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Title

Assessment of the *in vitro* digestibility of Cry3Bb1 protein purified from corn event MON 863 and Cry3Bb1 protein purified from *E. coli*.

Authors

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Study Completed On

July 13, 2001

Performing Laboratory

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Laboratory Project ID

MSL Number: 17292
Study Number: 01-01-39-13

Statement of No Data Confidentiality Claim

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)(l)(A), (B), or (C).

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

Company: _____ Monsanto Company _____

Company Agent: _____

Title: _____

Signature: _____ Date: _____

Statement of Compliance

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

The characterization of the test substances was initiated prior to their use in this study and was conducted concurrent with this study. All characterization was completed prior to the end of this study.

Submitter: _____ Date: _____

Sponsor: Ravinder S Sidhu Date: July 13, 2001

Study Director: John Leavitt Date: July 13, 2001

Quality Assurance Statement

Study Title: Assessment of the *in vitro* digestibility of Cry3Bb1 protein purified from corn event MON 863 and Cry3Bb1 protein purified from *E. coli.*

Study Number: 01-01-39-13

Reviews conducted by the QAU 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 AG Regulatory QAU on the study reported herein.

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
05/30/2001	Digestive Fate	06/13/2001	06/13/2001
07/11/2001	Draft Report Review	07/13/2001	07/13/2001
07/11/2001	Raw Data Audit	07/13/2001	07/13/2001

Barbara Foley — July 13, 2001

Quality Assurance Unit — **Date**
Monsanto Regulatory, Monsanto Company

Signatures of Approval

Study Number: 01-01-39-13

Title: Assessment of the *in vitro* digestibility of Cry3Bb1 protein purified from corn event MON 863 and Cry3Bb1 protein purified from *E. coli*.

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Records Retention: All study specific raw data, protocols, final reports and facility records will be retained at Monsanto-St. Louis.

Ravinder S Sidhu — July 13, 2001
Sponsor — Date

John Leach — July 13, 2001
Study Director — Date

Director of Product Safety Center — July 13, 2001
Date

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Abbreviations

aa	amino acid
$A_{280\ nm}$	Absorbance of light at a wavelength of 280 nm
B.	*Bacillus*
B.t.	*Bacillus thuringiensis*
B.t.t.	*Bacillus thuringiensis tenebrionis*
CFR	Code of Federal Regulations
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
GLP	Good Laboratory Practice
kDa	kilodalton
mA	milliampere
mM	millimolar
N0	Negative control treatment with zero incubation time
N9	Negative control treatment incubated for as long as T=9
P0	Positive control treatment with zero incubation time
P9	Positive control treatment incubated for as long as T=9
PAGE	Polyacrylamide gel electrophoresis
P/N	Product number, same as catalog number
SDS	Sodium dodecyl sulfate
SGF	Simulated gastric fluid
SOP	Standard operating procedure
T	Time point
TCA	Trichloroacetic acid
Tricine	N-[tris(hydroxymethyl)methyl]glycine
Tris	Tris(hydroxymethyl)aminomethane
v/v	solute volume to solution volume
w/o	without
w/v	solute weight to solution volume

1.0 Summary

Monsanto has developed, through the use of biotechnology, corn event MON 863 that produces a variant of the wild type Cry3Bb1 protein (Hileman and Astwood, 2001) from *Bacillus thuringiensis* (*B.t.*). The Cry3Bb1 variant protein has insecticidal activity against coleopteran species such as corn rootworm (*Diabrotica* spp.), thereby protecting corn plants against feeding damage from these pests.

Certain food, feed and environmental safety evaluations that utilize purified protein require relatively large (i.e., gram) quantities of the protein. Because of the relatively low levels of the Cry3Bb1 variant protein in tissues from corn event MON 863 (Dudin et al., 1999), it was not feasible to isolate sufficient quantities of this 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 Cry3Bb1 proteins were identical.

The purpose of this study was to assess the *in vitro* digestibility of Cry3Bb1 protein purified from corn grain from event MON 863 and the identical Cry3Bb1 protein purified from *E. coli* using a simulated gastric fluid (SGF) mammalian digestion model. SDS-PAGE and colloidal blue staining were used to assess the extent of Cry3Bb1 protein digestion and the formation of any incomplete digestion peptide fragments over time.

The results of this study showed that the Cry3Bb1 proteins (MW ~74 kDa) produced from corn and from *E. coli* were rapidly digested below the limit of detection of the assay (LOD; ≤ 17 ng and ≤ 10 ng, respectively) within 15 seconds. Digestion of both proteins in SGF produced a faint band corresponding to a low molecular weight peptide (MW ~3 kDa), within 15 seconds. This fragment of the Cry3Bb1 protein was digested to below the detection limit of the SDS-PAGE assay within 15 minutes in the case of the corn-produced Cry3Bb1 protein and within two minutes in the case of the *E. coli*-produced Cry3Bb1 protein. These differences in peptide fragment digestion rates were possibly due to impurities present in the corn-produced Cry3Bb1 protein which has a lower purity (53.9%) relative to the *E. coli*-produced Cry3Bb1 protein (97.5%).

The rapid digestibility of Cry3Bb1 protein described herein is consistent with results of previous studies of Cry1, Cry2, Cry3 and other highly homologous Cry3Bb1 variant proteins (Leach et al., 2001). These results, in combination with the lack of sequence similarity of Cry3Bb1 protein to known allergens (Hileman et al., 2001), the lack of reported allergy to microbial pesticides that contain Cry3Bb1 protein, and the low level of Cry3Bb1 protein in corn grain, suggest that there is no increased risk of food allergy due to the presence of Cry3Bb1 protein in corn grain. Likewise, these results, combined with

the lack of sequence similarity of Cry3Bb1 protein to known toxins, the known mode of action of Cry proteins, the history of safety of microbial pesticides that contain Cry3Bb1 protein, and the lack of mammalian toxicity of Cry3Bb1 protein, confirm that there should be no significant risk of toxicity associated with Cry3Bb1 protein.

2.0 Introduction

Genetically modified corn event MON 863 produces a variant of the *Bacillus thuringiensis* (*B.t.*) Cry3Bb1 protein (Hileman and Astwood, 2001). Corn plants producing this Cry3Bb1 protein variant are resistant to larval feeding damage from the coleopteran insect, corn rootworm (Coleoptera, Chrysomelidae, *Diabrotica* sp). The rapid digestibility of Cry3Bb1 protein described herein was consistent with results of previous studies of Cry1, Cry2, Cry3 and other highly homologous Cry3Bb1 variant proteins (Leach et al., 2001).

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 (Dudin et al, 1999), 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 Cry3Bb1 proteins were identical (Hileman et al., 2001a).

This report describes the digestibility of the corn-produced Cry3Bb1 protein and *E. coli*-produced Cry3Bb1 protein *in vitro*. Models of digestion are commonly used to assess the digestibility of proteins. A correlation between digestibility in simulated gastric fluid (SGF) and food safety has been previously validated using this model (Astwood et al., 1996). In SGF, proteins which have been safely consumed were observed to be rapidly degraded and known allergens were observed to be relatively stable to proteolysis by pepsin. Although the SGF model demonstrates the digestibility of a protein by mammalian digestive enzymes, it is not intended to predict the half-life of a protein *in vivo*. However, specific parameters in the SGF model are representative of human digestion and are widely used in nutrition studies. Proteins which are nutritionally desirable tend to be relatively digestible and will have greater bioavailability of amino acids than stable proteins. In addition, proteins that are highly digestible would be expected to have less opportunity to exert adverse health effects when consumed. Simulated gastric models provide supportive data which can be used in combination with direct toxicity testing (i.e. acute oral toxicity in rodents) (Hammond and Fuchs, 1999) or in combination with additional parameters used to assess allergenic potential (Metcalfe et al., 1996).

