7) Hileman, R. E., J. N. Leach and J. D. Astwood (2001). Assessment of the in vitro digestibility of the Cry3Bb1.11098(Q349R) protein in simulated intestinal fluid. MSL-17530, an unpublished study conducted by Monsanto Company.

Study Title

Assessment of the *in vitro* digestibility of the Cry3Bb1.11098(Q349R) protein in simulated intestinal fluid

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Study Completed On

December 14, 2001

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Laboratory Project ID

MSL Number: 17530
Study Number: 01-01-62-11

Statement of No Data Confidentiality

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

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Statement of Compliance

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

The equipment records for the densitometer used in this study do not include an SOP, however, calibration and maintenance has been performed and documented on a routine basis, and a draft SOP was followed for this procedure. All supporting documentation is archived with this study.

The equipment records for the spectrophotometer used in this study do not include an SOP, calibration or maintenance documentation.

_____ Date: _____
Submitter

Ravinder S Sidhu Date: Dec 14, 2001
Sponsor

R W E Hahn Date: 14 DEC 2001
Study Director

Quality Assurance Unit Statement

Study Title: Assessment of the *in vitro* digestibility of Cry3Bb1.11098(Q349R) protein in simulated intestinal fluid

Study Number: 01-01-62-11

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
11/26/2001	SDS-PAGE	11/28/2001	11/28/2001
12/13/2001	Raw Data Audit	12/14/2001	12/14/2001
12/13/2001	Draft Report Audit	12/14/2001	12/14/2001

Barbara A. Foy

Date: 14 Dec. 2001

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Experimental Completion Date: December 13, 2001

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Records Retention: All study specific raw data, protocols, electronically stored files and final report will be retained at Monsanto-St. Louis. Specimens and remaining test substance will be stored in the original containers in a –20 °C freezer for one year, after which they may be disposed.

Signatures of Final Report Approval:

Ravinder S Sidhu
Sponsor Representative — Date: Dec 14, 2001

Director, Product Safety Center — Date: Dec 14, 2001

John Leach
Author — Date: Dec. 14, 2001

Author, Study Director — Date: 14 DEC 2001

Table of Contents

Table

Figures

Appendices

Abbreviations and Definitions

aa	amino acid
B.t.	*Bacillus thuringiensis*
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
GenBank	A public genetic database maintained by the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, MD
HRP	Horseradish peroxidase
kDa	Kilodalton
NFDM	Non-fat dried milk
PBST	Phosphate buffered saline containing Tween-20
SDS	Sodium dodecylsulfate
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SOP	Standard operating procedure
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl)aminomethane

1.0 Summary

Genetically enhanced 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 purpose of this study was to investigate the *in vitro* digestibility of the *E. coli*-produced Cry3Bb1.11098(Q349R) protein in simulated intestinal fluid (SIF). This protein was previously characterized and shown to be physicochemically and functionally equivalent to the Cry3Bb1 protein produced in corn event MON 863.

Digestibility in SIF was evaluated at selected time points using immunoblot analysis. As expected, the Cry3Bb1 protein was observed to rapidly degrade (within 1 minute) from a size of approximately 74 kDa to smaller fragments with approximate molecular weights of 68 and 57 kDa. Continued exposure to SIF (≥5 minutes) resulted in the formation of a single stable fragment at a molecular weight of approximately 57 kDa. This fragment persisted to the last time point tested, 24 hours.

2.0 Introduction

Genetically enhanced 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). Studies designed to evaluate the food and feed safety of genetically modified plants include assessment of the digestibility of each engineered protein (Astwood *et al.*, 1996). The *E. coli*-produced Cry3Bb1.11098(Q349R) protein investigated in this study, was previously characterized and shown to be physicochemically and functionally equivalent to the Cry3Bb1 protein produced in corn event MON 863 (Hileman and Astwood, 2001; Hileman *et al.*, 2001a). The purpose of this study was to investigate the *in vitro* digestibility of the *E. coli*-produced Cry3Bb1.11098(Q349R) protein in simulated intestinal fluid (SIF).

The digestibility of Cry3 proteins has been previously evaluated in SGF (Keck *et al.*, 1993; Leach *et al.*, 2001a; Leach *et al.*, 2001b) and SIF (Leach *et al.*, 2001b). The SGF digestion pattern of these Cry3 proteins was consistent with the SGF digestibility pattern of safely consumed proteins. In SGF, full-length Cry3 polypeptides were rapidly degraded within 15 seconds. In SIF, the full-length Cry3 polypeptides were rapidly degraded to smaller, stable polypeptides. Stability to trypsin and trypsin-like proteases is a predictable phenomenon for Cry proteins (Aronson and Shai, 2001; Höfte and Whiteley, 1989; Rajamohan *et al.*, 1998; Rukmini *et al.*, 2000). The relationships between the Cry3 proteins referred to in this report are described in Table 1.

Table 1. Comparison of Cry3 proteins.

Cry3 Protein	% Amino Acid Identity (% Amino Acid Similarity)[a]						
	Cry3Aa4	Wild type Cry3Bb1	*B.t.* strain EG11231 Cry3Bb1	Corn event MON 853 Cry3Bb1	*B.t.* strain EG11098 Cry3Bb1	*E. coli* 11098(Q349R) Cry3Bb1	Corn event MON 863 Cry3Bb1
Cry3Aa4[b]	—						
Wild type Cry3Bb1[c]	69.3 (76.9)	—					
B.t. strain EG11231 Cry3Bb1[d]	69.1 (76.9)	99.4 (99.4)	—				
Corn event MON 853 Cry3Bb1[e]	68.9 (76.7)	99.2 (99.2)	99.8 (99.8)	—			
B.t. strain EG11098 Cry3Bb1[f]	69.3 (77.0)	99.2 (99.2)	99.8 (99.8)	99.7 (99.7)	—		
E. coli 11098(Q349R) Cry3Bb1[g]	68.9 (76.7)	98.9 (98.9)	99.5 (99.5)	99.7 (99.7)	99.7 (99.7)	—	
Corn event MON 863 Cry3Bb1[h]	68.9 (76.7)	98.9 (98.9)	99.5 (99.5)	99.7 (99.7)	99.7 (99.7)	100.0 (100.0)	—

[a] Percent similarity corresponds to the sum of identical and similar amino acids divided by the total amino acids. A similar amino acid was defined as any non-identical but physicochemically related amino acid. Similar amino acids are structurally related and share polar, hydrophobic, or charged states. Such substitutions are referred to as "conservative" since they are unlikely to change the structures, and by inference, the functions of homologous proteins.

