Study Title

Assessment of the *In Vitro* Digestibility of the Cry1Ac Protein in Simulated Gastric and Simulated Intestinal Fluids.

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Report Completed On

December 16, 2008

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Laboratory Project ID

Monsanto Study Number: REG-08-136
MSL: 0021376

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

Submitter — Date

Eddie Zhu — Dec 16, 2008

Sponsor Representative
Eddie Zhu, Ph.D. — Date

Brian Goertz — 12/16/08

Study Director
Brian E. Goertz — Date

Quality Assurance Unit Statement

Study Title: Assessment of the *In Vitro* Digestibility of the Cry1Ac Protein in Simulated Gastric and Simulated Intestinal Fluids.

Study Number: REG-08-136

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 characterization plan reported herein.

Dates of Inspection / Audit	Phase	Date Reported To:	
		Study Director	Management
3/25/2008	Digestive Fate	3/28/2008	3/28/2008
7/9/2008	Raw Data Audit	7/31/08	7/31/08
7/9/2008	Draft Report Audit	7/31/08	7/31/08

Michael J. Finkes

12/16/08

Michael J. Finkes
Quality Assurance Specialist
Monsanto Regulatory
Monsanto Company

Date

Study Certification Page

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

Signatures of Final Report Approval:

Brian E. Goertz — 12/16/08
Study Director — Date

Elena A. Rice — 12/16/2008
Protein and Emerging Technologies Lead — Date

Elena A. Rice, PhD.

Study Information

Study Number:	REG-08-136
MSL Number:	0021376
Title:	Assessment of the *In Vitro* Digestibility of the Cry1Ac Protein in Simulated Gastric and Simulated Intestinal Fluids.
Testing Facility:	Monsanto Company Product Characterization and Detection Methods Team 800 North Lindbergh Boulevard St. Louis, Missouri 63167
Team Lead:	Elena A. Rice, Ph.D.
Study Director:	Brian E. Goertz
Contributors:	Richard Thoma and Erin Bell, Ph.D.
Study Initiation Date:	March 20, 2008
Study Completion Date:	December 16, 2008
Specimen Retention:	Specimens will be retained at Monsanto Company, St. Louis, as specified in Section 7.0
Records Retention:	All study protocol specific raw data, electronically stored Atlas files, the study protocol, amendments, and final report will be retained at Monsanto Company, St. Louis.

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Abbreviations and Definitions[1]

Bt	*Bacillus thuringiensis*
CFR	Code of Federal Regulations
DF	Dilution Factor
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
GLP	Good Laboratory Practice
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
ILSI	International Life Science Institute
LB	Laemmli buffer [62.5mM Tris-HCl, 5% (v/v) 2-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate, 0.005% (w/v) bromophenol blue, 10% (v/v) glycerol, pH 6.8]
5× LB	Five times concentrated 1× LB
LOD	Limit of detection
MSL	Monsanto Technical Report
NFDM	Non-fat dried milk
PBST	Phosphate buffered saline - Tween® 20
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SOP	Standard operating procedure
T	Time
TCA	Trichloroacetic acid
U	Units
U.S.	United States

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

1.0 Summary

Monsanto Company has developed insect-protected soybean MON 87701 that produces the Cry1Ac insecticidal crystal (Cry) protein (δ-endotoxin) derived from *Bacillus thuringiensis* (*B.t.)* var. *kurstaki*. The Cry1Ac protein provides protection from feeding damage caused by targeted lepidopteran pests.

The purpose of this study was to assess the *in vitro* digestibility of the Cry1Ac protein from *Bacillus thuringiensis* in simulated gastric fluid (SGF) containing a proteolytic enzyme called pepsin and in simulated intestinal fluid (SIF) containing a mixture of proteolytic enzymes called pancreatin. The digestibility of the Cry1Ac protein was also assessed in a sequential enzymatic digestion of the protein in SGF followed by digestion in SIF. Digestibility of the Cry1Ac protein was assessed using stained SDS-PAGE and western blot analysis.