3.0 Gene and Protein Nomenclature

Cry3Bb1 was previously referred to as CryIIIB2 (or Cry3B2) as well as Cry3Bb or CryIIIC. According to the most recent and accepted nomenclature, this protein should be referred to as Cry3Bb1 (Crickmore et al., 1998). The Cry3Bb1 nomenclature will be used in this report. Wild type Cry3Bb1 protein is one of the insecticidal proteins present in the microbial product Raven™ [1], that has been sold in the United States since 1995 to control coleopteran insect pests (Baum et al., 1996). The Cry3Bb1 protein also shares approximately 67% amino acid identity to the Cry3A class protein, Cry3Aa4 (GenBank accession number M30503), also referred to as CryIIIA (or Cry3A) and *B.t.t.* (McPherson et al., 1988). Genetically modified potatoes that produce the Cry3Aa4 protein and provides control of Colorado potato beetle (Perlak et al., 1993) has been in commerce within the United States and Canada since 1995 and 1996, respectively.

4.0 Materials

4.1 *Test Substances.* The test substances for this study were the Cry3Bb1 protein purified from corn grain containing event MON 863 (lot # 6957088) and Cry3Bb1 protein purified from *E. coli* (lot # 6962478). The concentration of the corn-produced Cry3Bb1 protein was estimated to be 0.46 mg/ml total protein with a purity of 53.9% (Hileman et al., 2001a). The concentration of the *E. coli*-produced Cry3Bb1 protein was estimated to be 0.65 mg/ml total protein with a purity of 97.5% (Pyla et al., 2001). The limit of detection for the *E. coli*-produced Cry3Bb1 protein was determined from material of the same lot number as that used in the digestion experiment (lot # 6962478), however, this material had a concentration of 49.1 mg/ml total protein with a purity of 92.6% (Hileman et al., 2001a). After sample transfer, these materials were stored in a -20 °C freezer or a -80 °C freezer until used in this study. These materials have been shown to be stable under these storage conditions (Hileman et al., 2001a). After analysis, excess volumes from each digestion trial were retained in a -20 °C freezer. Cry3Bb1 protein not used in this study was returned to the providers and stored in a -80 °C freezer for sample retention.

4.2 *Control Substance.* There was no control substance for this study. A description of how control samples were prepared to assist in the interpretation of results can be found in section 5.2.2.

[1] Registered trademark of Ecogen, Inc.

4.3 *Reference Substance.* There was no reference substance for this study. A description of how reference samples were prepared to assist in the interpretation of results can be found in section 5.2.3.

Analytical references were used. Hemoglobin was used as a reference protein to assay the activity of SGF and determine its suitability for use in this study. See section 5.1 for details. A commercially available set of molecular weight standards was used to estimate the molecular weights of bands detected within the SDS-PAGE gels.

4.4 *T/C/R Characterization.* Characterization of the test materials used in this study were conducted concurrently with this study. Preliminary estimates were used during the progress of this study. The methods used for characterization and the final assessments for concentration, purity and stability were reported for the corn-produced Cry3Bb1 protein used in this study (Hileman et al., 2001a). Characterization of *E. coli*-produced Cry3Bb1 protein used in this study to determine the limit of detection and the digestion pattern in SGF were also reported (Hileman et al., 2001a; Pyla et al., 2001, respectively). To be consistent with the digestion of the corn-produced Cry3Bb1 protein, the *E. coli*-produced Cry3Bb1 protein, at an initial concentration of 0.65 mg/ml, was diluted to a final concentration of 0.46 mg/ml total protein before incubation in SGF.

4.5 *Test System.* The test system for this study was simulated gastric fluid (SGF).

SGF was prepared according to SOP No. BR-ME-0460-01 and is based on the methods described in The United States Pharmacopoeia (2000). The activity of SGF was confirmed to be suitable for use in this study, according to SOP No. BR-ME-0460-01. The volume used to deliver Cry3Bb1 protein to SGF was kept at 20% of the SGF volume to reduce significant changes in the pH of the reaction. To maintain a ratio of 1.0 g of total protein to ≈ 17.7 g of pepsin powder, the SGF containing pepsin powder at 3.2 mg/ml was diluted with SGF prepared without pepsin to provide a final concentration of 1.6 mg/ml pepsin powder.

4.6 *Justification of Test System. In vitro* digestion models are used widely to assess the digestibility of ingested substances. SGF was prepared based on the method described in The United States Pharmacopoeia (2000) and is frequently used for *in vitro* digestibility studies.

The time course and experimental parameters used in this study are based on previously published work (Astwood et al., 1996). A common characteristic of many allergens is stability in SGF. The relevance of the correlation between

digestibility in the SGF model and allergen potency was confirmed by experimental results. Chemical reduction of disulfide bonds in milk β-lactoglobulin resulted in enhanced digestibility and significantly reduced allergenicity in milk allergic dogs (del Val et al., 1999).

Appropriate experimental controls were prepared to assess the digestion pattern of the Cry3Bb1 protein over time as a result of exposure to SGF. These controls are called control and reference treatments and are described in detail in sections 5.2.2 and 5.2.3 respectively.

Tricine gels are useful for the detection of small molecular weight bands and Colloidal Brilliant Blue G stain is an appropriate method for detection of low levels of non-specific proteins.

4.7 *Reagents / Buffers / Materials.* The following reagents and materials were obtained:

Source	Reagent
Sigma Chemical Company (St. Louis, MO)	Pepsin (porcine, P/N P-7000)
	Hemoglobin (bovine, P/N H-2625)
	Brilliant Blue G-Colloidal (P/N B-2025)
NOVEX Corporation (Indianapolis, IN)	10→20% gradient pre-cast tricine mini gels (P/N EC6625)
	Tris-Glycine transfer buffer (P/N LC1675)
GibCo	Molecular weight markers (P/N 16001-018)

All other reagents were reagent grade or better and were obtained from commercial sources.

5.0 Methods

5.1 *SGF Activity Assay.* Simulated gastric fluid was prepared and confirmed to be functionally active within an expected range on the day of use. SGF activity was measured in accordance with the assay described in SOP No. BR-ME-0460-01. This assay relates the amount of pepsin activity to the rate of hemoglobin digestion. Hemoglobin digestion was quantified by the change in absorbance (280 nm) after TCA (trichloroacetic acid) precipitation. Small peptides that remain in suspension after centrifugation result in greater absorbance. Photometric

absorbance of the suspension is thus directly related to the activity of the digestive fluid and serves as a measure for activity. An acceptable range has been designated in SOP No. BR-ME-0460-01.

5.2 *Preparation of Digestion Trials.* Timed incubations were initiated by the addition of corn-produced or *E. coli*-produced Cry3Bb1 protein to individual incubation tubes containing SGF. Incubations were conducted in a shaking waterbath pre-warmed to approximately 37 °C. Due to concern that SGF activity may be lost if stored at warm temperatures for prolonged periods of time, SGF was kept at room temperature until addition of the test substance. Trials that were initiated late in the experiment were stored at 4 °C and then allowed to equilibrate to room temperature prior to use. Since the time required to heat SGF to 37 °C was proportionally more significant for short verses long digestion time points, samples incubated for five minutes are less were preheated to 37 °C prior to addition of the test substance. These measures to preserve the activity of SGF before use have been demonstrated to be effective (Astwood et al., 1996; Leach et al., 2001).

After incubation, SGF digestions were quenched by the addition of sodium carbonate (0.53 M), diluted with 5× Laemmli sample loading buffer, and heated at approximately 100 °C for at least 5 min.

The zero incubation time trials were quenched prior to the addition of the test substance by adding sodium carbonate before the test substance.