[b] *B.t.*-derived protein present in NewLeaf varieties of genetically modified potatoes (GenBank Accession No. M30503). This protein contains a total of 644 amino acids.

[c] The wild type sequence (GenBank Accession No. M89794) that encodes a total of 652 amino acids.

[d] A *B.t.* Cry3Bb1 variant (Astwood *et al.*, 2001) with enhanced activity towards corn rootworm. The sequence (Hileman and Astwood, 2001) contains a total of 652 amino acids. This protein was shown to be physicochemically and functionally equivalent to the Cry3Bb1 protein produced in corn event MON 853 (Holleschak *et al.*, 2001b).

[e] The Cry3Bb1 protein produced in corn event MON 853 that contains a total of 653 amino acids (Hileman and Astwood, 2001).

[f] A *B.t.* Cry3Bb1 variant (Astwood *et al.*, 2001) with enhanced activity towards corn rootworm. The sequence (Hileman and Astwood, 2001) contains a total of 652 amino acids. This protein was shown to be physicochemically and functionally equivalent to the Cry3Bb1 protein produced in corn event MON 863 (Holleschak *et al.*, 2001a; Holleschak *et al.*, 2001b).

[g] The Cry3Bb1 protein produced in *E. coli*, which is physicochemically and functionally equivalent to the Cry3Bb1 protein produced in corn event MON 863 (Hileman *et al.*, 2001a). The sequence (Hileman and Astwood, 2001) contains a total of 653 amino acids.

[h] The Cry3Bb1 protein produced in corn event MON 863 that contains a total of 653 amino acids (Hileman and Astwood, 2001).

3.0 Purpose

The purpose of this study was to characterize the *in vitro* digestibility of the Cry3Bb1.11098(Q349R) protein isolated from *E. coli* using a simulated intestinal fluid (SIF) mammalian digestion model. Immunoblot analysis was used to assess the extent of Cry3Bb1 protein degradation as well as the formation of incompletely degraded peptide fragments over time.

4.0 Materials

4.1 *Test substance.* The test substance, *E. coli*-produced Cry3Bb1.11098(Q349R) protein (lot 6962478), used in this study was previously shown to be the same variant of the wild type Cry3Bb1 protein produced in corn event MON 863 (Hileman *et al.*, 2001a). This protein was isolated using chromatographic methods from a large-scale fermentation of *E. coli* containing the pET24d(+)/25097 expression plasmid.

4.2 *Control substance.* There was no control substance used in this study. However, experimental controls were included to assess the stability of the test substance in the test system lacking pancreatin and to characterize the test system (see Section 5.3).

4.3 *Reference substance.* There was no reference substance used in this study. Analytical reference standards (molecular weight markers) were used to aid interpretation of the immunoblots. Hemoglobin was used as a reference protein to assess the activity of SIF prior to, and after, digestion of the test substance (see Section 5.1).

4.4 *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 of the expression plasmid, as well as characterization and stability of the *E. coli*-produced protein, are archived in study 01-01-39-30. Protein purity was previously determined to be 92.6% (Hileman *et al.*, 2001a). Prior to initiation of this study, Cry3Bb1.11098(Q349R) protein (lot 6962478) was clarified by centrifugation (approximately 100,000×g, 1 h, 4 °C). Total protein concentration (0.34 mg/mL) was determined as an average of three replicate determinations using amino acid analysis. Records pertaining to the centrifugation and protein concentration were archived in study 01-01-62-06 (in progress). The purity of the test substance was verified after, rather than before, the digestions were performed. While this constituted a protocol deviation (archived in this study), there was no impact to the study.

4.5 *Test system.* The test system used for this study was simulated intestinal fluid (SIF). SIF was prepared according to SOP GEN-PRO-058-01 and is based on a

published method (The Unites States Pharmacopoeia, 1995). The activity of SIF was determined both before and after its use to digest the test substance, according to SOP GEN-PRO-058-01.

4.6 *Justification of the test system.* *In vitro* digestion models are widely used to assess the digestibility of ingested substances, including plant proteins (Marquez and Lajolo, 1981; Nielson, 1988), animal proteins (Zikakis *et al.*, 1977) and food additives (Tilch and Elias, 1984). In addition, simulated digestion fluids are used to assess protein quality (Akeson and Stahmann, 1964); to study digestion in pigs and poultry (Fuller, 1991); to measure tablet dissolution rates to assess bioavailability for pharmaceuticals (Alam *et al.*, 1980); and to investigate the controlled release properties of experimental pharmaceuticals (Doherty *et al.*, 1991).

