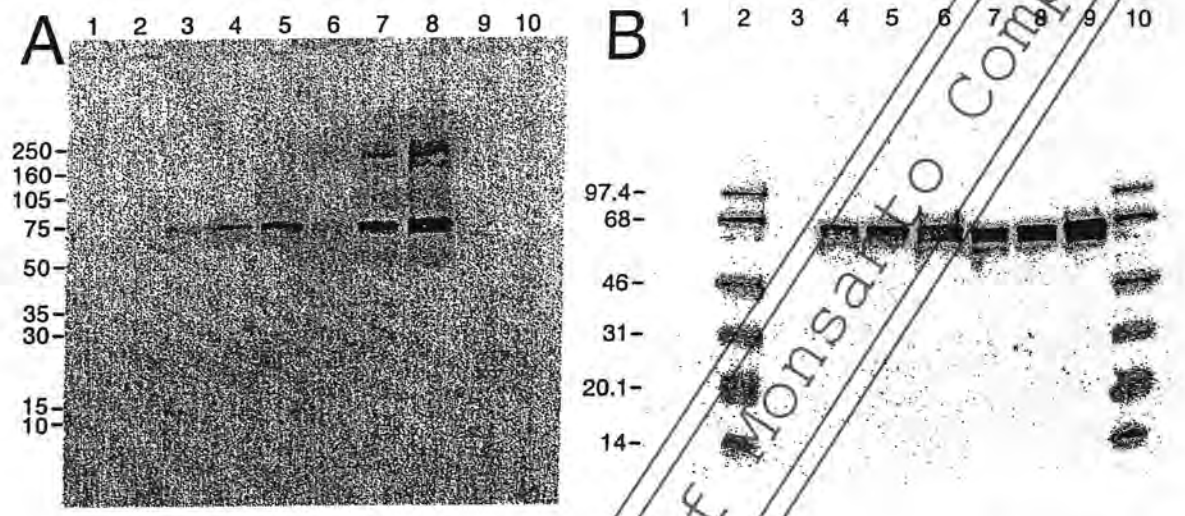


Figure 2. Summary of MALDI-TOF mass spectrometric analyses. The *E. coli*- and corn-produced protein sequences shown were deduced from their respective coding regions and are identical (Figure 1). The shaded regions correspond to identified peptides (see Table 1). Approximately 69% and 72% of the expected protein sequences were identified for the *E. coli*- and corn-produced proteins, respectively.

Figure 3. N-terminal amino acid sequence analysis. The expected amino acid (residues 1-50 of 653) sequence and observed sequences obtained from the *E. coli*- and corn-produced Cry3Bb1 proteins are shown. Undesignated amino acid assignments are shown as an "X" and tentative amino acid assignments are shown in parentheses. Multiple residues were observed in most cycles, indicating that the proteins had undergone degradation. These were interpreted as separate N-termini and are shown using the single letter amino acid code[a].

Expected	^{1}M A N P N N R S E H D T I K V T P N S E L Q T N H N Q Y P L A D N P N S T L E E L N Y K E F L R M T•••
E. coli (1)	^{2}A N P N N R S E H D(T)I K V T^{16}
E. coli (2)	^{32}D N P N S T L E X L N Y K E F^{46}
MON 863 (1)	^{19}S E L Q T N H X Q Y P L A D X^{33}
MON 863 (2)	^{36}S T L E X L N Y K E F L X M T^{50}
MON 863 (3)	^{25}X N Q Y P L A D X P N S X X X^{39}

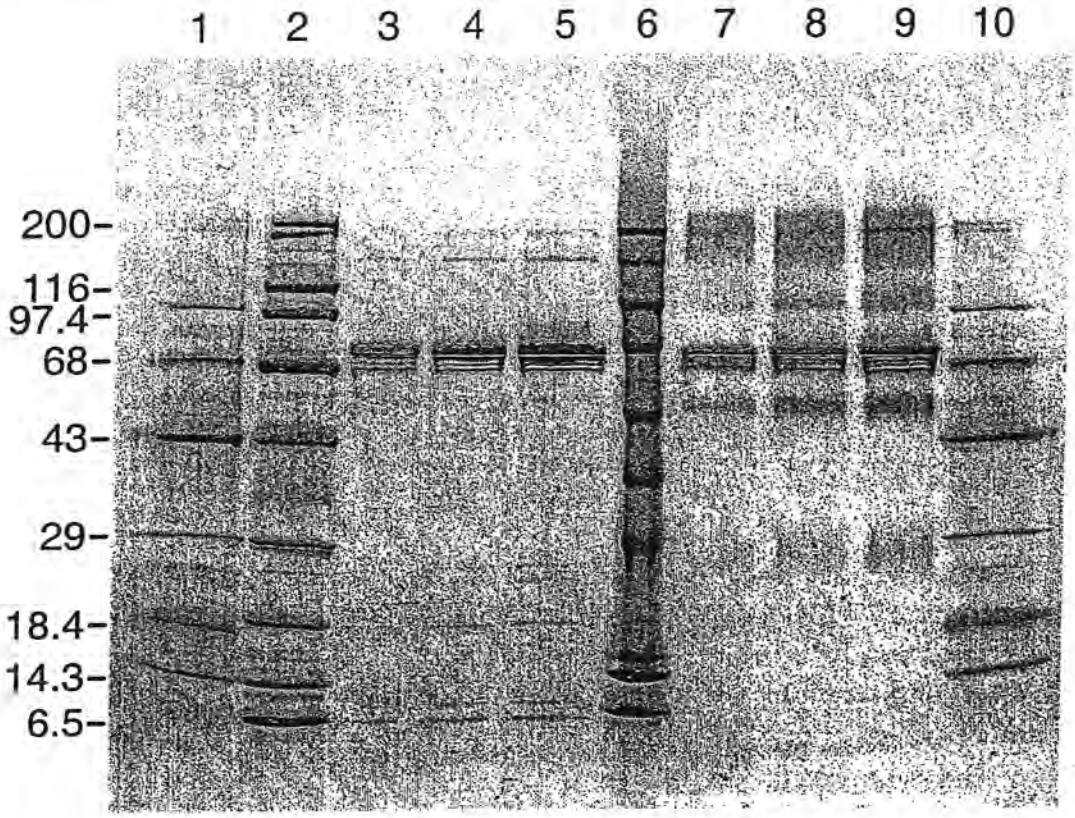
[a] The single letter IUPAC-IUB amino acid code is A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, Asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.



Lane	Sample	Amount (ng)
1	pre-stained MW marker	750/band
2	MW marker	–
3	*E. coli* Cry3Bb1	~4.9
4	*E. coli* Cry3Bb1	~25
5	*E. coli* Cry3Bb1	~49
6	MON 863 Cry3Bb1	~8.3
7	MON 863 Cry3Bb1	~41
8	MON 863 Cry3Bb1	~83
9	MW marker	–
10	blank	–