Separate treatments were easily identified by color coding and individual time points were redundantly labeled 0 through 9. Reference treatments were identified by the letter "P" and control treatments were identified by the letter "N". All trials were stored frozen in a –20 °C freezer until analyzed using SDS-PAGE. A schematic of the digestive fate experimental procedure is included as Appendix 1 in this report.

5.2.1. *Test Treatments.* Test treatments were prepared by the addition of corn-produced or *E. coli*-produced Cry3Bb1 protein to SGF. These treatments were prepared as described in section 5.2. The incubation times were 0, 15, 30 seconds and 1, 2, 4, 8, 15, 30, and 60 minutes.

5.2.2 *Control Treatments.* Sodium carbonate (100 mM) was added to SGF in place of the corn-produced or *E. coli*-produced Cry3Bb1 protein to generate control treatments. These trials were prepared in a similar manner as described above in section 5.2.1. Volumes were the same as those used to prepare test treatments.

Incubation times were 0 and 60 minutes. This addition of sodium carbonate (100 mM) has been tested to ensure that the pH of the test system does not rise above 2 and should not be confused with the greater concentration of sodium carbonate (0.53 M) used to quench pepsin activity.

5.2.3 *Reference Treatments.* The test substances were also used in this study to generate reference treatments. Reference treatments were prepared by adding either the corn-produced or *E. coli*-produced Cry3Bb1 protein to tubes containing SGF w/o pepsin. These trials were prepared in a similar manner as described above in section 5.2.1. Volumes were the same as those used to prepare test treatments, but the incubation times were 0 and 60 minutes.

SGF w/o pepsin (lot # 6558456-A) consisted of 2 mg/ml sodium chloride adjusted to pH 1.2 using hydrochloric acid.

5.3 *SDS-PAGE/Colloidal Blue Staining.* Separation of proteins on tricine gels by SDS-PAGE followed by gel staining was performed following SOP No. BtC-PRO-026-01 and manufacturer specifications for gel staining. This is a sensitive method for detection of proteins. The limit of detection by Colloidal Brilliant Blue G staining for corn-produced Cry3Bb1 protein was demonstrated, as shown in Figure 3, to be ≤ 17 ng of Cry3Bb1 protein/lane or less. The limit of detection for *E. coli*-produced Cry3Bb1 protein was demonstrated, as shown in Figure 6, to be ≤ 10 ng of Cry3Bb1 protein/lane or less. The low selectivity of this method was useful for the detection of digestion products that may not be immunoreactive to antibodies. The use of tricine gels with tricine running buffers provided resolution of small molecular weight bands ≥ 2 kDa in size (Schagger and von Jagow, 1987).

Proteins were separated using pre-cast gradient tricine SDS-PAGE mini gels (Laemmli, 1970). Proteins were fixed in the gel matrix by incubation in a fixing solution [40%(v/v) methanol and 7%(v/v) glacial acidic acid in water] at room temperature on a shaking table for at least 30 min. The fixing solution was replaced with a colloidal blue dye staining solution. Gels were stained overnight using Colloidal Brilliant Blue G (Neuhoff et al., 1988), shaking at room temperature. The staining solution was replaced with post wash solution [25%(v/v) methanol in water] and shaken at room temperature for several hours to wash away excess background staining. Several changes of the post wash solution was used to reveal blue bands against a clear background. Permanent records of the results were made by photographing the gels using Polaroid 667 film.

6.0 Control of Bias and Quality Control Measures

Comparison of the reference treatments to the test treatments demonstrated: 1) that the experimental quenching method was effective at inactivating SGF activity and 2) that the test substance was stable under the conditions of the test system in the absence of pepsin. Control treatments were prepared to determine effects of the test system on the analytical methods applied and demonstrated that the pepsin band was stable for the duration of the experiment. 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 trial and provided an opportunity to eliminate errors during the experiment.

7.0 Results and Discussion

7.1 *Effectiveness of the Test System.* The proteolytic activity of SGF prepared for digestion of corn-produced Cry3Bb1 protein was 18,883 U/ml and the activity of SGF prepared for digestion of *E. coli*-produced Cry3Bb1 protein was 18,317 U/ml ($1U = \Delta 0.001$, $A_{280\ nm}\ min^{-1}$ at 37 °C). These activity values were within the acceptability requirements of SOP No. BR-ME-0460-01.

7.2 *Digestibility of Cry3Bb1 Protein in SGF.* Both the corn-produced and the *E. coli*-produced Cry3Bb1 proteins were rapidly degraded in SGF. Cry3Bb1 protein purified from corn grain containing event MON 863 was degraded to a small transient peptide fragment (MW ≈ 3 kDa) within 15 seconds in SGF (Figure 1, lane 5). This transient peptide fragment persisted for 15 minutes before it was degraded to a level below the limit of detection (Figure 2, lane 8). Cry3Bb1 protein purified from *E. coli* was also degraded to a small transient peptide fragment (MW ≈ 3 kDa) within 15 seconds in SGF (Figure 4, lane 5) and degraded to a level below the limit of detection after 2 minutes in SGF (Figure 5, lane 8).

The presence of a low molecular weight (MW ≈ 3 kDa) fragment was consistent with the previously reported digestibility studies using highly homologous Cry3Bb1 proteins (Leach et al., 2001). Differences in the rate at which the transient peptide fragments degrade below the limit of detection were possibly due to impurities present in the corn-produced Cry3Bb1 protein (53.9%) relative to the *E. coli*-produced Cry3Bb1 protein (97.5%).

7.3 *Test Substance Stability.* The ability to recover the corn-produced and *E. coli*-produced Cry3Bb1 proteins from the SGF test system was demonstrated by

comparison of reference treatments. Preparation of reference treatments is detailed in section 5.2.3 of this report.

SDS-PAGE/Colloidal Blue staining method demonstrated that the corn-produced and *E. coli*-produced Cry3Bb1 proteins were stable in an inactive test system for the duration of the experiment (lanes 2 and 3 in Figures 2 and 5). Thus, all observed Cry3Bb1 protein degradation was attributed to the proteolytic activity of SGF.

7.4 *Limit of detection.* Figures 3 and 6 demonstrate that as little as 17 ng of undegraded corn-produced Cry3Bb1 protein per lane and 10 ng of undegraded *E. coli*-produced Cry3Bb1 protein per lane can be detected by the method used to determine the rate of digestion. Since 827 ng of corn-produced Cry3Bb1 protein and 526 ng of *E. coli*-produced Cry3Bb1 protein were loaded per lane based on predigestion estimates, digestion below the limit of detection represents greater than 98% degradation.

The gel loadings for corn-produced and *E. coli*-produced Cry3Bb1 proteins were intended to be the same, but purity estimates for these proteins changed after reassessment of the characterization data (Hileman, et al., 2001a; Pyla et al., 2001). The reassessed purity estimates were used to calculate corrected values for this report.

7.5 *Rejected Data.* The first digestion of *E. coli*-produced Cry3Bb1 protein in SGF yielded an unexpected prolonged rate of digestion. This observation was consistent with a previous study with Cry3Bb1 protein (Leach et al, 2001). Due to the high protein concentration of the sample transferred for use in this study, and the need to warm the sample to 37 °C in order to reduce viscosity and obtain a portion for dilution, the formation of protein aggregates was a highly probable cause. However, even with differences attributed to aggregation of the *E. coli* sample, both the corn-produced Cry3Bb1 protein and the *E. coli*-produced Cry3Bb1 protein were rapidly degraded after 2 minutes or less in SGF. To remove aggregates, a sample of *E. coli*-produced Cry3Bb1 protein was clarified using ultracentrifugation. This material was used to repeat the digestion experiment on the *E. coli* produced material and the results were reported in this study.