5.0 Methods

5.1 *SIF activity assay.* The activity of SIF was evaluated before and after digestion of the test substance according to SOP GEN-PRO-058-01. In this assay, proteolytic activity is directly proportional to the amount of trichloroacetic acid (TCA)-soluble resorufin-labeled peptides released from a sample of resorufin-labeled casein (Roche Molecular Biochemicals, Mannheim, Germany). Solutions containing resorufin-labeled casein were incubated with SIF for 15 min at approximately 37 °C and the reactions quenched by addition of 5% (v/v) TCA. Soluble material recovered after centrifugation was neutralized by the addition of a buffer and the activity determined as an average of triplicate samples, measured using a Beckman DU-650 spectrophotometer ($A_{574\ nm}$). The control sample utilized deionized water in place of SIF and was also determined in triplicate.

5.2 *Digestion of Cry3Bb1.1098(Q349R) protein in SIF.* Incubations were performed using a shaking water bath pre-warmed to approximately 37 °C. Incubation times included T0 (0 min), T1 (1 min), T2 (5 min), T3 (15 min), T4 (30 min), T5 (1 h), T6 (2 h), T7 (4 h), T8 (8 h), T9 (16 h) and T10 (24 h). Timed incubations were initiated by the addition of the test substance to individual incubation tubes containing the test system. Reactions were quenched by addition of Laemmli sample buffer followed by heating at approximately 5 min, 100 °C. The zero (T0) time point was quenched by addition of Laemmli sample buffer and heated approximately 5 min, 100 °C prior to the addition of the test substance. The 1 and 5 min (T1 and T2) time points were pre-warmed to approximately 37 °C prior to addition of the test substance.

5.3 *Experimental controls.* Experimental controls were included to assess the stability of the test substance in SIF lacking pancreatin at two incubation times, 0 min

(P0) and 24 h (P10). These samples were used to evaluate the extent of degradation of the test substance in the absence of proteases (pancreatin) and to demonstrate the effectiveness of the quenching method by comparison to the 0 min (T0) time point. The 0 min (P0) time point was quenched by addition of Laemmli sample buffer and heated approximately 5 min, 100 °C prior to addition of test substance.

Experimental controls were included to evaluate the extent of background signal in the detection method at two incubation times, 0 min (N0) and 24 h (N10). These samples were prepared by addition of buffer (10 mM phosphate buffer, pH 7.0) to SIF in place of the test substance. The zero (N0) time point was quenched by addition of Laemmli sample buffer and heated approximately 5 min, 100 °C prior to addition of buffer.

5.4 *SDS-PAGE and immunoblot analysis.* All quenched samples contained 1× Laemmli (Laemmli, 1970) sample buffer [62.5 mM Tris-HCl, pH 6.8, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 10% (v/v) glycerol and 0.005% (w/v) Bromophenol Blue]. Digestion samples containing the test substance were prepared at a final concentration of 2 ng/µL, based on pre-digestion estimates. Separation of the pre-stained molecular weight markers (Amersham Life Science, Buckinghamshire, England) were visually confirmed and used as an accept criterion for SDS-PAGE. Electrotransfer of the pre-stained molecular weight markers to membranes were visually confirmed and used as an acceptance criterion for immunoblot analysis.

The limit of detection of Cry3Bb1.11098(Q349R) protein was assessed by dilution of the T0 digestion sample with 1× Laemmli sample buffer. Analyses for digestibility and limit of detection were run in the same gel apparatus and electrotransfer apparatus. Although placed in separate trays, membranes were incubated with approximately the same volumes of primary and secondary antibody solutions and detected on a single sheet of film.

All samples were heated at approximately 100 °C for 5 min and applied to pre-cast NOVEX® 10→20% polyacrylamide gradient tricine-buffered mini-gels (Invitrogen™, Carlsbad, CA). Samples (10 ng total protein) were loaded into separate lanes. Electrophoresis was performed under reducing conditions according to SOP BtC-PRO-026-01 at constant voltage (125 V) for approximately 100 min (the dye front was retained at the bottom of the gel).

Immunoblotting was performed according to SOP GEN-PRO-002-03. Gels were incubated with filter papers and nitrocellulose membranes (NOVEX® 0.45 µm pore size, Invitrogen™) in transfer buffer (24 mM Tris, 192 mM glycine containing 20% (v/v) methanol) for approximately 20 min. The transfer sandwich was assembled, submerged in transfer buffer and proteins electrotransferred at 25 V (constant voltage) for 90 min

using a NOVEX® Xcell II blot module. Membranes were removed and blocked by incubation with gentle shaking in PBST (1 mM KH_2PO_4, 100 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 5% (w/v) NFDM for 30 min. Immunoblots were probed with a 1:5000 dilution of rabbit polyclonal anti-Cry3Bb1 antibody (lot 6199830B) prepared in PBST containing 1% (w/v) NFDM for 1 h.

The polyclonal primary antibody (lot 6199830B) was produced by repeated immunization of a rabbit with wild type Cry3Bb1 protein (EG 11037). Characterization of this antibody has been previously described (Dudin *et al.*, 1999). The wild type *cry3Bb1* 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 *E. coli* containing pET24d(+)/25097 expression plasmid (Hileman and Astwood, 2001; Hileman *et al.*, 2001a). As these modified Cry3Bb1 proteins were variants of the wild type protein (Astwood *et al.*, 2001), antibody raised against either one of the variants was expected to cross-react with the other variant. Therefore the use of this antibody preparation did not impact the results or interpretation of the immunoblot analyses.

Excess primary antibody was removed by repeated washes with PBST. 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 1% (w/v) NFDM for 1 h. Excess secondary antibody was removed by repeated washes with PBST. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL™) detection system (Amersham Pharmacia, Buckinghamshire, England) and exposed to Hyperfilm™ ECL™ high performance chemiluminescence film (Amersham Pharmacia). Films were developed using a Konica SRX101A automated film processor (Tokyo, Japan).