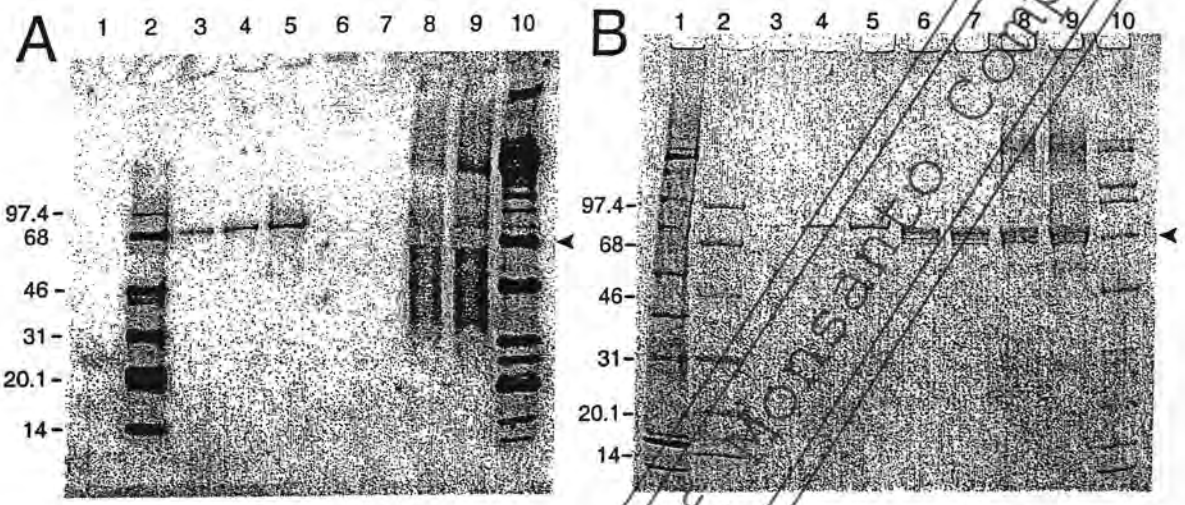
Lane	Sample	Amount (ng)
1	pre-stained MW marker	750/band
2	MW marker	2000 total
3	blank	–
4	*E. coli* Cry3Bb1	~98
5	*E. coli* Cry3Bb1	~196
6	*E. coli* Cry3Bb1	~491
7	MON 863 Cry3Bb1	~165
8	MON 863 Cry3Bb1	~331
9	MON 863 Cry3Bb1	~827
10	MW marker	2000 total

Figure 4. Immunoblot analysis. Samples of *E. coli*- and corn-produced Cry3Bb1 proteins were separated by SDS-PAGE, electroblotted to nitrocellulose membrane, detected using Cry3Bb1 antisera followed and developed using an ECL system. Amounts loaded correspond to purity corrected values. Approximate molecular weights (kDa) are shown on the left of each panel. Panel **A**, detection using polyclonal antisera (lot 6199830B) raised against wild type Cry3Bb1 protein (15 s exposure). Panel **B**, detection using monoclonal antibody (lot 6199915) raised against *B.t.* strain EG11231 Cry3Bb1.11231 protein (4 s exposure).



Lane	Sample	Amount (µg)
1	MW marker	1/band
2	MW marker	1/band
3	*E. coli* Cry3Bb1	1
4	*E. coli* Cry3Bb1	2
5	*E. coli* Cry3Bb1	3
6	MW marker	1/band
7	MON 863 Cry3Bb1	1
8	MON 863 Cry3Bb1	2
9	MON 863 Cry3Bb1	3
10	MW marker	1/band

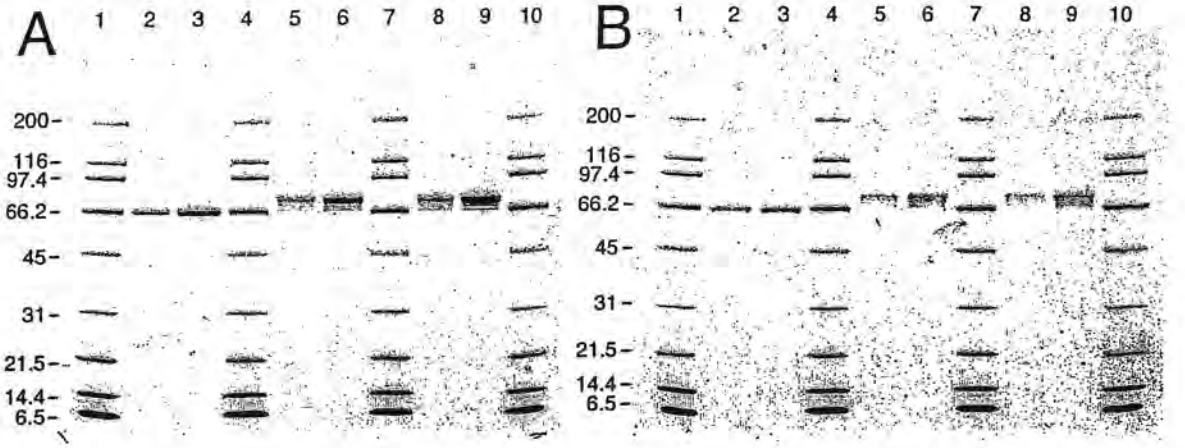
Figure 5. Gradient SDS-PAGE analysis. A 4→20% polyacrylamide gel was stained with Colloidal Brilliant Blue G. Samples of *E. coli*- and corn-produced Cry3Bb1 protein were analyzed at study initiation. Amounts loaded correspond to total protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lane 2.



Lane	Sample	Amount (µg)
1	pre-stained MW marker	1/band
2	ECL MW marker	5 total
3	Transferrin	0.02
4	Transferrin	0.1
5	Transferrin	0.5
6	*E. coli* Cry3Bb1	0.5
7	*E. coli* Cry3Bb1	1
8	MON 863 Cry3Bb1	0.5
9	MON 863 Cry3Bb1	1
10	Biotinylated MW marker	1.3 total

Lane	Sample	Amount (µg)
1	pre-stained MW marker	1/band
2	ECL MW marker	5 total
3	Transferrin	0.02
4	Transferrin	0.1
5	Transferrin	0.5
6	*E. coli* Cry3Bb1	0.5
7	*E. coli* Cry3Bb1	1
8	MON 863 Cry3Bb1	0.5
9	MON 863 Cry3Bb1	1
10	Biotinylated MW marker	5.2 total

Figure 6. Glycosylation analysis. Transferrin was used as a positive control. Amounts of *E. coli*- and corn-produced Cry3Bb1 proteins loaded correspond to total protein. Approximate molecular weights (kDa) to the left of each panel correspond to the markers loaded in Lane 2. Arrows indicate the 66.2 kDa marker protein in Lane 10. Panel **A**, ECL glycoprotein detection on PVDF membrane electroblotted with the indicated proteins (approximately 2 min exposure). Panel **B**, Gel stained using Colloidal Brilliant Blue G.



Lane	Sample	Amount (µg)
1	MW marker	1/band
2	*E. coli* Cry3Bb1, 4 °C	2
3	*E. coli* Cry3Bb1, 4 °C	1
4	MW marker	1/band
5	*E. coli* Cry3Bb1, –20 °C	2
6	*E. coli* Cry3Bb1, –20 °C	1
7	MW marker	1/band
8	*E. coli* Cry3Bb1, –80 °C	2
9	*E. coli* Cry3Bb1, –80 °C	1
10	MW marker	1/band

Lane	Sample	Amount (µg)
1	MW marker	1/band
2	MON 863 Cry3Bb1, 4 °C	2
3	MON 863 Cry3Bb1, 4 °C	1
4	MW marker	1/band
5	MON 863 Cry3Bb1, –20 °C	2
6	MON 863 Cry3Bb1, –20 °C	1
7	MW marker	1/band
8	MON 863 Cry3Bb1, –80 °C	2
9	MON 863 Cry3Bb1, –80 °C	1
10	MW marker	1/band

Figure 7. Storage stability analysis. Gradient SDS-PAGE (4→20%) analysis was performed on proteins stored at specified temperatures for 29 days. Gels were stained with Coomassie Brilliant Blue R. Amounts of *E. coli*- and corn-produced Cry3Bb1 proteins correspond to total protein. Approximate molecular weights (kDa) are shown on the left. Panel **A**, samples of *E. coli*-produced Cry3Bb1 protein. Panel **B**, samples of corn-produced Cry3Bb1 protein.