8.0 Conclusions

The results of this study showed that the Cry3Bb1 proteins were rapidly and completely digested after incubation in SGF. These results for the corn-produced and *E. coli*-

produced Cry3Bb1 proteins suggest that these proteins will be readily digestible in the mammalian digestive tract.

Cry3Bb1 proteins are highly digestible and would be expected to have less opportunity to exert adverse health effects when consumed relative to known toxins and allergens. Rapid digestion of the intact Cry3Bb1 proteins in SGF is a characteristic shared among proteins with a history of safe consumption. Conversely, the major food allergens that have been tested in the SGF model are stable to digestion (Astwood et al., 1996; del Val et al., 1999; Deshpande and Neilsen, 1987; Taylor and Lehrer, 1996). Comparison of these digestion patterns with the *in vitro* digestion model cited (Astwood et al, 1996) indicates that the Cry3Bb1 proteins fall within the digestion patterns observed for safe proteins.

The rapid digestibility of Cry3Bb1 protein described herein was consistent with results of previous studies of Cry1, Cry2, Cry3 and other homologous Cry3Bb1 variants. These results, in combination with the lack of sequence similarity of Cry3Bb1 protein to allergens, the lack of reported allergy to microbial pesticides that contain Cry3Bb1 protein, and the low level of Cry3Bb1 protein in corn grain (Dudin et al, 1999) suggest that there is no increased risk of food allergy due to the presence of Cry3Bb1 protein in corn grain. Likewise, these results combined with the lack of sequence similarity of Cry3Bb1 protein to toxins, the known mode of action of Cry proteins, the history of safety of microbial pesticides that contain Cry3Bb1 protein and the lack of mammalian toxicity of Cry3Bb1 protein, confirm that there should be no significant risk of toxicity associated with Cry3Bb1 protein.

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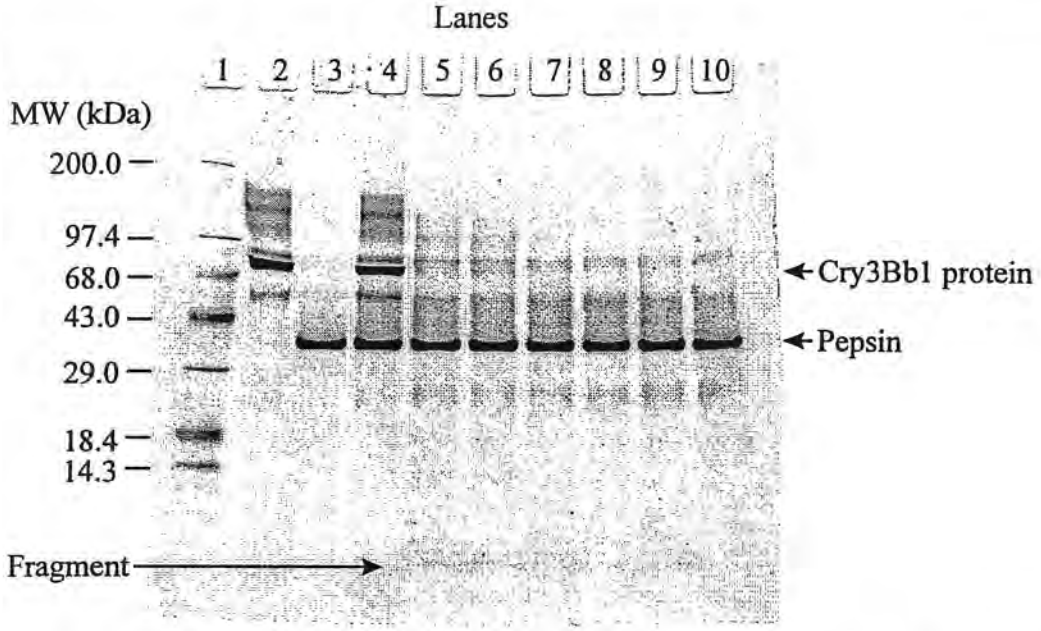


Figure 1. Colloidal Blue Stained Gel 1 Showing the Digestion of Cry3Bb1 Protein Purified from Corn grain containing event MON 863 in Simulated Gastric Fluid. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G stain. Corn-produced Cry3Bb1 protein was loaded at 827 ng per lane based on purity corrected and pre-digestion estimates. Molecular weight markers were loaded at 500 ng per band.

Lane	Description	Incubation time
1	Mid-range protein markers	
2	Corn-produced Cry3Bb1 protein reference treatment (P0)	0 s
3	SGF control treatment (N0)	0 s
4	Corn-produced Cry3Bb1 protein in SGF T = 0	0 s
5	Corn-produced Cry3Bb1 protein in SGF T = 1	15 s
6	Corn-produced Cry3Bb1 protein in SGF T = 2	30 s
7	Corn-produced Cry3Bb1 protein in SGF T = 3	1 min
8	Corn-produced Cry3Bb1 protein in SGF T = 4	2 min
9	Corn-produced Cry3Bb1 protein in SGF T = 5	4 min
10	Corn-produced Cry3Bb1 protein in SGF T = 6	8 min

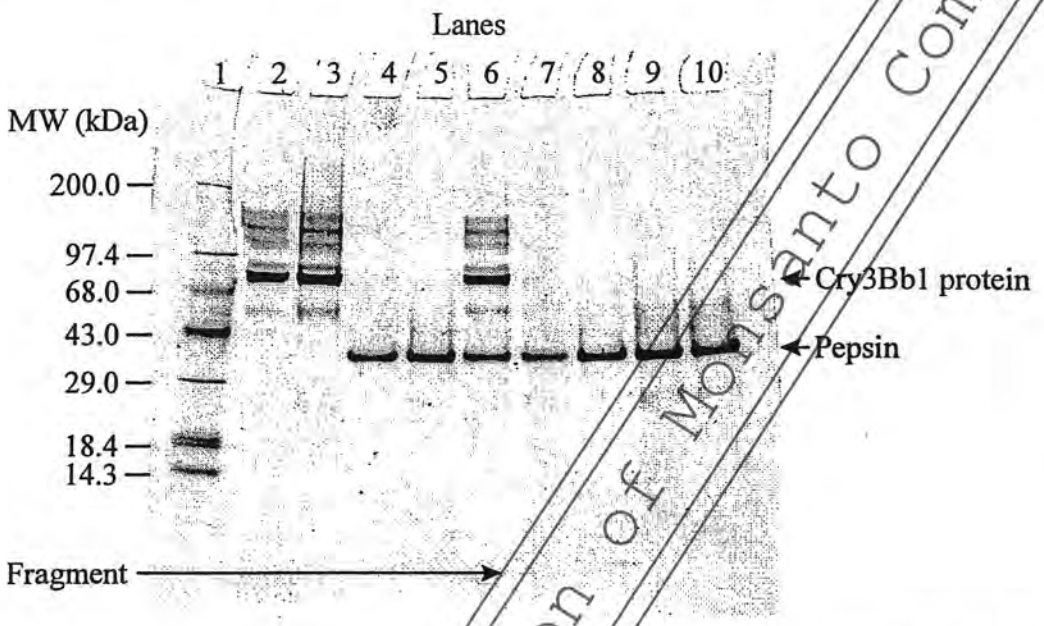


Figure 2. Colloidal Blue Stained Gel 2 Showing the Digestion of Cry3Bb1 Protein Purified from Corn grain containing event MON 863 in Simulated Gastric Fluid. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G stain. Corn-produced Cry3Bb1 protein was loaded at 827 ng per lane based on purity corrected and pre-digestion estimates. Molecular weight markers were loaded at 500 ng per band.