5.5 *Rejected data.* The SDS-PAGE and immunoblot procedures were performed twice. Data obtained from the first analysis were rejected. The molecular weight marker used (broad range pre-stained markers, Bio-Rad Laboratories, Hercules, CA) ran aberrantly. Samples were immediately frozen in a –20 °C freezer after their use in the first analysis. These procedures were repeated (as described in Section 5.4) on the following day using markers from a separate source (pre-stained molecular weight markers, Amersham Life Science, Buckinghamshire, England), which ran correctly. The digestion pattern of the repeated analyses was identical to the first. Changing the source of the molecular weight markers used had no impact on the assessment of digestibility.

6.0 Control of Bias and Quality Control Measures

Comparison of the results of the experimental controls prepared without pancreatin added to the test substance digestion samples demonstrated that the quenching method was effective at inactivating SIF activity and that the test substance was stable under conditions of the test system in the absence of pancreatin. Experimental controls prepared without added test substance demonstrated that the test system did not contribute to the immunoreactive signal observed in the test substance digestion samples. Separate tubes were used for each time point in order to provide replicates that fall within a certain range. Systematic positioning and handling of the tubes was also used to track the progress of each digestion sample while providing the opportunity to eliminate errors during the experiment.

7.0 Results and Discussion

7.1 *SIF activity.* Simulated intestinal fluid contains pancreatin, a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease. The proteolytic activity of SIF was measured prior to (13,749 U/mL), and after digestion of the test substance (12,795 U/mL). One unit of activity is defined as an increase in absorbance of 0.001 $A_{574\,nm}$ per min at 37 °C. These values were within the acceptability requirements of SOP GEN-PRO-058-01.

7.2 *Digestibility of Cry3Bb1.11098(Q349R) protein in SIF.* The Cry3Bb1.11098(Q349R) protein used in this study was produced using an *E. coli* heterologous protein production system using the same *cry3Bb1* DNA sequence present in corn event MON 863. That is, the DNA sequence and deduced amino acid sequence of the *E. coli*- and corn-produced proteins were identical (Hileman and Astwood, 2001). These proteins were shown to be physicochemically and functional equivalent (Hileman *et al.*, 2001a).

As expected, exposure of Cry3Bb1.11098(Q349R) protein to simulated intestinal fluid resulted in the formation of a stable digestion product that persisted for at least 24 h (Figure 1). The position of the single band observed in lanes 2, 4 and 15 is consistent with the predicted molecular weight of approximately 74 kDa for Cry3Bb1.11098(Q349R) protein (Hileman *et al.*, 2001a). At T1 (1 min, lane 5), the Cry3Bb1.11098(Q349R) protein appeared as two bands having approximate molecular weights of 68 and 57 kDa. These molecular weights are very similar to those observed previously for Cry3Bb1 variants isolated from *B.t.*, which corresponded to N-terminally truncated forms of the full-length proteins (Hileman *et al.*, 2001b). Time points greater than 1 min appeared as a single predominant band (≈57 kDa). The intensity of this band remained essentially unchanged from the 5 min to 24 h time points, indicating that it is

stable to digestion by pancreatin. A similar molecular weight (59 kDa) was reported for Cry3Bb1 variants exposed to SIF (Leach *et al.*, 2001b). When tested for insecticidal activity against the larvae of Colorado potato beetle (*Leptinotarsa decemlineata*), the lower molecular weight band (59 kDa) was shown to retain biological activity (Leach *et al.*, 2001b).

Experimental controls were used to evaluate the stability of Cry3Bb1.11098(Q349R) protein in the test system lacking pancreatin (P0 and P10, Figure 1, lanes 2 and 15). No degradation was observed in the absence of proteases, demonstrating that degradation observed in lanes 5 to 14 are due to pancreatic activity. No bands were observed where Cry3Bb1.11098(Q349R) protein was absent (N0, Figure 1, lane 3), demonstrating that the immunoreactive signal observed on this blot was due solely to Cry3Bb1 protein and protein fragments.

The N-termini of Cry3Bb1 variants have repeatedly been shown to be sensitive to proteases (Hileman *et al.*, 2001a; Hileman *et al.*, 2001b; Leach *et al.*, 2001b). 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 Cry3Aa4 was also shown to produce a stable, insecticidally active degradation product (molecular weight ≈55 kDa) when exposed to SIF (Keck *et al.*, 1993).

Proteolytic conversion of the full-length protoxin to an active toxin has been described for all members of the Cry1, Cry2, Cry3 and Cry4 class proteins of the *B.t.* δ-endotoxin family (Rukmini *et al.*, 2000). When exposed to trypsin or trypsin-like insect midgut proteases, Cry proteins are degraded to a stable "tryptic core" (Höfte and Whiteley, 1989). Indeed, this has been observed for Cry3Bb1 variants (Leach *et al.*, 2001b) and other Cry3 proteins (Carroll *et al.*, 1989; Keck *et al.*, 1993). These studies demonstrated that while Cry3 proteins are unstable when exposed to pepsin (Leach *et al.*, 2001a; Leach *et al.*, 2001b), they degrade to a stable product (molecular weight of 55-59 kDa) when exposed to trypsin or trypsin-like proteases. Not surprisingly, due to the method of classification of Cry proteins (Crickmore *et al.*, 1998), these Cry3 proteins share extensive amino acid identity with the Cry3Bb1.11098(Q349R) protein used in this study (Table 1). These proteins are expected to share similar structures and are in fact homologs. Therefore, the results shown in Figure 1 were expected.

7.3 *Limit of detection.* Dilutions prepared from T0 (0 min) were used to determine the limit of detection of Cry3Bb1.11098(Q349R) protein (Figure 2, lanes 3-11) under the same conditions used to detect protein in Figure 1. A single band (approximate molecular weight of 74 kDa) was observed. An amount corresponding to 0.2 ng (Figure 2, lane 10) was the lowest amount detectable using these conditions. This demonstrated

that even if 98% of the sample were digested, a band would still be detected [that is, 0.2 ng represents 2% of the total protein loaded (10 ng)].