Appendix 1

Pre-study Protein Purification

The synthesis of these test substances was performed prior to initiation of this study and were not required to meet the GLP requirements. However, procedures were documented in Monsanto Notebooks and, where applicable, SOP's were used.

Isolation of the corn event MON 863 Cry3Bb1 protein

Preparation of the immunogen. A synthetic peptide (74-3B1-PAb) corresponding to amino acids 1-58 of the Cry3Bb1.11098 protein sequence was produced at New England Peptide Inc. (Fitchburg, MA). N-terminal sequence analysis and MALDI-TOF mass spectrometry was performed to confirm the identity of the peptide prior to immunization of rabbits.

N-terminal sequencing results of the synthetic peptide (74-3B1-PAb).

Position	Abundance	Sequence
expected		MANPNNRSEHDTIKVTPNSELQTNHNQYPLADNPN S TL E ELNYKE F LR
1	50 - 60 %	MANPNNRSEHDTIKVTPNSELQTNHNQYPLADNPX(S)TL(E)XXNYKX(F)
4	20 - 30 %	PNNRSEHDTIKVXPNSELQTNHNXYPLAD
34	30 - 40 %	PN S TL E XLNYKE F LR

Observed mass values for the synthetic peptide (74-3B1-PAb) and corresponding sequence obtained using MALDI-TOF mass spectrometry.

MH+ Average Mass	Sequence
expected	[1]MANPNNRSEHDTIKVTPNSELQTNHNQYPLADNPNSTLEELNYKEFLRMTEDSSTEVL[58]
6693.3	MANPNNRSEHDTIKVTPNSELQTNHNQYPLADNPNSTLEELNYKEFLRMTEDSSTEVL
6376.9	PNNRSEHDTIKVTPNSELQTNHNQYPLADNPNSTLEELNYKEFLRMTEDSSTEVL
2947.3	PNSTLEELNYKEFLRMTEDSSTEVL
2850.1	NSTLEELNYKEFLRMTEDSSTEVL
2736.0	STLEELNYKEFLRMTEDSSTEVL
3990.0	No matching mass found

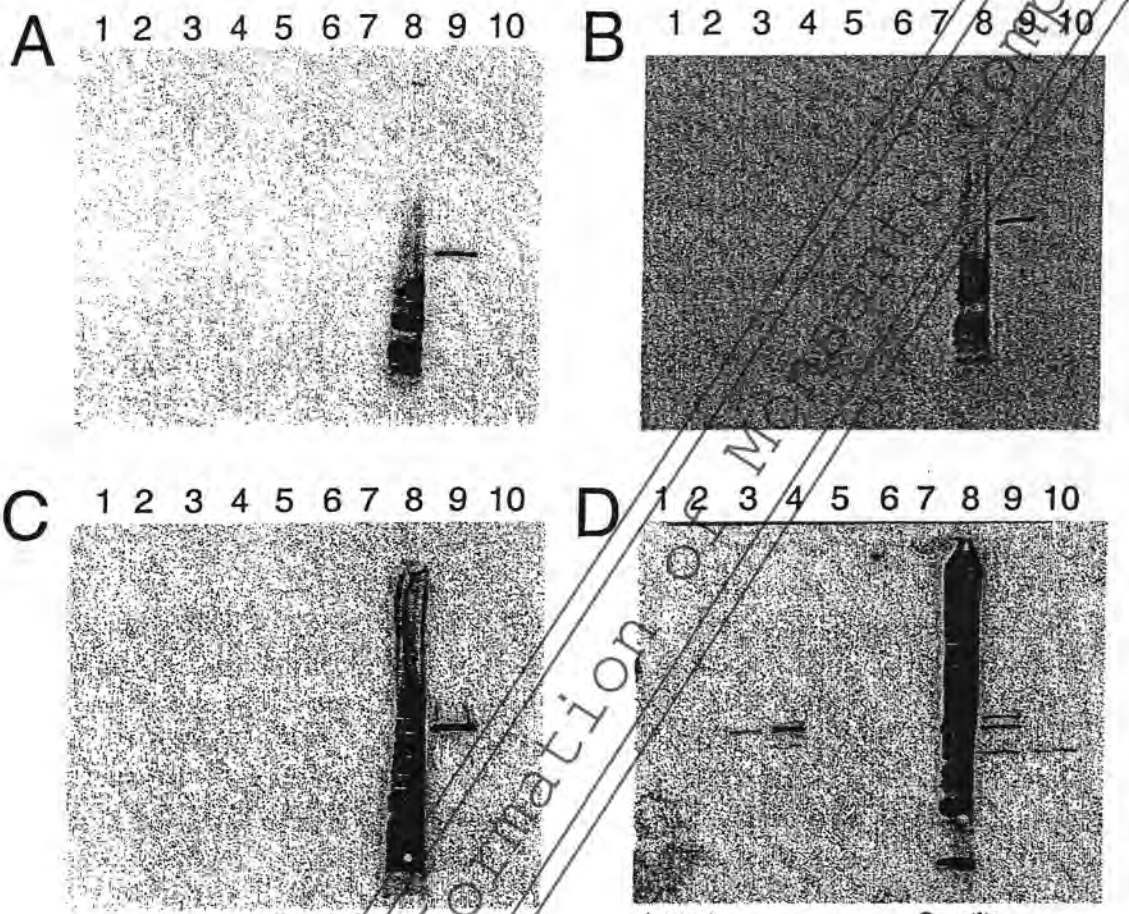
The N-terminal sequencing results confirmed that the majority (>50%) of this sample matched the expected sequence for the N-terminus of corn event MON 863 Cry3Bb1 protein variant. A mass was identified that matched the expected mass of residues 1-58. The results of these analyses confirmed the identity of the synthetic peptide (74-3B1-PAb).

Polyclonal antibody (PAb) production. Polyclonal antibody production was performed at Harlan Bioproducts for Science, Inc. (Madison, WI). Three rabbits (MR 876, MR 877 and MR 878) were immunized with ~1 mg of the 74-3B1-PAb synthetic peptide. After the initial immunization, the rabbits were boosted every 28 days until the final bleed on

day 112. Sera from the final bleeds were tested for anti-Cry3Bb1 antibodies by immunoblot analysis.

Immunoblot analysis. Cry3Bb1.11098 protein purified from *Bacillus thuringiensis* strain EG11098, 74-3B1-PAb peptide, extracts of the microbial pesticide Raven™, MON 863 and MON 846 grain were separated on SDS-polyacrylamide gels and electroblotted onto 0.45 µm nitrocellulose membranes. In separate containers, each membrane was blocked by incubation with 10% (w/v) NFDM in PBST for 45 min followed by a 45 min incubation with a dilution of the primary antibody in PBST containing 5% NFDM. Four polyclonal antibody preparations were compared: rabbit anti-74-3B1-PAb (MR 876, MR 877 and MR 878) at a 1:500 dilution and rabbit anti-Cry3Bb1 MR-783 at a 1:5000 dilution. Membranes were washed for approximately 3 min each in PBST and incubated for 45 min in a 1:7500 dilution of goat anti-rabbit IgG HRP conjugate. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL™) detection system and exposed to Hyperfilm™.