The results of the study demonstrated that greater than 99% of the full-length Cry1Ac protein was digested in SGF within 30 s when analyzed using Colloidal Brilliant Blue G stained SDS-PAGE, and at least 95% of the full-length Cry1Ac protein was digested within 30 s when analyzed by western blot with a Cry1Ac-specific antibody. At least 95% of the full-length Cry1Ac protein was digested, as expected, to the trypsin-resistant core (~55 kDa) within 5 min during incubation in SIF alone (Figure 5A). A transiently stable protein fragment migrating at ~4 kDa was observed during SGF digestion when analyzed using a Colloidal Brilliant Blue G stained polyacrylamide gel, but neither this fragment, nor any other immunoreactive peptides were detected by western blot analysis. The identity of the ~4 kDa fragment was determined by N-terminal sequencing to be a mixture of two degradation peptides from the Cry1Ac protein. The two identified peptides matched Cry1Ac sequence starting at amino acid positions 415 and 882. When the Cry1Ac protein was subjected to the sequential enzymatic digestion, i.e. digestion in SGF followed by a short digestion in SIF, the ~4 kDa fragment degraded in less than 1 min upon exposure to SIF.

These results show that full-length Cry1Ac protein is rapidly digested in the gastro-intestinal digestive system similar to the digestive fate of other well-characterized *Bt* Cry1 proteins. Rapid digestion of the full-length Cry1Ac protein in SGF and SIF, together with rapid degradation of the small Cry1Ac fragment in SIF following SGF indicates that it is highly unlikely that the full-length Cry1Ac protein or its fragment will pose an allergenic risk to humans.

2.0 Introduction

Currently, proteins introduced into commercial food crops using biotechnology are evaluated for their safety for human and animal consumption. One aspect of this assessment is an evaluation of a protein's intrinsic sensitivity to proteolytic digestion with the enzymes of the gastrointestinal tract. One characteristic of many allergens is their ability to withstand proteolytic digestion by enzymes present in the gastrointestinal tract

(Astwood et al., 1996; Vassilopoulou et al., 2006; Moreno et al., 2005; Vieths et al., 1999). When allergens or their fragments that are resistant to digestion are presented to the intestinal immune system, they can lead to a variety of gastrointestinal and systemic manifestations of immune-mediated allergy.

A correlation between *in vitro* protein digestibility with pepsin at acidic pH in SGF and the likelihood of the protein being an allergen has been previously reported (Astwood et al., 1996), but this correlation is not absolute (Fu et al., 2002). The SGF assay was standardized based on results obtained from an international, multi-laboratory ring study (Thomas et al., 2004). This ring study showed that the results of *in vitro* pepsin digestion assays were reproducible when a standard protocol was followed. Using this protocol, the pepsin digestion assay was used to assess the susceptibility of the Cry1Ac protein to pepsin digestion *in vitro.*

To reach the intestinal mucosa, where antigen processing cells reside, protein or protein fragments must first pass through the stomach, where they are exposed to pepsin, and then through the duodenum where they are exposed to pancreatic fluid containing a mixture of enzymes called pancreatin. The digestion of a protein by pepsin in the gastric system greatly reduces the possibility that an intact protein or protein fragment will reach the absorptive epithelium of the small intestine. In instances where transient stability of the protein or protein fragment is observed in SGF, further degradation of the fragment in SIF can be evaluated to better understand the fate of the protein during digestion *in vivo*. For example, if following exposure to SGF, a stable protein or protein fragment(s) is completely digested during exposure to SIF then, the probability of the protein or protein fragment(s) reaching the epithelial cells of the small intestine would be extremely low (Moreno, 2006; McClain et al., 2007).

Digestibility of protein in SIF alone is also used as a test system to assess the digestibility of food components *in vitro* (Yagami et al., 2000; Okunuki et al., 2002). The relationship between protein allergenicity and protein stability in the *in vitro* stand-alone SIF study is limited, because the protein has not been first exposed to the acidic, denaturing conditions of the stomach, as would be the case *in vivo* (FAO/WHO, 2001).

3.0 Purpose

The purpose of this study was to assess the *in vitro* digestibility of the Cry1Ac protein in SGF, SIF, and in a sequential enzymatic digestion where the protein was first digested in SGF followed by digestion in SIF.