Based on visual inspection of the digestion time course (Figure 1), the immunoreactive signal remained unchanged, relative to T0. These data suggest that all of the 74 kDa Cry3Bb1.11098(Q349R) protein was degraded to an intermediate product with a molecular weight of approximately 68 kDa, and finally to a stable product with a molecular weight of approximately 57 kDa.

8.0 Conclusions

As expected, a stable degradation product (approximately 57 kDa) was produced when Cry3Bb1.11098(Q349R) protein was exposed to SIF. The molecular weight of this polypeptide is consistent with the expected size of the tryptic core for Cry3 proteins.

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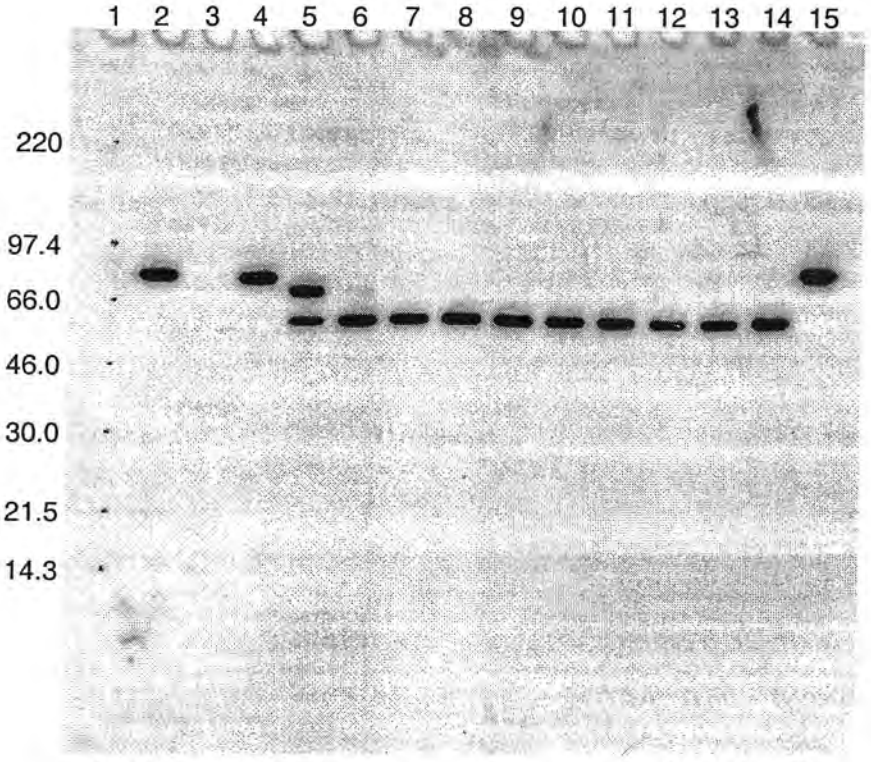
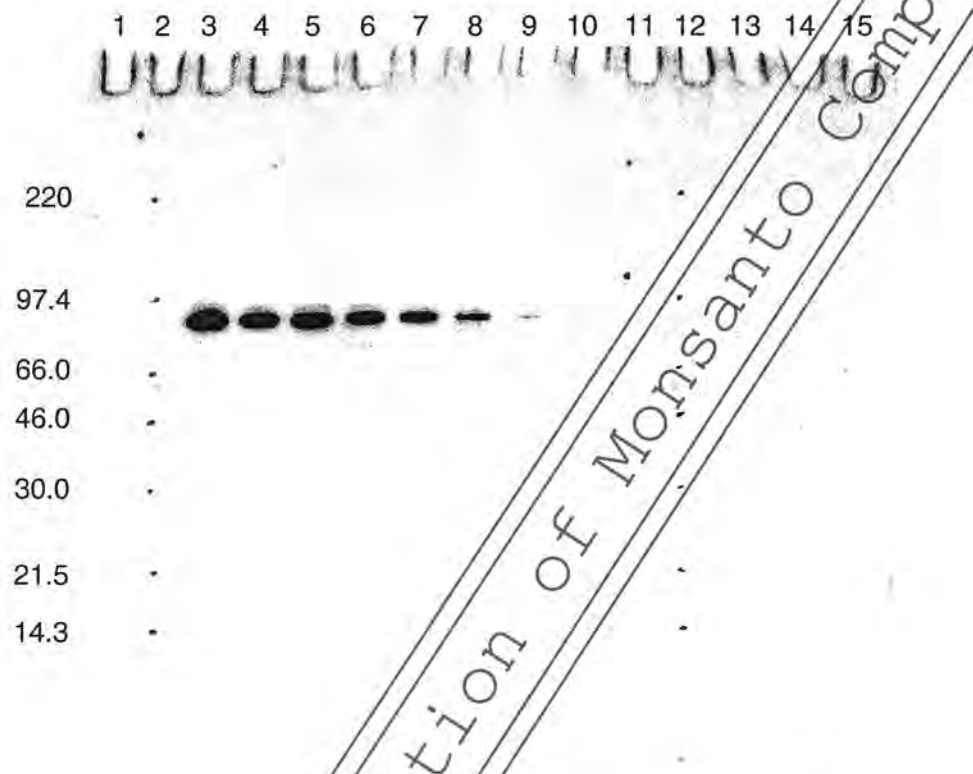