The immunoblot results (Figure shown on next page) demonstrated that the three polyclonal antibody preparations (MR 876, MR 877 and MR 878) were immunospecific for the full length Cry3Bb1 protein (~74 kDa). *B.t.* strain EG11098 Cry3Bb1.11098 protein (Lanes 2-4) was previously characterized (Hileman *et al.*, 2001). This *B.t.* isolated protein was N-terminally truncated (missing residues 1-57, ~66 kDa) and was thus not expected to be recognized by rabbit anti-74-3B1-PAb antibody (Panels **A-C**). Varying amounts of synthetic peptide (74-3B1-Pab, Lanes 5-7) were not detected using antibody preparations MR 876, MR 877 and MR 878 (Panels **A-C**), suggesting that these antibody preparations may not be highly immunoreactive (20 ng was not detected). The extract of Raven™ [a microbial pesticide that contains Cry3Bb1 and Cry3Aa protein (Holleschak *et al.*, 2001b)], was apparently over-loaded and appeared as a dark smear in each of the immunoblots (Lane 8). Extract of grain from corn event MON 863 clearly demonstrated the immunospecificity of the MR 876, MR 877 and MR 878 antibody preparations. A single immunoreactive band was observed (Lane 9, Panels **A-C**). Comparison of the electrophoretic mobility of these immunoreactive bands (Panels **A-C**) to those observed in Panel **D** indicate that the immunoreactive band was the full length (~74 kDa) Cry3Bb1.11098 protein. The anti-Cry3Bb1 MR 783 polyclonal antibody (Panel **D**) was expected to recognize the full length protein as well as N-terminally truncated forms (~66 kDa).



Lane	Description	Amount	Quantity
1	Amersham marker RPN 800	2 µL	—
2	Cry3Bb1.11098 (*B.t.*)	5 µL	5 ng
3	Cry3Bb1.11098 (*B.t.*)	10 µL	10 ng
4	Cry3Bb1.11098 (*B.t.*)	20 µL	20 ng
5	74-3B1-PAb synthetic peptide	5 µL	5 ng
6	74-3B1-PAb synthetic peptide	10 µL	10 ng
7	74-3B1-PAb synthetic peptide	20 µL	20 ng
8	Raven™	10 µL	—
9	MON 863 corn grain extract	30 µL	—
10	MON 846 corn grain extract	30 µL	—

Immunoblot analysis of Cry3Bb1 protein extracts produced from *Bacillus thuringiensis*, IPC event MON 863, and 74-3B1-PAb synthetic peptide. Immunoblots were detected using (**A**) anti-74-3B1-PAb MR 876 serum; (**B**) anti-74-3B1-PAb MR 877 serum; (**C**) anti-74-3B1-PAb MR 878 serum; (**D**) anti-Cry3Bb1 MR 783 polyclonal antibody.

Preparation of immunoaffinity column. Rabbit anti-74-3B1-PAb was covalently immobilized to protein A in the following manner. A 5 mL protein A agarose column was washed with 50 mL buffer B (100 mM Tris-HCl, pH 8.0) and 6 mL rabbit serum MR 877 was applied. Unbound material was removed by washing with 10 mL buffer B followed by an additional 50 mL of buffer C (200 mM sodium borate, pH 9.0). Antibody coupling to protein A agarose was achieved by addition of dimethylpimelimidate (DMP). Approximately 5.2 mg DMP (20 mM final concentration) was added to a slurry of 10 mL of protein A agarose in buffer C. The slurry was mixed at room temperature for 30 min. The coupling reaction was stopped by washing the column with 30 mL of buffer D (200 mM ethanolamine, pH 8.0). The slurry was further incubated with 20 mL of buffer D for approximately 2 h at room temperature. Finally, the column was washed with 10 mL of 100 mM triethylamine, pH 11.2 and stored in 10 mL of PBS at 4 °C.

Preparation of corn event MON 863 grain extract. Corn hybrid RX 670 grain containing event MON 863 was obtained from plants grown under Production Plan 00-01-39-18 and 00-01-39-16. Prior to extraction of the Cry3Bb1 variant protein, the grain was ground in liquid nitrogen and de-fatted in hexane at 55 °C. Soluble protein was extracted from the processed grain tissue using two approximately 30 sec bursts at 13,000 rpm with a polytron PT-MR 3000 homogenizer (Littau, Switzerland). Processed grain was mixed at a tissue to volume ratio of 1 g to 10 mL extraction buffer [100 mM sodium carbonate, pH 10, 5 mM EDTA, 1 mM CHAPS containing plant protease inhibitor cocktail (Sigma, cat No. P-9599)]. Insoluble plant tissue was removed by filtration through 3 layers of Mira-Cloth and centrifugation (38,000 ×g, 20 min at 4 °C using a Beckman JLA 16.250 rotor).

Immunoaffinity Chromatography. Isolation of Cry3Bb1 protein from grain extract was achieved by recirculating the plant extract over the protein A-antibody column at a flow rate of ~1 mL/min overnight. Bound Cry3Bb1 protein was eluted with 6 mL of 100 mM triethylamine and concentrated to ~2 mL using a Millipore 30 kDa ultrafree centrifugal device (Bedford, MA). Concentrated sample was diluted with 10 mL buffer B containing 0.5 M $(NH_4)_2SO_4$ and again concentrated to ~600 µL.

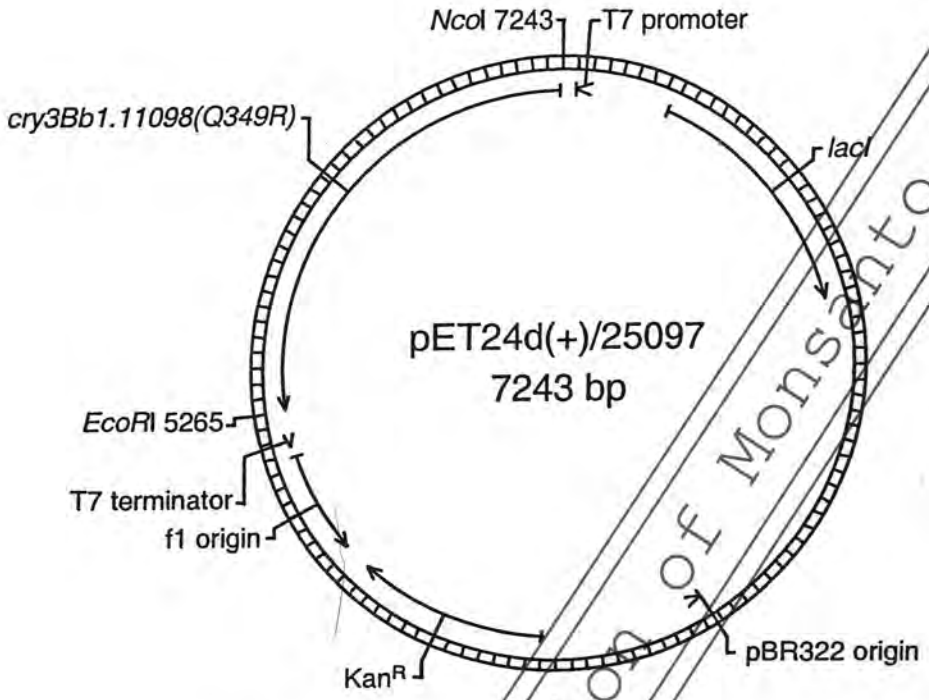
Hydrophobic Interaction Chromatography. Partially purified Cry3Bb1 protein sample from the previous chromatographic step was applied to a 4.6 × 100 mm Source 15 PHE column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer B containing 0.5 M $(NH_4)_2SO_4$. Retained protein was eluted at 1.5 mL/min, using a linear ammonium sulfate gradient from 0.5 to 0 M $(NH_4)_2SO_4$ (15 mL) and a final isocratic step at 0 M $(NH_4)_2SO_4$ (7.5 mL) in buffer B, followed by a wash with water (7.5 mL). Eluant was collected in 1 mL fractions. Fractions that corresponded to the Cry3Bb1 protein (22-30) were pooled and concentrated to a final volume of ~150 µL using a Millipore 30 kDa ultrafree centrifugal device. Concentrated sample was diluted with ~10 mL 100 mM

sodium carbonate, pH 10, containing 1 mM EDTA and again concentrated to ~150 µL. These described methods yielded the highest purity Cry3Bb1 protein (qualitatively assessed by visual comparison of samples separated using SDS-PAGE) from grain extracts of corn event MON 863. Other similar extraction methods were utilized to isolate the Cry3Bb1 protein from MON 863 grain and are archived with this study. The final pooled Cry3Bb1.11098 protein (lot 6957088) represented multiple rounds of purification from MON 863 grain using these described methods.