4.0 Materials

4.1 Test Substance

The test substance was the *Escherichia coli*-produced full-length Cry1Ac protein (historical APS lot 20-100133, current Orion lot 10000804), which was purified from the fermentation of *E. coli* transformed with plasmid pMON 107800. The construct for the *E. coli*-produced Cry1Ac was engineered to encode the same Cry1Ac protein

as that found in MON 87701, which contains four amino acids from the C-terminal end of a chloroplast transit peptide preceding the anticipated N-terminus of the protein (Bell et al., 2008).

The DNA sequence encoding the Cry1Ac protein was confirmed both prior to and following fermentation of *E. coli*. Records pertaining to the purification of this *E. coli*-produced Cry1Ac protein are archived under APS lot 20-100133. The Cry1Ac protein is stored in a -80°C freezer in a buffer containing 50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, and 1 mM benzamidine HCl.

4.2 Characterization of Test Substance

The characterization of the physicochemical and functional properties of the test substance was performed under characterization plan 20-100133 and is described in the current Certificate of Analysis under Orion lot number 10000804. The Cry1Ac protein has a total protein concentration of 1.4 mg/ml as determined by amino acid analysis, a purity of 80%, and an apparent molecular weight of 131.7 kDa as determined by SDS-PAGE. The N-terminal sequence of the *E. coli* produced full-length Cry1Ac protein was also confirmed during characterization.

5.0 Test Systems

Two test systems, SGF and SIF, were utilized independently to test stability of the full-length Cry1Ac protein and then in a sequential digestibility assay where the Cry1Ac protein was first exposed to SGF followed by exposure to SIF.

5.1 Simulated Gastric Fluid (SGF)

SGF contained the proteolytic enzyme pepsin in a buffer adjusted to an acidic pH. The SGF was prepared using a highly purified form of pepsin (catalog number P-6887, Sigma Company, St. Louis, MO) according to the current version of BR-ME-0460. The SGF was formulated so that ten units of pepsin activity per µg of the Cry1Ac protein would be present in the digestion reactions. The amount of pepsin powder used to prepare SGF was calculated from the specific activity reported on the product label. Activity was assessed using the SGF activity assay according to BR-ME-0460-02 where one unit of activity is defined as a change in $A_{280\ nm}$ of 0.001 per min at 37 °C, and is measured as trichloroacetic acid (TCA) soluble products using hemoglobin as the substrate. The SGF activity assay was used to confirm the activity of the preparation before initiating the digestions of the Cry1Ac protein. The digestion of the Cry1Ac protein was monitored by SDS-PAGE stained gels and western blot analysis using a Cry1Ac specific antibody.

5.1.1 Justification for Selection of the SGF Test System

In vitro digestion models are used widely to assess the nutritional value of ingested proteins based on their amino acid bioavailability. The correlation between protein allergenicity and protein stability in an *in vitro* pepsin digestion

assay has been previously established (Astwood et al., 1996). The pepsin digestibility assay protocol that was used in this study was standardized by the International Life Science Institute (ILSI) in a multi-laboratory test and the results demonstrated that the *in vitro* pepsin digestion assay is reproducible when a common protocol is followed (Thomas et al., 2004).

5.2 Simulated Intestinal Fluid (SIF)

SIF contained a mixture of enzymes, known as pancreatin, in a buffer adjusted to neutral pH. The SIF was prepared using pancreatin (catalog number P1500, Sigma Company, St. Louis, MO) according to the current version of BR-ME-0461. The SIF was formulated so that 55.3 µg of pancreatin powder would be present per µg of Cry1Ac protein in the digestion reactions. The activity of the SIF was confirmed using an SIF activity assay (SOP BR-ME-0461-03) prior to initiating the digestions of the Cry1Ac protein. The digestion of the Cry1Ac protein was monitored by western blot analysis using a Cry1Ac specific antibody.

5.2.1 Justification for Selection of the SIF Test System

In vitro digestion models are used widely to assess the digestibility of ingested substances. SIF is frequently used for *in vitro* studies to assess the digestibility of food components (Yagami et al., 2000; Okunuki et al., 2002).