Lane	Sample	Incubation Time
1	pre-stained MW marker	–
2	P0, Cry3Bb1 protein without pancreatin	0 min
3	N0, SIF without Cry3Bb1 protein	0 min
4	T0, Cry3Bb1 in SIF	0 min
5	T1, Cry3Bb1 in SIF	1 min
6	T2, Cry3Bb1 in SIF	5 min
7	T3, Cry3Bb1 in SIF	15 min
8	T4, Cry3Bb1 in SIF	30 min
9	T5, Cry3Bb1 in SIF	1 h
10	T6, Cry3Bb1 in SIF	2 h
11	T7, Cry3Bb1 in SIF	4 h
12	T8, Cry3Bb1 in SIF	8 h
13	T9, Cry3Bb1 in SIF	16 h
14	T10, Cry3Bb1 in SIF	24 h
15	P10, Cry3Bb1 protein without pancreatin	24 h

Figure 1. Digestibility of Cry3Bb1.11098(Q349R) protein in simulated intestinal fluid. Proteins were separated using a 10→20% polyacrylamide gradient tricine gel and electroblotted to nitrocellulose membrane. Digestion products were detected using Cry3Bb1 antisera and developed using an ECL system (60 s exposure shown). Approximately 10 ng protein (pre-digestion) was loaded per lane. Approximate molecular weights (kDa) for marker proteins (lane 1) are shown on the left.



Lane	Sample	Amount
1	empty	–
2	pre-stained MW marker	–
3	T0, Cry3Bb1 in SIF	10 ng, 0%
4	T0, Cry3Bb1 in SIF	5 ng, 50%
5	T0, Cry3Bb1 in SIF	4 ng, 60%
6	T0, Cry3Bb1 in SIF	3 ng, 70%
7	T0, Cry3Bb1 in SIF	2 ng, 80%
8	T0, Cry3Bb1 in SIF	1 ng, 90%
9	T0, Cry3Bb1 in SIF	0.5 ng, 95%
10	T0, Cry3Bb1 in SIF	0.2 ng, 98%
11	T0, Cry3Bb1 in SIF	0.1 ng, 99%
12	pre-stained MW marker	–
13	N0, SIF without Cry3Bb1 protein, 0 min	–
14	N10, SIF without Cry3Bb1 protein, 24 h	–
15	empty	–

Figure 2. Limit of detection immunoblot. Proteins were separated using a 10→20% polyacrylamide gradient tricine gel and electroblotted to nitrocellulose membrane. Proteins were detected using Cry3Bb1 antisera and developed using an ECL system (60 s exposure shown). Dilutions of the T0 sample were prepared in Laemmli sample buffer. Amounts (ng) loaded per lane and corresponding expected percent digestion of Cry3Bb1.11098(Q349R) protein are indicated. Approximate molecular weights (kDa) for marker proteins (lane 2, 12) are shown on the left.

Appendix 1

List of Applicable SOPs

SOP Number	SOP Title
GEN-PRO-058-01	Assay for Proteolytic Activity in Simulated Gastric Fluid
BtC-PRO-026-01	SDS Polyacrylamide Gel Electrophoresis (PAGE) using Pre-Cast Gels in Mini Gel Electrophoresis Apparatus
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)

Appendix 2

Protocol and Amendment

Monsanto Study #: **01-01-62-11**

Study Title: Assessment of the *in vitro* digestibility of the Cry3Bb1.11098(Q349R) protein in simulated intestinal fluid

Sponsor:
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St. Louis, MO 63198

Primary Testing Facility:
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Study Director:
Ronald E. Hileman
Monsanto Company
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Approved By:

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Reviewed By:

Barbara Joey

Quality Assurance Specialist
Monsanto Company
Monsanto Regulatory

19 NOV 2001
Date

1.0 Regulatory Compliance

1.1 GLP Compliance

This study will be conducted in compliance with the United States EPA FIFRA Good Laboratory Practice Regulations (40 CFR Part 160). Monsanto Regulatory Quality Assurance Unit (QAU) personnel will provide oversight for this study, and will distribute reports according to Monsanto Regulatory QAU Standard Operating Procedures (SOPs).

2.0 Introduction

Genetically modified corn, containing corn event MON 863, produces a variant of the *Bacillus thuringiensis* Cry3Bb1 protein. Corn plants producing this Cry3Bb1 protein variant are resistant to larval feeding damage from the coleopteran insect, corn rootworm (Coleoptera, Chrysomelidae, *Diabrotica* species). Using standard molecular biology methods, the DNA encoding the modified Cry3Bb1 protein and used to transform corn event MON 863 (Cavato *et al.*, 2001) was also used to engineer a protein production system in *Escherichia coli*. The isolated *E. coli*-produced Cry3Bb1.11098(Q349R) protein (lot 6962478) was shown to be physicochemically and functionally equivalent to the Cry3Bb1 protein produced in corn event MON 863 (Hileman *et al.*, 2001).

3.0 Purpose

The purpose of this study is to assess the *in vitro* digestibility of purified *E. coli*-produced Cry3Bb1.11098(Q349R) protein (lot 6962478) in simulated intestinal fluid (SIF).

4.0 Timelines

4.1	Proposed Experimental Start Date:	November 2001
4.2	Proposed Experimental Termination Date:	November 2001

5.0 Test, Control and Reference Substances

5.1 Test Substance

The test substance for this study is the *E. coli*-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. The test substance is stored in 10 mM phosphate buffer, pH 7 (Hileman *et al.*, 2001). Concentration and purity of the test substance will be verified prior to initiation of this study. The test substance will be stored in a –80 °C freezer until use in this study.

5.2 Control Substance

There is no control substance for this study.

However, experimental controls will be included in the experimental design to assess the stability of the test substance in the test system lacking pancreatin and to characterize the test system (see Section 6.2).

5.3 Reference Substance

There is no reference substance for this study.

Analytical reference standards used in the study will be documented in the results and will be described in the final report for each procedure employed. These will include, but are not limited to, molecular weight markers.