Lane	Description	Incubation time
1	Mid-range protein markers	
2	Corn-produced Cry3Bb1 protein reference treatment (P0)	0 s
3	Corn-produced Cry3Bb1 protein reference treatment (P9)	60 min
4	SGF control treatment (N0)	0 s
5	SGF control treatment (N9)	60 min
6	Corn-produced Cry3Bb1 protein in SGF T = 0	0 s
7	Corn-produced Cry3Bb1 protein in SGF T = 6	8 min
8	Corn-produced Cry3Bb1 protein in SGF T = 7	15 min
9	Corn-produced Cry3Bb1 protein in SGF T = 8	30 min
10	Corn-produced Cry3Bb1 protein in SGF T = 9	60 min

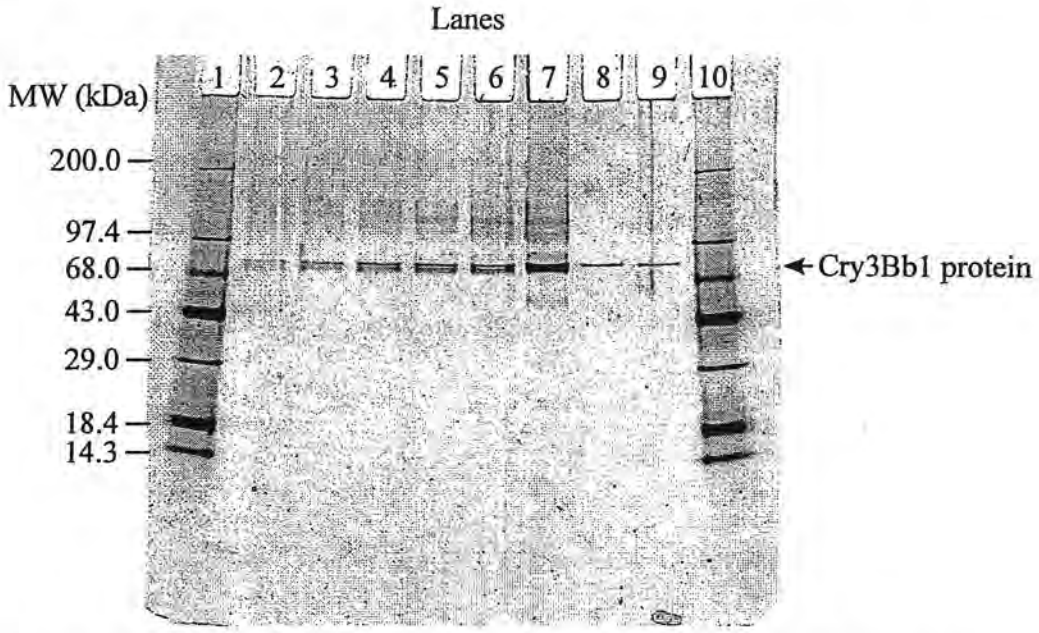


Figure 3. Colloidal Blue Stained Gel Showing the Lower Limit of Detection for Cry3Bb1 Protein Purified from Corn grain containing event MON 863. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G stain. Molecular weight markers were loaded at 500 ng per band.

Lane	Description	Cry3Bb1 protein loading*
1	Mid-range protein markers	
2	Corn-produced Cry3Bb1 protein	17 ng
3	Corn-produced Cry3Bb1 protein	33 ng
4	Corn-produced Cry3Bb1 protein	50 ng
5	Corn-produced Cry3Bb1 protein	66 ng
6	Corn-produced Cry3Bb1 protein	83 ng
7	Corn-produced Cry3Bb1 protein	165 ng
8	*E. coli*-produced Cry3Bb1 protein**	49 ng
9	*E. coli*-produced Cry3Bb1 protein**	29 ng
10	Mid-range protein markers	

* Cry3Bb1 protein loadings shown above were corrected for purity.

** *E. coli*-produced Cry3Bb1 protein was included for comparison to corn-produced Cry3Bb1 protein.

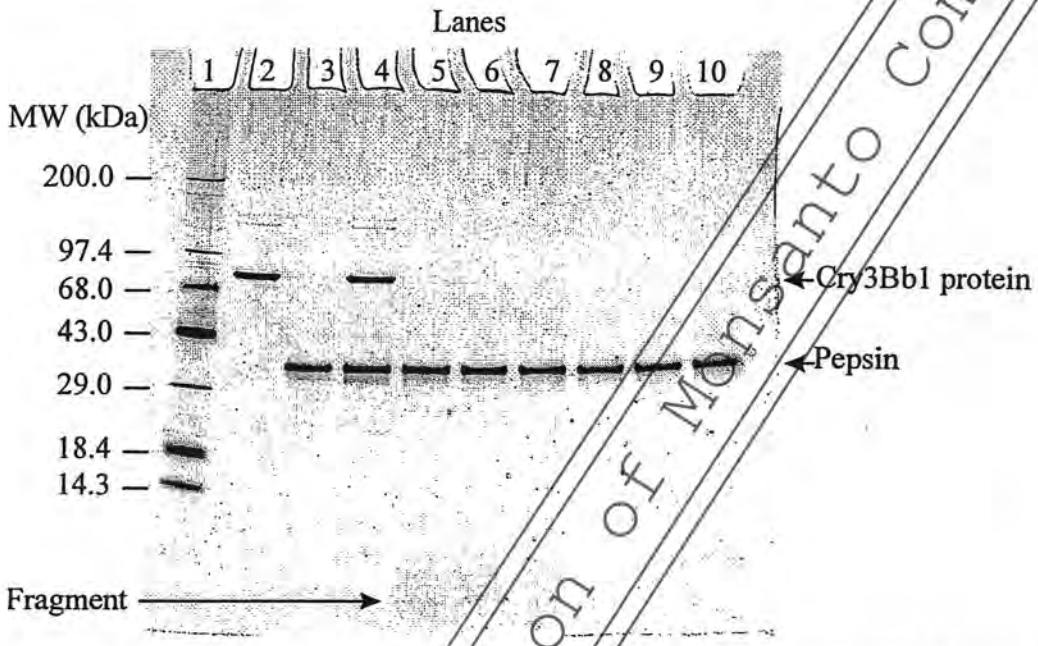


Figure 4. Colloidal Blue Stained Gel 1 Showing the Digestion of Cry3Bb1 Protein Purified from *E. coli* in Simulated Gastric Fluid. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G stain. *E. coli*-produced Cry3Bb1 protein was loaded at 526 ng per lane based on purity corrected and pre-digestion estimates. Molecular weight markers were loaded at 500 ng per band.

Lane	Description	Incubation time
1	Mid-range protein markers	
2	*E. coli*-produced Cry3Bb1 protein reference treatment (P0)	0 s
3	SGF control treatment (N0)	0 s
4	*E. coli*-produced Cry3Bb1 protein in SGF T = 0	0 s
5	*E. coli*-produced Cry3Bb1 protein in SGF T = 1	15 s
6	*E. coli*-produced Cry3Bb1 protein in SGF T = 2	30 s
7	*E. coli*-produced Cry3Bb1 protein in SGF T = 3	1 min
8	*E. coli*-produced Cry3Bb1 protein in SGF T = 4	2 min
9	*E. coli*-produced Cry3Bb1 protein in SGF T = 5	4 min
10	*E. coli*-produced Cry3Bb1 protein in SGF T = 6	8 min