Purification of the *Escherichia coli* produced Cry3Bb1 protein

Preparation of expression vector: The plasmid expression system used to generate the full length Cry3Bb1.11098(Q349R) protein was constructed for this study using the coding region of the *cry3Bb1.11098(Q349R)* gene inserted into the commercially available pET24d(+) expression vector from Novagen (Madison, WI). The plasmid used to transform corn, pZMIR13L, resulting in corn event MON 863, was obtained from the Product Characterization Center of Monsanto Company (Figure shown on next page). This plasmid was subsequently transformed to STBL2 *E. coli* competent cells (GibcoBRL Life Technologies, Rockville, MD) and selected on a Kanamycin containing LB/Agar media. Amplification of the plasmid DNA was accomplished by inoculating a Fernbach flask containing 1 L TB media with kanamycin using a single positive transformant and incubating overnight at 37 °C with agitation. Plasmid DNA was purified from the resulting *E. coli* paste using a QIAfilter Plasmid Maxi Kit (Qiagen Inc., Valencia, CA). After digestion with restriction endonucleases *Nde*I and *Eco*RI (Roche Molecular Biochemicals, Mannheim, Germany), the *cry3Bb1.11098* coding region was agarose gel purified and subcloned into an *NdeI/EcoR*I digested, alkaline phosphatase (Roche Molecular Biochemicals) treated, pET24d(+) expression vector (Novagen) using T4 DNA ligase (GibcoBRL Life Technologies).

DNA sequence verification. Plasmid DNA nucleotide sequence of the Cry3Bb1.11098(Q349R) expression system was verified using primers (Integrated DNA Technologies, Coralville, IA) generated to sequence the entire length of the *cry3Bb1.11098(Q349R)* insertion. Primers were designed in such a way that multiple, overlapping layers of nucleotide sequence data were generated in both the sense and antisense directions to ensure 100% sequence identity. This was accomplished through the creation of complementary forward and reverse primers that would generate nucleotide sequence beginning at approximately 300 nucleotide intervals in both the sense and antisense directions of the *cry3Bb1.11098(Q349R)* insert. All sequence data was generated by the Research Sample Core of Monsanto's Genomics Sequencing Center using ABI 3700 automated capillary sequencers. A summary of these results were archived with this study.



Expression vector containing the Cry3Bb1 coding region.

Optimization of fermentation. After verification of the presence of the correct nucleotide sequence of the pET24d(+)/25097 construct, the Cry3Bb1.11098(Q349R) expression plasmid was transformed to BL21(DE3) *E. coli* competent cells (Novagen, Madison, WI) for expression of the Cry3Bb1.11098(Q349R) protein. BL21(DE3) *E. coli* transformants containing the pET24d(+)/25097 plasmid were grown under varying conditions in order to optimize expression of soluble Cry3Bb1.11098(Q349R) protein. Variables in fermentation included the use of TB, LB and M9 minimal *E. coli* growth media, concentrations of 0.4 and 1.0 mM IPTG during induction, length of IPTG induction time of 0-8 h and induction incubation temperatures of 20, 30 and 37 °C.

After comparison of the multiple fermentation variables, it was determined that the pET24d(+)/25097 *E. coli* expression system performed best in M9 minimal media with an IPTG induction concentration of 1 mM at 30 °C for 6-8 h. These conditions yielded the most soluble Cry3Bb1 protein per unit fermentation volume.

Large-scale fermentation. The ~110 L fermentation (lot MON105) was performed at the Center for Bioprocessing Research, Inc., State College, PA. Briefly, an initial seed culture was prepared by inoculating 1 L LB media containing ~12.5 µg/mL kanamycin

with 2-3 colonies of BL21(DE3) *E. coli* competent cells transformed with the pET24d(+)/25097 expression plasmid. The seed culture was incubated for 9.5 h at 37 °C in a rotary shaker. The reactor containing approximately 94 L minimal (M9) basal media with Ucon625 antifoam was sterilized in place. Feed solutions (magnesium/trace metal and glucose solutions) and feed cans were sterilized by autoclaving and cooled. Feed cans containing glucose, magnesium/trace metal and ammonium hydroxide solutions were aseptically attached to the reactor. Kanamycin was added, the reactor inoculated with the seed culture and the fermentation allowed to proceed for approximately 24 h at 37 °C while monitoring temperature, air addition, agitation, dissolved oxygen, pressure and pH. The reactor was cooled to 30 °C and induced by addition of 25 g IPTG. Induction was carried out for 6 h at 30 °C.

Cells were harvested using a Sharples centrifuge at 2 L per min, 16,800 rpm. Paste was scraped from the bowl and packaged in ~1 kg freezer bags, labeled and shipped to Monsanto Company on dry ice. Approximately 6.04 kg of paste was received.

Protein extraction. Cell paste (~2 kg) was thawed directly in buffer A (50 mM sodium carbonate, pH 10, 1 mM EDTA) at an approximate cell paste to buffer ratio of 1:1. The thawed cell suspension was homogenized using a Microfluidics International Corp. Model M-110F microfluidizer (Newton, MA) equipped with a Dynisco UPR 700 microprocessor-based pressure indicator (Franklin, MA) at 15,000-18,000 psi. The entire microfluidizer tubing assembly, interaction chamber and auxiliary processing module was covered in ice. Product was collected into a stainless steel bucket chilled on ice. The cell suspension (approximately 4 L) was effectively lysed by two passes through the microfluidizer. To remove cell debris, lysed cells were centrifuged at ~15,000 ×g for 1 h, ~4 °C. Approximately 3 L of supernatant was recovered.

E. coli-produced Cry3Bb1 protein was enriched by performing ammonium sulfate fractionation. Ammonium sulfate was slowly added to 20% saturation and clarified by centrifugation at ~15,000 ×g for 30 min at ~4 °C using a Beckman JLA 8.1000 rotor. Additional ammonium sulfate was added to the supernatant to 60% saturation and protein again recovered by centrifugation. Protein pellets were suspended in ~2.5 L buffer A and dialyzed overnight in 100 L buffer A prior to chromatography.

Anion exchange chromatography. Chromatography was performed in a 4 °C coldroom. A Millipore Corporation (Bedford, MA) Vantage A2 Biochromatography column VA130×500 equipped with an air pack controller and bubble trap assembly was packed with a bed volume of ~5.5 L Q-Sepharose Fast Flow. The media was pre-equilibrated with approximately 50 L buffer A. Flow rate (~50 mL/min) was maintained with a Cole-Parmer Masterflex model 7518-10 peristaltic pump (Chicago, IL). Absorbance was

monitored using a REC 112 chart recorder, UV-1 optical and control unit (Amersham Pharmacia Biotech). Approximately 2.5 L of the 20→60% ammonium sulfate cut was loaded at a flow rate of ~53 mL/min and washed with buffer A until the effluent reached an $A_{280\,nm}$ value of <0.1. A 40 L linear gradient of 0→0.5 M NaCl in buffer A was used to elute protein from the column at a flow rate of ~53 mL/min. Fractions (~450 mL) were collected using an LKB Bromma 2211 Superrac fraction collector (Uppsala, Sweden). Conductivity of fractions was measured using a VWR Scientific, model 1054 EC meter to monitor the gradient. SDS-PAGE analysis was used to locate fractions containing the majority of the ~74 kDa *E. coli*-produced Cry3Bb1 protein. Based on these results, fractions #52-58 (total volume of ~2750 mL) were pooled.