6.0 Experimental Design

6.1 Digestibility of the Cry1Ac Protein in SGF

Digestibility of the Cry1Ac protein in SGF was evaluated over time by analyzing specimens from targeted incubation time points. A numerical code using the numbers 0 through 7 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Specimen Code(s)
0 min	SGF T0, SGF P0, SGF N0
0.5 min	SGF T1
2 min	SGF T2
5 min	SGF T3
10 min	SGF T4
20 min	SGF T5
30 min	SGF T6
60 min	SGF T7, SGF P7, SGF N7

SGF was prepared to contain approximately 2279 U/ml of pepsin activity by dilution of a stock SGF solution with SGF buffer lacking pepsin (10 mM HCl, 2 mg/ml NaCl, pH ~1.2). The digestion mixture was prepared by adding 126 µl of the solution containing Cry1Ac protein to a tube containing 774 µl of pre-heated (36.5 ºC, 10 min) SGF which corresponds to 176.4 µg of Cry1Ac protein and 1764 U of pepsin, respectively. The tube contents were mixed by vortexing then placed immediately in

a 36.6 ºC water bath. Specimens (100 µl) were removed at 0.5, 2, 5, 10, 20, 30 and 60 min (corresponding to specimen codes SGF T1 through SGF T7). Each 100 µl specimen was placed immediately in a tube containing quenching mixture, consisting of 35 µl of 0.7 M sodium carbonate buffer (0.7 M Na_2CO_3, pH 11), and 35 µl of 5× Laemmli buffer [5× LB, 312.5 mM Tris-HCl, 25% (v/v) 2-mercaptoethanol, 10% (w/v) sodium dodecyl sulfate, 0.025% (w/v) Bromophenol Blue, and 50% (v/v) glycerol, pH 6.8].

The SGF T0 specimen was prepared in a separate tube. The SGF T0 sample used 86 µl of SGF (196 U of pepsin) quenched by the addition of 35 µl of 0.7 M sodium carbonate buffer, and 35 µl of 5× LB prior to the addition of 14 µl (19.6 µg) of the Cry1Ac protein.

All specimens, after the addition of the corresponding quenching buffers, were heated to 75-100 ºC for 5-10 minutes, frozen on dry ice and stored in a –80 ºC freezer until analysis.

6.1.1 SGF Experimental Control Specimens

Experimental control specimens were prepared to determine the stability of the Cry1Ac protein in the test system buffer lacking pepsin (10 mM HCl, 2 mg/ml NaCl, pH ~1.2). The SGF P0 control was prepared in a similar manner as described in Section 6.1 for SGF T0 with an incubation time of 0 min. The SGF P7 control was prepared in a manner similar to SGF P0, except the protein and 10 mM HCl, 2 mg/ml NaCl were incubated for 60 min before quenching with carbonate buffer and 5× LB.

Experimental control specimens were also prepared to determine the stability of the test system lacking the Cry1Ac protein. Protein storage buffer (50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, 1 mM Benzamidine-HCl) was added to SGF in place of the Cry1Ac protein. The SGF N0 control was prepared in a similar manner as described in Section 6.1 for SGF T0 with an incubation time of 0 min. The SGF N7 control was also prepared in a manner similar to SGF N0, except storage buffer and SGF were incubated for 60 min before quenching with carbonate buffer and 5× LB.

All quenched specimens were heated to 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a –80 ºC freezer until analysis.

6.2 Digestibility of the Cry1Ac Protein in SGF Followed by SIF

Digestibility of the full-length Cry1Ac protein in SGF followed by SIF was evaluated over time by analyzing specimens at targeted incubation time points. The Cry1Ac protein was digested first in SGF (phase I) as described in Section 5.1 for 2 min at which point the reaction was stopped by quenching with 0.7 M sodium carbonate buffer. The SGF digested and quenched Cry1Ac was then placed in the SIF assay

(phase II) and digested as described in Section 5.2. A numerical code using the numbers 0 through 7 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Specimen Code(s)
SGF system (Phase I)	
0 min	SEQ 0min
2 min	SEQ 2min
SIF system (Phase II)	
0 min	SEQ T0, SEQ P0, SEQ N0
0.5 min	SEQ T1
2 min	SEQ T2
5 min	SEQ T3
10 min	SEQ T4
30 min	SEQ T5
1 h	SEQ T6
2 h	SEQ T7, SEQ P7, SEQ N7

For phase I, the SGF was prepared to contain approximately 2632 U/ml of pepsin activity. The digestion in SGF was prepared by adding 143 µl of the Cry1Ac protein to a tube containing 760 µl of pre-heated (36.6 ºC, 5 min) SGF, corresponding to 200 µg of Cry1Ac protein and 2000 U of pepsin, respectively. The tube contents were mixed by vortexing and immediately placed in a 36.5 ºC water bath. The tube was removed after 2 min, and the reaction was immediately quenched by adding 316 µl of 0.7 M sodium carbonate buffer. After quenching, an aliquot of 120 µl was removed for analysis, and mixed with 30 µl of 5× LB, and heated to 75-100 ºC for 5-10 min, frozen on dry ice, and designated as SEQ 2min.