5.4 Characterization of the Test Substance

Characterization and stability of the test substance was conducted prior to this study (Hileman *et al.*, 2001). Any further preparation of this substance before use in this study will be documented and discussed in the final report.

5.5 Characterization of the Analytical Reference Standards

When available, copies of certificates of analysis confirming the characterization of reference standards and other materials used in this study will be archived with the study files.

6.0 Test System

The test system is simulated intestinal fluid (SIF). SIF will be prepared according to the standard operating procedure (SOP) GEN-PRO-058-01 and is based on the methods described in The United States Pharmacopoeia (1995). Pancreatin used for preparation of SIF will be from Sigma Company catalog number P-1500. The activity of SIF will be confirmed according to SOP GEN-PRO-058-01.

6.1 Justification for Selection of the Test System

In vitro digestion models are used widely to assess the digestibility of ingested substances. The digestion pattern of the test substance as a result of exposure to SIF will be evaluated at selected time points. Time points will be evaluated using western blot analysis. SIF is prepared based on the method described in The United States Pharmacopoeia (1995), and is frequently used for *in vitro* digestibility studies.

The time course and experimental parameters proposed in this study are similar to conditions used in a previously published study (Astwood *et al.*, 1996).

6.2 Experimental Controls

Experimental controls will be prepared to characterize the stability of the test substance in the test system (SIF) lacking pancreatin for the duration of the digestion time. These experimental controls will be designated with the letter "P". Conversely, experimental controls will be prepared to characterize the test system (SIF) lacking the test substance for the duration of the digestion time. These experimental controls will be designated with the letter "N".

6.3 Specimens

Specimens will be generated to represent various lengths of time at which the test substance will be incubated in the test system. Section 7.0 describes the preparation and analysis of specimens.

6.4 Procedure for Identification of Specimens

A numerical code using the numbers 0 through 10 will be used to distinguish assay time points.

7.0 Analytical Methods and Experimental Design

A schematic of the digestibility experimental procedure is shown in Attachment 2. All assay tubes will be frozen on dry ice and transferred to a –20 °C freezer until analyzed.

7.1 Digestibility of the Test Substance in SIF

Cry3Bb1.11098(Q349R) protein (stored in 10 mM phosphate buffer, pH 7) will be added to assay tubes containing SIF. The ratio of test substance to pancreatin powder in SIF will be 1 mg total protein to approximately 55 mg pancreatin powder. Digestions will be incubated at 37 ± 2 °C in separate tubes for each of the targeted incubation times. SIF digestions will be quenched by dilution with Laemmli sample loading buffer followed by heating at approximately 100 °C for approximately 5 min as previously described (Astwood *et al.*, 1996).

The zero incubation time point (T = 0) will be quenched by diluting SIF with Laemmli sample loading buffer followed by heating at approximately 100 °C for approximately 5 min prior to addition of the test substance.

The targeted incubation times will be 0, 1, 5, 15, 30 min, and 1, 2, 4, 8, 16, and 24 h. Actual incubation times will be recorded in the data file.

7.2 Experimental Controls

The stability of Cry3Bb1.11098(Q349R) protein in SIF lacking pancreatin will be assessed at two time points, 0 and 24 h. Additional time points

will be used if necessary. SIF without pancreatin consists of 6.8 mg/mL potassium phosphate monobasic, pH 7.5 (adjusted with sodium hydroxide). These experimental controls will be prepared in separate tubes containing the same volume used in the test substance digestibility assay tubes.

Experimental controls prepared by omitting Cry3Bb1.11098(Q349R) protein will be assessed at two time points, 0 and 24 h. Additional time points will be used if necessary. These experimental controls will be prepared in separate tubes using the same volumes used to prepare the test substance digestibility assay tubes. However, 10 mM phosphate buffer, pH 7, will be added to SIF in place of the test substance. Bands observed from western blot analysis of these samples will be attributed to the test system.

7.3 SDS-PAGE and Western Blot Analysis

Samples of all assay tubes will be separated using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with pre-cast 10-20% tricine mini-gels. This procedure is described in SOP No. BtC-PRO-026-01 with the following modifications. All SDS-PAGE runs conducted during this study will use tricine gels run with tricine buffers. Gels will be electrophoresed using 100 mM tricine, 100 mM Tris and 0.1%(w/v) SDS, pH ≈ 8.25 in the cathode (–) reservoir and 200 mM Tris, pH ≈ 8.9 in the anode (+) reservoir. Tricine SDS-PAGE gels will be used because they have been shown to provide optimum resolution of low molecular weight proteins (Schägger and von Jagow, 1987).

Based on predigestion concentrations and purity corrections, approximately 500 ng of *E. coli*-produced Cry3Bb1.11098(Q349R) protein will be loaded per lane. Samples of the experimental controls will be loaded using the same volume determined for the test substance digestibility samples.

Western blot analysis will be performed according to SOP No. GEN-PRO-002-03. Bound antibody will be detected using an appropriate secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins will be visualized using an enhanced chemiluminescence technique.

7.4 Statistical Methods

No statistical analysis will be performed.

8.0 Control of Bias

Measures taken to control bias in this study will include, but are not limited to, the analysis of samples in sets to eliminate run-to-run variations and the inclusion of

appropriate controls to account for any effects due to the model in the absence of test substance. Exact replicates will not be used in this study. Instead, individual digestion time points generated from the time course will serve as replicates that fall within a certain range.

9.0 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.

10.0 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.

11.0 References

Astwood, J. D., Leach, J. N., and Fuchs, R. L. (1996). Stability of food allergens to digestion *in vitro*. *Nat Biotechnol* **14**:1269-1273.

Cavato, T. A., Rigden, E. C., Mittanck, D. W., and Lirette, R. P. (2001). Amended report for MSL-16505: Molecular analysis of corn event MON 863. (Monsanto Company, St. Louis, MO) Study 99-01-39-27, Technical Report MSL-17152.