Buffer exchange and concentration of protein. Protein was precipitated by addition of ammonium sulfate to 60% saturation and recovered by centrifugation at 8000 rpm for 45 min at 4 °C using a Beckman JLA 8.1000 rotor. Pellets were suspended in a total volume of ~0.5 L buffer B (10 mM phosphate buffer, pH 7.0) and dialyzed overnight against ~100 L buffer B, followed by an additional 8 h against 20 L buffer B using 14 k MWCO cellulose membrane in a 4 °C coldroom. Protein was concentrated by covering the entire membrane with Aquacide I (carboxymethylcellulose, MW ~70 kDa, Calbiochem) for 6 h at 4 °C. Approximately 150 mL *E. coli*-produced Cry3Bb1.11098(Q349R) protein was recovered and stored as a suspension in a polypropylene tub at 4 °C until the study was initiated.

Appendix 2

List of Applicable SOPs

SOP Number	SOP Title
BR-EQ-0265-01	Applied Biosystems 494 Procise Protein Sequencing System
BR-EQ-0376-01	Hitachi L-8800 Amino Acid Analysis System
BR-ME-0044-02	Diet Incorporation Insect Bioassay for the Activity Measurement of *Bacillus thuringiensis* & Other Insecticidal Proteins
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)
GEN-PRO-015-00	Bio-Rad Protein Assay
PB-EQP-005-01	SDS Polyacrylamide Gel Electrophoresis

Appendix 3

Protocol, Amendments and Deviations

Monsanto Study #: 01-01-39-30

Study Title: Characterization of the Cry3Bb1.11098(Q349R) Protein Produced by Fermentation of *E. coli*, the Cry3Bb1.11098 Protein Produced in Corn Event MON 863 and Assessment of the Physicochemical and Functional Equivalence of these Proteins

Sponsor:
Monsanto Company
700 Chesterfield Parkway North
St. Louis, MO 63198

Primary Testing Facility:
Monsanto Company
Product Safety Center
700 Chesterfield Parkway North
St. Louis, MO 63198

Additional Testing Facility
Monsanto Company
Eco Technology Center
800 North Lindbergh Blvd
St. Louis, MO 63197

Study Director:
Ronald E. Hileman
Monsanto Company
Product Safety Center
700 Chesterfield Parkway North
St. Louis, MO 63198
Phone: (636) 737-5499
Fax: (636) 737-6189
email: ronald.e.hileman@monsanto.com

Principal Investigators:

Gyula Holleschak
Monsanto Company
Product Safety Center
700 Chesterfield Parkway North
St. Louis, MO 63198
Phone: (636) 737-5446
email: gyula.holleschak@monsanto.com

Larry A. Turner
Monsanto Company
Product Safety Center
700 Chesterfield Parkway North
St. Louis, MO 63198
Phone: (636) 737-7424
email: larry.a.turner@monsanto.com

Christopher R. Brown
Monsanto Company
Eco Technology Center
800 North Lindhberg
Phone: (314) 694-2467
email: christopher.r.brown@monsanto.com

Approved By:

Patrick T. Weston
Testing Facility Management Representative
Monsanto Company
Biotechnology Regulatory Sciences

May 3, 2001
Date

Ravinder S. Sidhu
Sponsor Representative
Monsanto Company
Biotechnology Regulatory Affairs

May 3, 2001
Date

James D. Astwood
Director, Product Safety Center
Monsanto Company
Product Safety Center

May 4, 2001
Date

Ronald E. Hileman
Study Director
Monsanto Company
Product Safety Center

3 MAY 2001
Date

Reviewed By:

5/3/01
Date

Gyula Holleschak
Monsanto Company
Product Safety Center
Principal Investigator

5/3/01
Date

Larry A. Turner
Monsanto Company
Product Safety Center
Principal Investigator

5/4/01
Date

Christopher R. Brown
Monsanto Company
Eco Technology Center
Principal Investigator

May 3, 2001
Date

QA Specialist
Monsanto Regulatory

[redacted]

1.0 Regulatory Compliance

1.1 *GLP Compliance.* This is a product characterization study as defined by section §160.135(b) of the United States Environmental Protection Agency (EPA) Federal Insecticide, Fungicide and Rodenticide Act (FIFRA); Good Laboratory Practice Standards (40 CFR Part 160) intended to characterize the physical and/or chemical properties of a potential commercial product. This study will be conducted in compliance with all requirements of section §160.135(b).

2.0 Purpose

The purpose of this study is to first, characterize the physicochemical and functional properties of the *Escherichia coli* (*E. coli*) produced Cry3Bb1.11098(Q349R) protein and the corn event MON 863 Cry3Bb1.11098 protein and second, to evaluate the physicochemical and functional equivalence of these proteins. Demonstration of the physicochemical and functional equivalence justifies the substitution of the *E. coli* produced Cry3Bb1.11098(Q349R) protein in studies designed to evaluate the safety or other aspects of this protein.

Assessment of equivalence of these proteins will be judged by comparison of the results obtained from each of the analytical methods used in this protocol. These comparisons include (1) mass spectrometric analyses, (2) N-terminal sequence analyses, (3) immunoblot analyses, (4) insect bioassay analyses, (5) sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses, (6) glycosylation analyses and (7) amino acid compositional analyses.

3.0 Timelines

Proposed experimental start date:	May 3, 2001
Proposed experimental termination date:	June 1, 2001

4.0 Test, Control and Reference Substances

4.1 *Test Substances.* There are two test substances in this characterization study. Both test substances are considered variants of the wild type Cry3Bb1 protein.

4.1.1 The first test substance is the bacterially produced Cry3Bb1.11098(Q349R) protein (lot 6962478), isolated using chromatographic methods from a large-scale fermentation of *Escherichia coli* containing the pET24d(–)/25097 expression plasmid.

4.1.2 The second test substance is the plant produced Cry3Bb1.11098 protein (lot 6957088) isolated from corn hybrid RX 670 containing event MON 863. This protein was purified using chromatographic methods from the grain grown under Production Plans # 00-01-39-16 and # 00-01-39-18.

4.2 *Test substance characterization.* DNA sequence including the Cry3Bb1.11098(Q349R) coding region of the pET24d(–)/25097 expression plasmid was obtained prior to fermentation of *E. coli*. Isolation of the *E. coli* produced Cry3Bb1.11098(Q349R) protein will be described in the final report. Records pertaining to the identity and isolation of the *E. coli* produced Cry3Bb1.11098(Q349R) protein will be archived with this study and described in the final report.

The identity of corn event MON 863 grain from which the test substance (lot 6957088) was isolated will be confirmed by event specific polymerase chain reaction (PCR). Test substance characterization data will be archived under Production Plans # 00-01-39-16 and # 00-01-39-18. Records pertaining to the isolation of the Cry3Bb1.11098 protein (lot 6957088) from grain of corn event MON 863 will be archived with this study and described in the final report.

5.0 Description of Experimental Design

Analytical methods will be used to characterize the physical and functional properties of the bacterial- and plant-produced test substances. The identity of each test substance will be assessed using N-terminal sequencing, matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and immunoblotting with a Cry3Bb1 specific polyclonal antibody. The functional activity (strength) of each test substance will be assessed using a bioactivity assay with the larvae of a known susceptible insect, Colorado potato beetle (CPB, *Leptinotarsa decemlineata*). The purity of each test substance will be assessed using SDS-PAGE. Glycosylation analysis will be performed to assess for the presence of covalently bound carbohydrates. The amino acid composition of each test substance will be assessed using amino acid compositional analysis. Total protein concentration will be assessed using amino acid compositional analysis and protein assay.