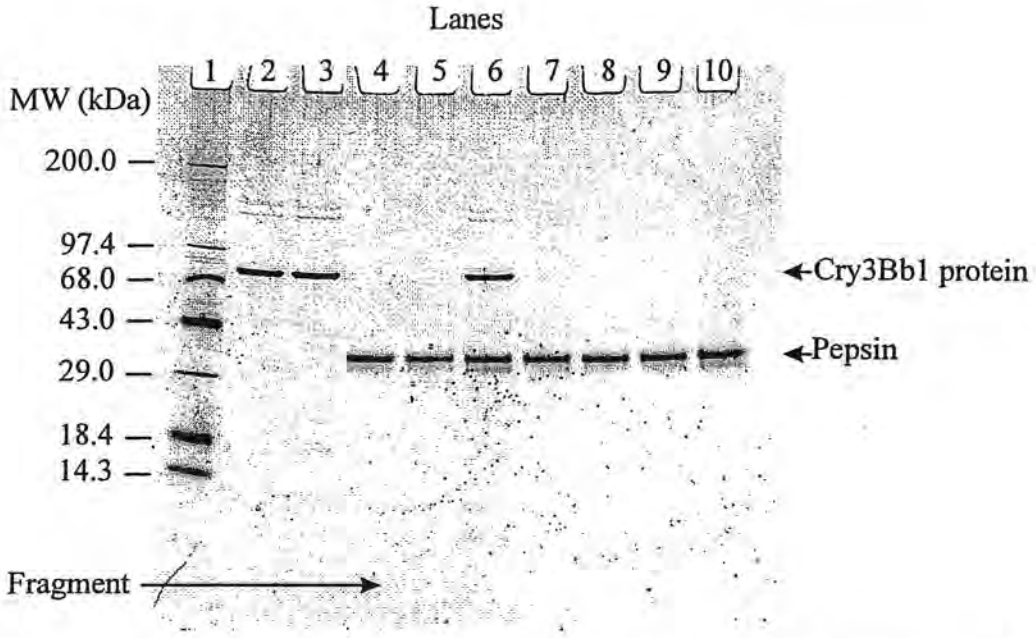


Figure 5. Colloidal Blue Stained Gel 2 Showing the Digestion of Cry3Bb1 Protein Purified from *E. coli* in Simulated Gastric Fluid. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G stain. *E. coli*-produced Cry3Bb1 protein was loaded at 526 ng per lane based on purity corrected and pre-digestion estimates. Molecular weight markers were loaded at 500 ng per band.

Lane	Description	Incubation time
1	Mid-range protein markers	
2	*E. coli*-produced Cry3Bb1 protein reference treatment (P0)	0 s
3	*E. coli*-produced Cry3Bb1 protein reference treatment (P9)	60 min
4	SGF control treatment (N0)	0 s
5	SGF control treatment (N9)	60 min
6	*E. coli*-produced Cry3Bb1 protein in SGF T = 0	0 s
7	*E. coli*-produced Cry3Bb1 protein in SGF T = 6	8 min
8	*E. coli*-produced Cry3Bb1 protein in SGF T = 7	15 min
9	*E. coli*-produced Cry3Bb1 protein in SGF T = 8	30 min
10	*E. coli*-produced Cry3Bb1 protein in SGF T = 9	60 min

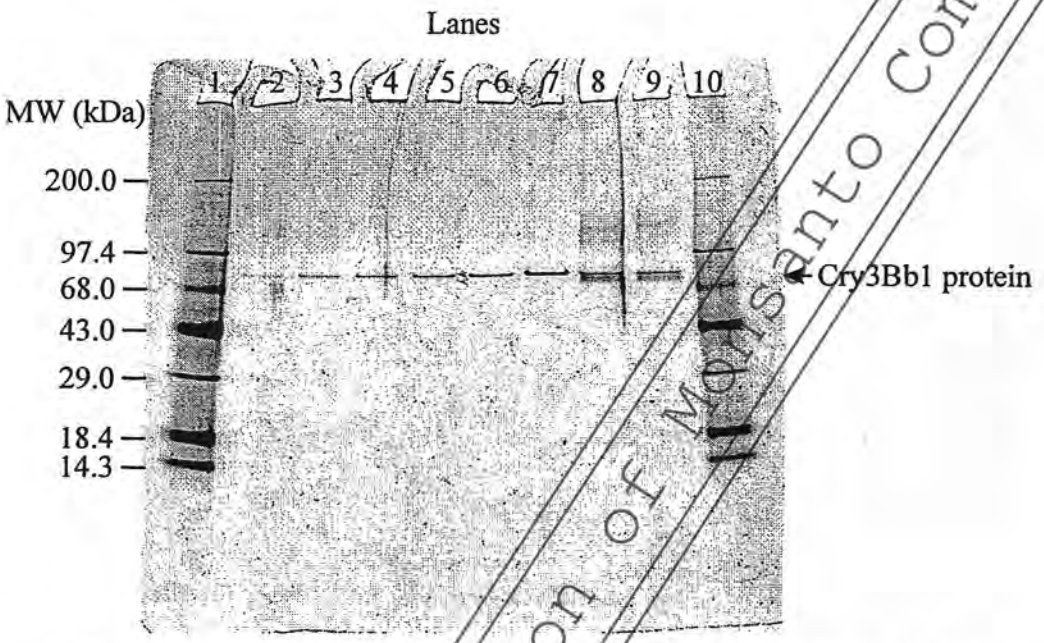


Figure 6. Colloidal Blue Stained Gel Showing the Lower Limit of Detection for Cry3Bb1 Protein Purified from *E. coli*. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a tricine buffered gel. Proteins were detected by staining with Brilliant Blue G stain. Molecular weight markers were loaded at 500 ng per band.

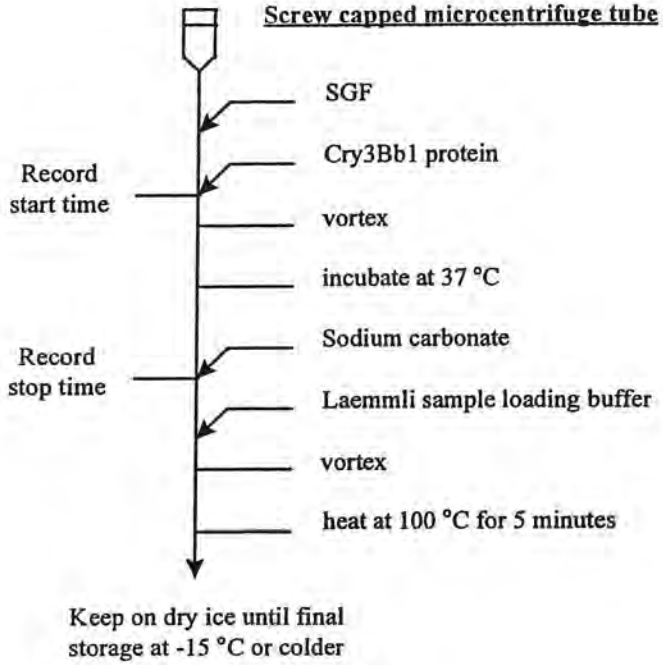
Lane	Description	Cry3Bb1 protein loading*
1	Mid-range protein markers	
2	*E. coli*-produced Cry3Bb1 protein	10 ng
3	*E. coli*-produced Cry3Bb1 protein	20 ng
4	*E. coli*-produced Cry3Bb1 protein	29 ng
5	*E. coli*-produced Cry3Bb1 protein	39 ng
6	*E. coli*-produced Cry3Bb1 protein	49 ng
7	*E. coli*-produced Cry3Bb1 protein	98 ng
8	Corn-produced Cry3Bb1 protein**	83 ng
9	Corn-produced Cry3Bb1 protein**	50 ng
10	Mid-range protein markers	

* Cry3Bb1 protein loadings shown above were corrected for purity.

** Corn-produced Cry3Bb1 protein was included for comparison to *E. coli*-produced Cry3Bb1 protein.

Appendix 1

SGF Digestive Fate Schematic



Control treatments were prepared by adding 100 mM sodium carbonate to SGF in place of Cry3Bb1 protein. Reference treatments were prepared by adding Cry3Bb1 protein to SGF without pepsin.