Hileman, R. E., Turner, L. A., Holleschak, G., Thoma, R. S., Brown, C. R., and Astwood, J. D. (2001). Characterization and equivalence of the Cry3Bb1 protein produced by *E. coli* fermentation and corn event MON 863. (Monsanto Company, St. Louis, MO) Study 01-01-39-30, Technical Report MSL-17274.

Schägger, H., and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166**:368-379.

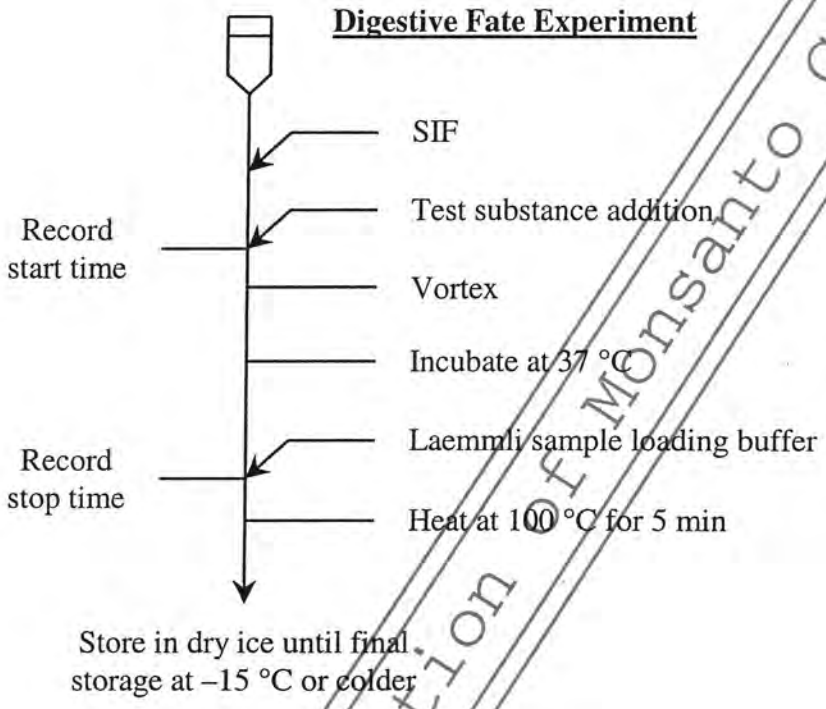
The United States Pharmacopoeia. 1995. Vol. 23, NF 18. United States Pharmacopoeial Convention, Inc., Rockville MD. p 2053.

Attachment 1: List of Applicable Method SOPs

SOPs cited in this protocol will be used in this study unless superseded by newer versions. In this event, the actual SOPs followed in this study will be reflected in the final report.

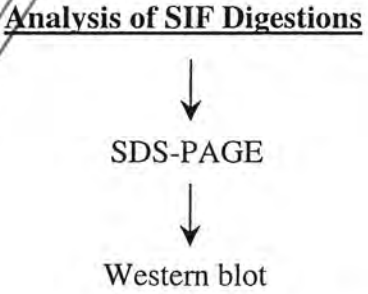
SOP	Title
GEN-PRO-058-01	Assay for Proteolytic Activity in Simulated Intestinal Fluid
BtC-PRO-026-01	SDS Polyacrylamide Gel Electrophoresis (PAGE) using Pre-Cast Gels in Mini Gel Electrophoresis Apparatus
GEN-PRO-002-03	Western Blot Analysis (Immunoblotting)

Attachment 2: Schematic of Experimental Procedure



Experimental Controls:
(1) Addition of protein buffer to SIF in place of test susbstance. (2) Stability of the test substance in the test system is evaluated by addition of Cry3Bb1.11098(Q349R) protein in SIF lacking pancreatin.

Pancreatin activity assay is conducted on SIF before and after the digestive fate experiment.



Protocol Amendment Form **Amendment #: 01**

Monsanto Study #: 01-01-62-11

Date changes implemented: November 26, 2001

Page number(s) and section(s): Page 7 of 10, Section 7.3

Protocol originally stated:

Based on predigestion concentrations and purity corrections, approximately 500 ng of *E. coli*-produced Cry3Bb1.11098(Q349R) protein will be loaded per lane. Samples of the experimental controls will be loaded using the same volume determined for the test substance digestibility samples.

Western blot analysis will be performed according to SOP No. GEN-PRO-002-03. Bound antibody will be detected using an appropriate secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins will be visualized using an enhanced chemiluminescence technique.

Protocol amended as follows

Based on predigestion concentrations and purity corrections, approximately 10 ng of *E. coli*-produced Cry3Bb1.11098(Q349R) protein will be loaded per lane. Samples of the experimental controls will be loaded using the same volume determined for the test substance digestibility samples.

Western blot analysis will be performed according to SOP No. GEN-PRO-002-03. Bound antibody will be detected using an appropriate secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins will be visualized using an enhanced chemiluminescence technique. A lower limit of detection will be determined for the test substance using western blot analysis.

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

The amount of test substance specified for western blot analysis was incorrect. This change will not have an impact on the study.

Omission of a description of the limit of detection from this protocol was an oversight. Addition of a limit of detection western blot will aid in the interpretation of the blot.

Protocol Amendment Form **Amendment #: 01**

Approved By:

Patrick T. Weston — Dec 4, 2001
Testing Facility Management Representative — Date

Ronald E. Hileman — 6 DEC 2001
Study Director — Date

Ravinder S. Sidhu — Dec 6, 2001
Sponsor Representative — Date

Reviewed By:

Quality Assurance Specialist — Dec. 4, 2001
Date