Relevant SOPs for these analytical methods are described in Attachment 1.

The equivalence of these two test substances will be evaluated by comparison of the results obtained from each of the described analytical methods.

5.1 Analytical Methods

5.1.1 *MALDI-TOF mass spectrometric analysis.* This analysis will be used to assess the identity of the test substances. Both the *E. coli* produced Cry3Bb1.11098(Q349R) protein and the corn event MON 863 Cry3Bb1.11098 protein will be separated by SDS-PAGE according to SOP PB-EQP-005-01 and visualized with Coomassie Brilliant blue G stain (Sigma, St. Louis, MO). Molecular weight markers will be used to calibrate the polyacrylamide gel. Stained bands corresponding to these Cry3Bb1 proteins (~74 kDa) will be excised from the gel, reduced, alkylated and digested with trypsin. Digested peptide samples will be analyzed using MALDI-TOF mass spectrometry. A peptide reference mixture will be used to calibrate the MALDI-TOF instrument. Observed masses will be compared to the expected tryptic digest of each of the test substances. The expected protein sequences will be deduced from the *cry3Bb1* coding region of the *E. coli* and plant nucleotide sequences. The identity of each of the test substances will be evaluated by comparison of the observed and expected masses.

5.1.2 *N-terminal sequence analysis.* This analysis will be used to assess the identity of the test substances. Both the *E. coli* produced Cry3Bb1.11098(Q349R) protein and the corn event MON 863 Cry3Bb1.11098 protein will be separated by SDS-PAGE according to SOP PB-EQP-005-01, electrotransferred to polyvinylidene difluoride (PVDF) and visualized using a Ponceau S or Coomassie Brilliant blue stain. Pre-stained molecular weight markers will be used to verify electrotransfer of proteins to the membrane. Bands corresponding to the Cry3Bb1 proteins (~74 kDa) will be excised from the membrane and sequenced according to BR-EQ-0265-01 using automated cycles of the Edman degradation reaction. Contaminant protein bands may also be sequenced using this method, if sufficient material (*e.g.* $\geq$ 10% of the total protein) is observed. An analytical reference protein, β-lactoglobulin, will be used prior to, and after test samples, to verify that the N-terminal sequencer is operating within specified limits. Results of these analyses will be compared to the expected protein sequence for each test substance. Identification of contaminant proteins, if necessary, will be accomplished by comparison of the observed sequence(s) to known sequences in a public protein database such as SwissProt or GenPept using an appropriate pairwise sequence comparison algorithm.

5.1.3 *Immunoblot analysis.* This analysis will be used to assess the identity as well as to compare the relative immunoreactivities of the test substances with a polyclonal antibody specific for Cry3Bb1 proteins. Data pertaining to the specificity of the antibody used will be archived in this study. Both the *E. coli* produced Cry3Bb1.11098(Q349R) protein and the corn event MON 863 Cry3Bb1.11098 protein will be separated by SDS-PAGE according to SOP PB-EQP-005-01 and electrotransferred to PVDF or nitrocellulose membrane. Pre-stained molecular weight markers will be used to verify electrotransfer of proteins. Immunoblot analysis will be performed according to SOP GEN-PRO-002-03. Enhanced chemiluminescence (ECL™) detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) will be used to visualize immunoreactive bands on x-ray film (Hyperfilm™ ECL). Films will be developed using an automated film processor.

5.1.4 *Insect bioassay.* This analysis will be used to estimate and compare the bioactivity (measured as an LC_{50} value) of the test substances incorporated into a diet fed to CPB larvae, a susceptible insect. The LC_{50} is defined as the concentration of protein (µg/mL diet) required to kill 50% of the test larvae relative to control. Insect bioassays will be conducted at the Ecological Technology Center laboratories, Creve Coeur campus of Monsanto Company. Both the *E. coli* produced Cry3Bb1.11098(Q349R) protein, corn event MON 863 Cry3Bb1.11098 protein and appropriate controls (vehicle solution) will be assayed according to SOP BR-ME-0044-02. A final diet volume of 300 µL per well (rather than the 500-1000 µL amount specified in SOP BR-ME-044-02) will be used to decrease overall quantity of sample needed for this procedure. Data will be analyzed using SAS Probit and/or logistic regression procedures.

5.1.5 *SDS PAGE and densitometric analysis.* This analysis will be used to assess purity (reported as a percentage) and apparent molecular weight (reported in kDa) of the test substances. Both the *E. coli* produced Cry3Bb1.11098(Q349R) protein and corn event MON 863 Cry3Bb1.11098 protein will be separated using SDS-PAGE according to SOP PB-EQP-005-01. Molecular weight markers will be used to calibrate polyacrylamide gels. Protein bands visualized with Colloidal Brilliant blue G (Sigma Chemical Co., St. Louis, MO) will be analyzed using a Bio-Rad model GS-710 imaging densitometer with the supplied software. A detailed description of the imaging and densitometric analysis will be archived with the raw data. Each visible band will be quantified as a percentage of the total protein observed within the gel lane for each test substance. The apparent molecular weight of each visible band will be estimated by comparison to molecular weight markers loaded on the same gel.

5.1.6 *Glycosylation analysis.* This analysis will be used to determine if any carbohydrate modifications are present. Many eukaryotic proteins are post-translationally modified with glycan structures, while prokaryotic proteins are not. Results of this assay will provide additional data to support the equivalence of the test substances. The test substances and appropriate glycosylated control proteins and molecular weight markers will be separated by SDS-PAGE according to SOP PB-EQP-005-01 and electrotransferred to PVDF or nitrocellulose membrane. Carbohydrate detection will be performed using a kit according to the method provided by the manufacturer and described in the final report.

5.1.7 *Amino acid compositional analysis.* This analysis will be used to assess the amino acid composition and total protein concentration of the test substances. Both the *E. coli* produced Cry3Bb1.11098(Q349R) protein and corn event MON 863 Cry3Bb1.11098 protein will be separately hydrolyzed and analyzed according to SOP BR-EQ-0376-01. Amino acid standards will be used to calibrate the amino acid analyzer and bovine serum albumin will be used to verify the vapor phase protein hydrolysis.

5.1.8 *Protein assay.* This analysis will be used as a secondary means of estimating the total protein concentration of the test substances. This colorimetric protein assay will be used to provide a rapid estimate of the total protein concentration. The Bio-Rad colorimetric protein assay will performed according to SOP GEN-PRO-015-00.

5.1.9 *Storage stability.* This analysis will be used to assess the extent of degradation of each of the test substances throughout the duration of the study. Samples (stored as solutions or suspensions) will be analyzed using SDS-PAGE at the beginning and end of the experimental phase of this study according to SOP PB-EQP-005-01 and visualized with Colloidal Brilliant blue G stain. The extent of degradation will be evaluated by using densitometric analysis, as described in Section 5.1.5.

6.0 Records to be Maintained

Records will be maintained of all sample transfers, analyses, the protocol and all deviations and amendments thereto and copies of all letters, memoranda and other correspondence related to this study. These documents may include: photocopies, computer generated hard copies or hand-written notes that describe the procedures used to generate data for this study. Upon completion of the study, all study records and final report will be archived at Monsanto Company.

7.0 Changes to the Protocol

Planned changes to the protocol will be documented in the form of written protocol amendments and signed by the Study Director. Amendments become part of the protocol and will be archived with the protocol. All other changes will be in the form of written protocol deviations and will be filed with the raw data. All changes to the protocol will be addressed in the final report.