Appendix 2

Protocol, Amendments, and Deviations

Protocol is attached as Pages 30 - 40

Protocol amendment is attached as Pages 41 - 42

and

Protocol deviation is attached as Page 43

Monsanto Study #: **01-01-39-13**

Study Title: Assessment of the *in vitro* digestibility of Cry3Bb1.11098 protein purified from corn grain event MON 863 and Cry3Bb1.11098(Q349R) protein purified from *E. coli*.

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St. Louis, MO 63198

Primary Testing Facility:
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Product Safety Center
700 Chesterfield Parkway North
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Study Director:
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Approved By:

Patrick T. Weston
Testing Facility Management Representative
Monsanto Company

May 14, 2001
Date

Ravinder S Sidhu

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John N. Leach
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James D. Astwood
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Reviewed By:

Barbara Fay

Quality Assurance Specialist
Monsanto Company
Monsanto Regulatory

May 14, 2001
Date

[redacted]

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).

2.0 Purpose

The purpose of this study is to assess the *in vitro* digestibility in simulated gastric fluid of Cry3Bb1.11098 protein purified from corn grain of event MON 863 and Cry3Bb1.11098(Q349R) protein purified from *E. coli*.

3.0 Timelines

3.1	Proposed Experimental Start Date:	May 15, 2001
3.2	Proposed Experimental Termination Date:	July 15, 2001

4.0 Test, Control and Reference Substances

4.1 Test Substance

The test substances for this study are Cry3Bb1.11098 protein purified from corn grain event MON 863 (lot # 6957088) and Cry3Bb1.11098(Q349R) protein purified from *E. coli* (lot # 6962478). These materials will be stored in a 4 °C refrigerator until use in this study.

4.2 Control Substance

There is no control substance for this study.

4.3 Reference Substance

There is no reference substance for this study.

Appropriate reference standards will be documented in the results and will be described in the final report for each analytical procedure employed. Reference standards will include but are not limited to molecular weight markers.

4.4 Characterization of Test, Control and Reference Substances

Characterization of the test substances (Study no. 01-01-39-30) will be conducted concurrently with this study. Any further preparation of these substances before use in this study will be documented and discussed in the final report.

Stability of corn event MON 863 Cry3Bb1.11098 protein and *E. coli* produced Cry3Bb1.11098(Q349R) protein will be assessed by an SDS-PAGE gel staining method performed before and after this study.

5.0 Description of Experimental Design

In this protocol, the term treatment is used to describe the selection of components for use in the test system and the sequential order in which components are added to the test system. Reference and control treatments will be prepared to assist interpretation of the results and are described below.

The test substances will be used in this study to generate an incubation time course of Cry3Bb1.11098 and Cry3Bb1.11098(Q349R) proteins in SGF. These treatments are described in greater detail as test treatments in section 5.1.1.

Control treatments will be used in this study to prepare at least two incubation timepoints without Cry3Bb1 protein. In analysis, this will assist in identification of SDS-PAGE bands due to pepsin and/or its degradation products. These treatments are described in greater detail in section 5.1.2.

Reference treatments will be used to determine whether degradation of Cry3Bb1.11098 or Cry3Bb1.11098(Q349R) proteins is due solely to pepsin activity or to other factors. Reference treatments will also be used to demonstrate the effectiveness of the quenching method, by comparison to the zero incubation test treatment. See section 5.1.3 for greater detail.

Samples will be prepared for SDS-PAGE prior to storage by dilution with Laemmli sample loading buffer and heating at ≈100 °C for ≈5 minutes. Samples will be frozen on dry-ice until they can be stored in a –20 °C freezer until analyzed. A schematic of the digestive fate experimental procedure is shown in Attachment 3.

5.1 Test system

The test system is simulated gastric fluid (SGF).

SGF will be prepared according to SOP No. BR-ME-0460-01 and is based on the methods described in "The United States Pharmacopoeia" (3).

5.1.1 Test treatments

The test substances will be used in this study to generate an incubation time course of Cry3Bb1.11098 protein and Cry3Bb1.11098(Q349R) protein in SGF. Trials will be prepared by adding Cry3Bb1.11098 protein or Cry3Bb1.11098(Q349R) protein to tubes containing SGF. The ratio of total protein from the test substance to pepsin powder in SGF will be 1.0 g total protein to ≈17.7 g pepsin powder. Digestions will be incubated at 37 ± 2 °C in separate tubes for each of the targeted time trials. SGF digestions will be quenched by addition of a sodium carbonate solution to the test system. This has been shown in prestudy experiments to be an appropriate method of quenching SGF activity (2).

Zero incubation time trials (T = 0) will be quenched by addition of sodium carbonate solution to SGF prior to addition of the test substance.

The targeted incubation times will be 0, 15, 30 sec, and 1, 2, 4, 8, 15, 30, and 60 min. Actual incubation times will be recorded in the data file.

5.1.2 Control treatments

100 mM sodium carbonate will be added to SGF to generate control treatments. These trials will be prepared in a similar manner as described above in section 5.1.1. Volumes will be the same as those used to prepare the test treatments. Targeted incubation times will be 0 and 60 minutes. Note: This addition of sodium carbonate has been tested to ensure that the pH of the test system will not raise above 2.0 and should not be confused with the greater concentration of sodium carbonate used to quench pepsin activity.

5.1.3 Reference treatments

The test substances will also be used in this study to generate reference treatments. Reference treatments will be prepared by adding Cry3Bb1.11098 protein or Cry3Bb1.11098(Q349R) protein to tubes containing SGF w/o pepsin. These trials will be prepared in a similar manner as described above in section 5.1.1. Volumes will be the same as those used to prepare the test treatments, but the targeted incubation times will be 0 and 60 minutes.

SGF w/o pepsin (lot # 6558456-A) consists of 2 mg/ml sodium chloride and pH adjusted to 1.2 with hydrochloric acid.

5.2 Justification for selection of the test system

In vitro digestion models are used widely to assess the digestibility of ingested substances. A previous study has demonstrated the correlation between the digestibility of proteins and the allergy safety assessment of proteins (2). Appropriate tests will be conducted to assess the digestion pattern of the Cry3Bb1 protein over time as a result of exposure to SGF. These tests include, but may not be limited to gel staining methods. SGF is prepared based on the method described in "The United States Pharmacopoeia" (3), and is frequently used for *in vitro* digestibility studies. The activity of SGF will be confirmed according to SOP No. BR-ME-0460-01.

The time course and experimental parameters used in this study are similar to conditions used in a previously published study (2).

Appropriate experimental controls will be prepared to provide a clear interpretation of the results. These controls are called control and reference treatments and are described in detail in Sections 5.0 through 5.1.3.

5.3 Procedure for identification of the test system

A numerical code using the numbers 0 through 9, will be used to distinguish time trials corresponding to the targeted incubation times. Reference treatments will be identified by the letter "P" and control treatments will be identified by the letter "N". A color code will also be used to distinguish trails containing Cry3Bb1.11098 protein purified from corn grain of event MON 863 from Cry3Bb1.11098(Q349R) protein purified from *E. coli*.

5.4 Analytical Methods

5.4.1 Digestive fluid activity assays

The enzymatic activity of SGF will be assessed within 24 hours of preparation according to SOP No. BR-ME-0460-01. This will demonstrate that the test system is appropriate for use in this study.

In this assay, the quality of SGF activity is measured in reference to hemoglobin digestibility. The degree of hemoglobin digestion is measured by the optical density (wavelength of 280 nm) of digestion products remaining in solution after TCA precipitation.

5.4.2 Analysis of SGF digested trials

Analysis of SGF trials will be performed using an SDS-PAGE gel staining method. Gel staining methods are commonly used and extensively referenced in the scientific literature relevant to the purposes for which they are being employed (4).