For phase II (SIF digestion), 625 µl of the quenched SGF reaction mixture was added to 550 µl of pre-heated (36.0 ºC, 5 min) SIF, corresponding to 100 µg SGF digested and quenched Cry1Ac protein (based on the pre-digested concentration) and 5.5 mg of pancreatin. The tube contents were mixed by vortexing and immediately placed in a 37.5 ºC water bath. Digestion aliquots (100 µl) were removed from the tube at 1, 2, 5, 10, 30 min, 1, and 2 h (corresponding to specimen codes SEQ T1 through SEQ T7) and immediately placed in a tube containing 25 µl of 5× LB, heated to 75-100 ºC for 5-10 min, and frozen on dry ice for complete quenching.

The SEQ 0min specimen for the SGF digestion phase was prepared in a separate tube by first quenching 76 µl of SGF (200 U of pepsin) with 32 µl of sodium carbonate buffer, and 31 µl of 5× LB buffer and heating to 75-100 ºC for 5-10 min prior to the addition of 14 µl (20 µg)of the Cry1Ac protein.

The SEQ T0 specimen for the SIF digestion phase was prepared in a separate tube by first quenching 55 µl of SIF (0.55 mg) with 30 µl of 5× LB buffer and heating to 75-

100 ºC for 5-10 min prior to the addition of 63 µl (10 µg, based on the pre-digestion concentration) of the SGF digested and quenched Cry1Ac protein.

6.2.1 SGF Followed by SIF Experimental Control Specimens

Experimental control specimens for the SIF digestion phase were prepared to determine the stability of the Cry1Ac protein fragment in the SIF test system buffer lacking pancreatin enzymes (50 mM potassium phosphate monobasic, pH adjusted to 7.5 with sodium hydroxide). The SEQ P0 control was prepared in a similar manner as described in Section 6.2 for SEQ T0 with an incubation time of 0 min. The SEQ P7 control was also prepared in a similar manner, except the SGF digested and quenched Cry1Ac protein and 50 mM KH_2PO_4 were incubated for 2h before quenching with 5× LB.

Experimental control specimens were also prepared to characterize the test system (SIF) lacking the SGF digested and quenched Cry1Ac protein. Protein storage buffer (50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, 1 mM Benzamidine-HCl) was added to SIF in place of the SGF digested Cry1Ac protein. The SEQ N0 control was prepared in a similar manner as described in Section 6.2 for SEQ T0 with an incubation time of 0 min. The SEQ N7 control was also prepared in a similar manner, except storage buffer and 1× SIF were incubated for 2h before quenching with 5× LB.

All quenched specimens were heated to 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a –80 ºC freezer until analysis.

6.3 Digestibility of the Cry1Ac Protein in SIF

Digestibility of the full-length Cry1Ac protein in SIF was evaluated over time by analyzing specimens at multiple incubation time points. A numerical code using the numbers 0 through 8 was used to distinguish incubation time points as follows:

Targeted Incubation Time Point	Specimen Code
0 min	SIF T0, SIF P0, SIF N0
5 min	SIF T1
15 min	SIF T2
30 min	SIF T3
1 h	SIF T4
2 h	SIF T5
4 h	SIF T6
8 h	SIF T7
24 h	SIF T8, SIF P8, SIF N8

The digestion was prepared by adding 200 µl of the test substance to a tube containing 1.55 ml of pre-heated (36.2 ºC, 5 min) SIF, corresponding to 280 µg of Cry1Ac protein and 15.5 mg of pancreatin, respectively. The tube contents were mixed by vortexing and immediately placed in a 36.1 ºC water bath. Digestion specimens (100 µl) were removed at 5, 15, 30 min, 1, 2, 4, 8, and 24 h (corresponding to specimen time points SIF T1 through SIF T8) and immediately placed in a tube containing 25 µl of 5× LB, heated to 75-100 ºC for 5-10 min, and frozen on dry ice for complete quenching.