Attachment 1: SOP List

SOP Number	SOP Title
BR-EQ-0265-01	Applied Biosystems 494 Procise Protein Sequencing System
BR-EQ-0376-01	Hitachi L-8800 Amino Acid Analysis System
BR-ME-0044-02	Diet Incorporation Insect Bioassay for the Activity Measurement of *Bacillus thuringiensis* & Other Insecticidal Proteins
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)
GEN-PRO-015-00	Bio-Rad Protein Assay
PB-EQP-005-01	SDS Polyacrylamide Gel Electrophoresis

Protocol Amendment Form **Amendment #: 01**

Monsanto Study #: **01-01-39-30**

Date changes implemented: May 17, 2001

Page number(s) and section(s): Page 8, Section 5.1.3

Protocol originally stated:

5.1.3 *Immunoblot analysis.* This analysis will be used to assess the identity as well as to compare the relative immunoreactivities of the test substances with a polyclonal antibody specific for Cry3Bb1 proteins. Data pertaining to the specificity of the antibody used will be archived in this study.

Protocol amended as follows:

5.1.3 *Immunoblot analysis.* This analysis will be used to assess the identity as well as to compare the relative immunoreactivities of the test substances with a polyclonal and monoclonal antibody specific for Cry3Bb1 proteins. Data pertaining to the specificity of the antibodies used will be archived in this study.

Reason for the amendment and what impact will result from this change:

Immunoblot analysis using a polyclonal antibody resulted in detection of a large molecular weight band as well as other minor bands in the test substance purified from corn event MON 863. Monoclonal antibodies are generally more specific and recognize a single epitope, rather than multiple epitopes as in polyclonal antibody preparations. Thus, use of a monoclonal antibody preparation is expected to be more immunospecific, relative to a polyclonal antibody. A monoclonal antibody will be used to determine if the bands detected at molecular weights other than that expected for the Cry3Bb1 protein test substances are immunoreactive with a separate antibody preparation. Results of this additional analysis will enable simpler interpretation regarding the distinction between observed specific and non-specific immunoreactive bands. Therefore this analysis will positively impact the study.

Protocol Amendment Form **Amendment #: 01**

Approved By:

Patrick T. Weston
Testing Facility Management Representative

May 17, 2001
Date

Ronald E. Hileman
Study Director

17 MAY 2001
Date

Ravinder S. Sidhu
Sponsor Representative

May 17, 2001
Date

Reviewed By:

Quality Assurance Specialist

May 17, 2001
Date

Protocol Amendment Form **Amendment #: 02**

Monsanto Study #: **01-01-39-30**

Date changes implemented: July 6, 2001

Page number(s) and section(s): Page 1, Study Title

Protocol originally stated:

Study Title: Characterization of the Cry3Bb1.11098(Q349R) Protein Produced by Fermentation of *E. coli*, the Cry3Bb1.11098 Protein Produced in Corn Event MON 863 and Assessment of the Physicochemical and Functional Equivalence of these Proteins

Protocol amended as follows:

Study Title: Characterization and Equivalence of the Cry3Bb1 Protein from *E. coli* Fermentation and Corn Event MON 863

Reason for the amendment and what impact will result from this change: The title of the protocol was changed to be consistent with the final report. There was no impact to the study.

Page number(s) and section(s): Page 1, Additional Testing Facility

Protocol originally stated:

Additional Testing Facility Monsanto Company
Eco Technology Center
800 North Lindbergh Blvd
St. Louis, MO 63197

Protocol amended as follows:

Additional Testing Facility Monsanto Company
Eco Technology Center
800 North Lindbergh Blvd
St. Louis, MO 63167

Protocol Amendment Form **Amendment #: 02**

Reason for the amendment and what impact will result from this change: Due to a typographical error an incorrect zip code was recorded. There was no impact to the study by correcting the zip code.

Page number(s) and section(s): Page 6, Section 4.1.1 and 4.2

Protocol originally stated:

4.1.1 The first test substance is the bacterially produced Cry3Bb1.11098(Q349R) protein (lot 6962478), isolated using chromatographic methods from a large-scale fermentation of *Escherichia coli* containing the pET24d(–)/25097 expression plasmid.

4.2 *Test substance characterization.* DNA sequence including the Cry3Bb1.11098(Q349R) coding region of the pET24d(–)/25097 expression plasmid was obtained prior to fermentation of *E. coli*. Isolation of the *E. coli* produced Cry3Bb1.11098(Q349R) protein will be described in the final report. Records pertaining to the identity and isolation of the *E. coli* produced Cry3Bb1.11098(Q349R) protein will be archived with this study and described in the final report.

Protocol amended as follows:

4.1.1 The first test substance is the bacterially produced Cry3Bb1.11098(Q349R) protein (lot 6962478), isolated using chromatographic methods from a large-scale fermentation of *Escherichia coli* containing the pET24d(+)/25097 expression plasmid.

4.2 *Test substance characterization.* DNA sequence including the Cry3Bb1.11098(Q349R) coding region of the pET24d(+)/25097 expression plasmid was obtained prior to fermentation of *E. coli*. Isolation of the *E. coli* produced Cry3Bb1.11098(Q349R) protein will be described in the final report. Records pertaining to the identity and isolation of the *E. coli* produced Cry3Bb1.11098(Q349R) protein will be archived with this study and described in the final report.

Reason for the amendment and what impact will result from this change: Due to a typographical error, the incorrect symbol was used. There was no impact to the study by correcting the symbol.

Protocol Amendment Form **Amendment #: 02**

Page number(s) and section(s): Page 6, Section 4.1.1 and 4.2

Protocol originally stated:

5.1.4 *Insect bioassay.* This analysis will be used to estimate and compare the bioactivity (measured as an LC_{50} value) of the test substances incorporated into a diet fed to CPB larvae, a susceptible insect. The LC_{50} is defined as the concentration of protein (µg/mL diet) required to kill 50% of the test larvae relative to control. Insect bioassays will be conducted at the Ecological Technology Center laboratories, Creve Coeur campus of Monsanto Company. Both the *E. coli* produced Cry3Bb1.11098(Q349R) protein, corn event MON 863 Cry3Bb1.11098 protein and appropriate controls (vehicle solution) will be assayed according to SOP BR-ME-0044-02. A final diet volume of 300 µL per well (rather than the 500-1000 µL amount specified in SOP BR-ME-044-02) will be used to decrease overall quantity of sample needed for this procedure. Data will be analyzed using SAS Probit and/or logistic regression procedures.

Protocol amended as follows:

5.1.4 *Insect bioassay.* This analysis will be used to estimate and compare the bioactivity (measured as an LC_{50} value) of the test substances incorporated into a diet fed to CPB larvae, a susceptible insect. The LC_{50} is defined as the concentration of protein (µg/mL diet) required to kill 50% of the test larvae relative to control. Insect bioassays will be conducted at the Ecological Technology Center laboratories, Creve Coeur campus of Monsanto Company. Both the *E. coli* produced Cry3Bb1.11098(Q349R) protein, corn event MON 863 Cry3Bb1.11098 protein and appropriate controls (water) will be assayed according to SOP BR-ME-0044-02. A final diet volume of 300 µL per well (rather than the 500-1000 µL amount specified in SOP BR-ME-044-02) will be used to decrease overall quantity of sample needed for this procedure. Data will be analyzed using SAS Probit and/or logistic regression procedures.

Reason for the amendment and what impact will result from this change: Added for clarification. There was no impact.

Protocol Amendment Form **Amendment #: 02**

Approved By:

Patrick T. Weston
Testing Facility Management Representative

July 6, 2001
Date

Ravinder S Sidhu

Ravinder S. Sidhu
Sponsor Representative

July 6, 2001
Date

Ronald E. Hileman
Study Director

6 JULY 2001
Date

Reviewed By:

Quality Assurance Specialist

July 6, 2001
Date