5.4.3 SDS-PAGE

Samples from the SGF *in vitro* digestion models will be analyzed by SDS-PAGE using pre-cast 10-20% tricine mini-gels (NOVEX, P/N EC66255). This procedure is described in SOP No. BtC-PRO-026-01 with specifics and differences cited below. All SDS-PAGE runs conducted during this study will use NOVEX brand tricine gels run with tricine buffers. For tricine gels, the upper buffer reservoir will contain 100 mM tricine, 100 mM Tris and 0.1%(w/v) SDS, pH ≈ 8.25 and the lower buffer reservoir will contain 200 mM Tris, pH ≈ 8.9. Tricine SDS-PAGE gels will be used because they have been shown to provide optimum resolution of low molecular weight proteins (5).

Based on predigestion concentrations, 500 ng of Cry3Bb1 protein will be loaded per lane. Control treatments will be loaded with the same volumes used for the test and reference treatments.

5.4.4 Colloidal blue staining

After separation of proteins by SDS-PAGE, gels will be stained using colloidal blue dye. Prior to staining, the gels are incubated in fix solution (40%(v/v) methanol, 7%(v/v) acetic acid) for at least 30 minutes at room temperature. Gels are then stained for at least one hour with Brilliant Blue G Colloidal dye (Sigma P/N B-2025) diluted 4:1 with methanol. Excess background is removed by washing the gels in 25%(v/v) methanol.

6.0 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, trials generated from the time course will serve as replicates that fall within a certain range.

7.0 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.

8.0 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.

9.0 References

(1) Hileman, R. E., Pyla, P. D., Lee, T. C. and Astwood, J. D. 1999. Characterization of *B.t.* protein 11098 and *B.t.* protein 11231 produced by fermentation. Study number 98-01-39-05, MSL-15531, an unpublished study conducted by Monsanto Company.

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

(3) The United States Pharmacopoeia. 2000. Vol. 24, NF 19. United States Pharmacopoeial Convention, Inc., Rockville MD. p 2235.

(4) Deutcher, M.P. 1990. Guide to Protein Purification, in: *Methods in Enzymology* 182. Academic Press, Inc., Harcourt Brace Jovanovich, Publishers, New York.

(5) 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. *Analytical Biochemistry*, 166: 368-379.

Attachment 1: Abbreviations

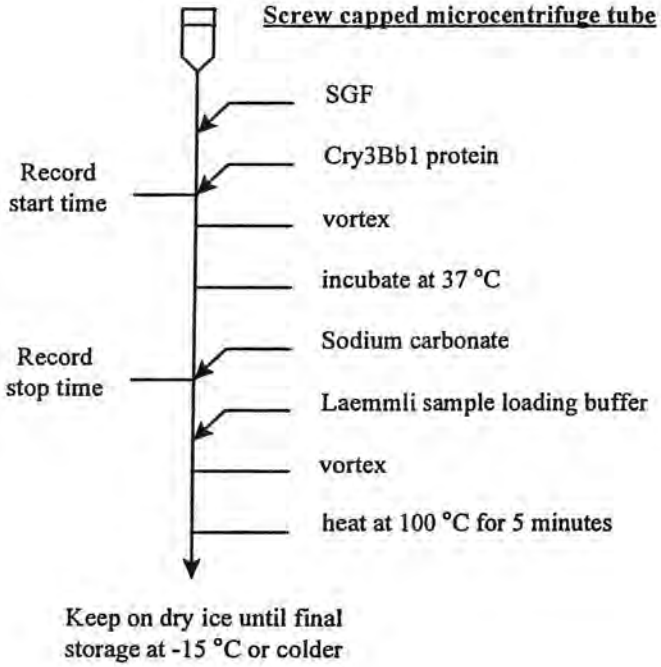
B.t.	*Bacillus thuringiensis*
CFR	Code of Federal Regulations
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
GLP	Good Laboratory Practice
PAGE	Polyacrylamide gel electrophoresis
P/N	Product number, same as catalog number
purified water	Water prepared using a Milli-Q filter purification system
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecylsulfate
SOP	Standard Operating Procedure
SGF	Simulated gastric fluid
T	Time
TCA	Trichloroacetic acid
Tricine	N-tris[hydroxymethyl]methyl glycine
Tris	Tris(hydroxymethyl)aminomethane

Attachment 2: List of Applicable Method SOPs

SOP	Title
BtC-PRO-026-01	SDS Polyacrylamide Gel Electrophoresis (PAGE) using Pre-Cast Gels in Mini Gel Electrophoresis Apparatus
BR-ME-0460-01	Assay for Pepsin Activity in Simulated Gastric Fluid

Attachment 3: Example Schematic of Experimental Procedure

SGF Digestive Fate Schematic



Control treatments are prepared by adding 100 mM sodium carbonate to SGF in place of Cry3Bb protein. Reference treatments are prepared by adding Cry3Bb1 protein to SGF without pepsin.

Protocol Amendment Form **Amendment #: 1**

Monsanto Study #: **01-01-39-13**

Date changes implemented: July 12, 2001

Page number(s) and section(s): Page 1, title

Protocol originally stated:
Title: Assessment of the *in vitro* digestibility of Cry3Bb1.11098 protein purified from corn grain event MON 863 and Cry3Bb1.11098(Q349R) protein purified from *E. coli*.

Protocol amended as follows:
Title: Assessment of the *in vitro* digestibility of Cry3Bb1 protein purified from corn event MON 863 and Cry3Bb1 protein purified from *E. coli*.

Reason for the amendment and what impact will result from this change:
The title of the protocol was amended to be consistent with the final report title. These changes reflect the current nomenclature used to refer to these proteins. There is no impact on this study.

Approved By:

Patrick T. Weston — July 12, 2001
Testing Facility Management Representative Date

John N. Leach — July 12, 2001
Study Director Date

Ravinder S Sidhu — July 12, 2001
Ravinder S. Sidhu Date
Sponsor Representative

Protocol Amendment Form

Amendment #: 1

Reviewed By:

Barbara Jody

Quality Assurance Specialist

July 12, 2001

Date

Protocol Deviation Form

Monsanto Study #: **01-01-39-13**

Date(s) deviation occurred: May 2001 - July 2001

Page number(s) and section(s): Page 7 of 11, section 5.4.3

Description of deviation:
The protocol states that based on predigestion estimates, SDS-PAGE gels will be loaded with 500 ng of Cry3Bb1 protein per lane. Instead, gels for the analysis of corn-produced Cry3Bb1 protein in SGF were loaded with 827 ng of Cry3Bb1 protein per lane and gels for the analysis of *E. coli*-produced Cry3Bb1 protein in SGF were loaded with 526 ng of Cry3Bb1 protein per lane.

Reason for deviation, how was deviation addressed and what impact will result from this deviation:
The reason for this deviation was reassessment of purity values reported in the characterization study for corn-produced and *E. coli*-produced Cry3Bb1 proteins (Hileman et al., 2001). When the newly reported purity values were used to calculate the actual amount of Cry3Bb1 protein loaded on each gel of this study, the loadings were found to be a deviation from the protocol. A note to file was added to the study folder to describe the corrected purity values and to provide the actual gel loadings used in this study.

There was no impact on this study. The specification in the protocol for loading 500 ng per lane was intended to ensure that enough protein would be loaded to observe >90% degradation. Since the corrected gel loadings for the limit of detection gels and for the gels showing Cry3Bb1 protein digestion in SGF demonstrated that >90% degradation could be observed, the intent of the protocol was met.

Reference:
Hileman, R. E., Holleschak, G., Turner, L. A., 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 St. Louis Technical Report, MSL-17274, an internal publication of Monsanto Company.

Acknowledged By:

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Date