The SIF TO specimen was prepared in a separate tube by first quenching 110 µl of SIF (1.1 mg) with 31 µl of 5× LB buffer and heating to 75-100 ºC for 5-10 min prior to the addition of 14 µl (20 µg) of the Cry1Ac protein.

6.3.1 SIF Experimental Control Specimens

Experimental control specimens were prepared to determine the stability of the Cry1Ac protein in the test system buffer lacking pancreatin (50 mM potassium phosphate, pH 7.5). The SIF P0 control was prepared in a similar manner as described in Section 6.3 for SIF T0 with an incubation time of 0 min. The SIF P8 control was also prepared in a similar manner, except the protein and 50 mM KH_2PO_4 were incubated for 24h before quenching with 5× LB.

Experimental control specimens were also prepared to characterize the test system (SIF) lacking the Cry1Ac protein. Protein storage buffer (50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, 1 mM Benzamidine-HCl) was added to SIF in place of the Cry1Ac protein. The SIF N0 control was prepared in a similar manner as described in Section 6.3 for SIF T0 with an incubation time of 0 min. The SIF N8 control was also prepared in a similar manner, except the protein storage buffer and 1× SIF were incubated for 24h before quenching with 5× LB.

All quenched specimens were heated to 75-100 ºC for 5-10 min, frozen on dry ice, and stored in a –80 ºC freezer until analysis.

7.0 Specimen Retention

All specimens will be retained in a –80 °C freezer for one year, after which it is concluded that they will no longer afford analytical evaluation and may be discarded.

8.0 Analytical Methods

Activities of the SGF and SIF were assessed using pepsin and pancreatin activity assays respectively. The digestibility of the Cry1Ac protein in SGF, and in SGF followed by SIF, were assessed using stained SDS-PAGE and western blot analysis. The digestibility of the Cry1Ac protein in SIF was assessed using western blot analysis. The limit of detection (LOD) of the Cry1Ac protein was visually estimated from stained SDS-PAGE and western blot data. The identity of a transiently stable fragment of ~4 kDa in SGF digestion was determined by N-terminal sequencing.

8.1 SGF Activity Assay

The SGF activity assay was conducted according to the current version of the SOP BR-ME-0460 to confirm the suitability of the test system before its use with the Cry1Ac protein. The assay is based on the ability of pepsin to digest denatured hemoglobin. Undigested hemoglobin was precipitated with TCA, and the amount of soluble peptides was estimated by measuring the absorbance at 280 nm. The amount of soluble peptides is directly proportional to the amount of protease activity. One unit of pepsin activity in this assay is defined as the amount of pepsin that will produce a change in absorbance at 280 nm of 0.001 per min at pH 1.2-2.0 at 37 ± 2 ºC. The SGF solution was formulated to contain 0.03 mg of powder per ml of SGF buffer. Acceptable specific activity (units/mg pepsin powder) for the SGF was equal to the specific activity determined by the manufacturer, ±1000 units/mg.

Because digestions of the Cry1Ac protein in SGF and in SGF followed by SIF were performed on the same day, only one SGF activity assay was conducted to confirm the suitability of the SGF.

The SGF activity assay was conducted as follows: The SGF solution was diluted to 0.03 mg of solid material (pepsin) per ml of SGF [the dilution factor (DF) was 26.7]. Acidified hemoglobin [2% (w/v), 5 ml] was added to each of three replicates of the test sample and blank samples and pre-warmed at 37 ± 2 °C for 5-10 min prior to starting the reactions. Diluted SGF (1 ml) was added to each test sample and both test and blank samples were incubated at 36.9°C for an additional 10 min. The reactions were stopped by the addition of 10 ml of 5% (v/v) chilled TCA to the test and blank samples. Diluted SGF (1 ml) was then added to the blank samples. Samples were mixed and then incubated for another 5-10 min at 36.9°C. Precipitated protein was removed by filtering the test and the blank samples using 0.8 µm syringe filters. Samples of the clarified test and blank samples were read at 280 nm in a Beckman DU-650 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) The activities of pepsin were calculated using the following equation:

$$\frac{Mean\,Test_{A280nm} - Mean\,Blank_{A280nm}}{0.001 \times 10\,\text{min} \times 1ml} \times DF$$

where 0.001 is the change in the absorbance at 280 nm per min at pH 1.2-2.0 and 37 ± 2 ºC produced by one unit of pepsin activity; 10 min is the reaction time, 1 ml is the amount of SGF added to the reaction; and, DF is the dilution factor for the SGF.

8.2 SIF Activity Assay

The SIF activity assay was conducted according to the current version of BR-ME-0461 to confirm the suitability of the test system before its use with the Cry1Ac protein. One unit of pancreatin activity in this assay is defined as an increase in the absorbance at 574 nm of 0.001 per min at 37 ± 2 ºC. An acceptable specific activity for the SIF was defined as 11,000 ± 3,000 U/ml.

The assay is based on the estimation of the amount of soluble peptides present in a trichloroacetic acid (TCA) solution after pancreatin digestion of resorufin-labeled casein (Roche Molecular Biochemicals, Mannheim, Germany). Undigested resorufin-labeled casein is precipitated with TCA and the amount of soluble peptide is estimated in the supernatant by measuring the absorbance at 574 nm. The amount of soluble peptide is directly proportional to the amount of proteolytic activity.

Because digestions of Cry1Ac in SIF and in SGF followed by SIF were performed on two separate days, an SIF activity assay was conducted on the day of each digestion to confirm the suitability of the SIF.

The SIF activity assay was conducted as follows: 50 µl of 0.4% (w/v) resorufin-labeled casein and 50 µl of incubation buffer (200 mM Tris, pH 7.8, 20 mM $CaCl_2$) was added to each of three activity replicates and three blank replicates and preheated for 1 – 3 minutes at 36.9 and 37.7°C for Cry1Ac Pancreatin Activity 1 and Cry1Ac Pancreatin Activity 2, respectively. To initiate the reaction, each of the three activity replicates were incubated with 100 µl 0.05× SIF (1× SIF was diluted to 0.05× SIF before the activity assay was initiated), and the three blank replicates were incubated with 100 µl 50 mM KH_2PO_4, pH 7.5 in place of SIF for 15 min at 36.9 and 37.7°C for Cry1Ac Pancreatin Activity 1 and Cry1Ac Pancreatin Activity 2, respectively. The reaction was quenched by the addition of 480 µl of chilled 5% (v/v) TCA to activity and blank replicates. The supernatants recovered after centrifugation (400 µl) were neutralized by the addition of 600 µl assay buffer (500 mM Tris-HCl, pH 8.8), and the absorbance of the clarified activity and blank replicates was read at 574 nm using a Beckman DU-650 spectrophotometer. The activity of SIF was calculated using the following equation:

$$\frac{Mean\,Activity_{A574nm} - Mean\,Blank_{A574nm}}{0.001 \times 15\,\text{min} \times 0.1ml \times 0.05}$$

where 0.001 is the change in the absorbance at 574 nm per min at 37 ± 2 ºC produced by one unit of pancreatin activity, 15 min is the reaction time, 0.1 ml is the amount of 0.05× SIF added to the reaction, and 0.05 is the SIF dilution factor.

8.3 SDS-PAGE and Colloidal Brilliant Blue G Staining

Specimens containing 1× LB from the SGF and from SGF followed by SIF digestions of the Cry1Ac protein were separated by SDS-PAGE using pre-cast tricine 10-20% polyacrylamide gradient mini-gels and tricine running buffer (Invitrogen, Carlsbad, CA) according to the current version of BR-ME-0388. The Cry1Ac protein was loaded at 0.8 µg per lane based on pre-digestion total protein concentration. All experimental controls were loaded at the same volumes as those containing Cry1Ac protein so that they would be comparable. Prior to loading on the gels, all specimens were heated for 5 min at 95.6 ºC and 95.4 ºC for SGF and SGF followed by SIF digestions of the Cry1Ac protein, respectively. Mark 12 molecular weight markers (Invitrogen, Carlsbad, CA) were loaded to estimate the relative molecular weight of proteins and peptides visualized by staining. Electrophoresis was performed at a constant voltage of 125 V for 90 (SGF digestion) and 75 (SGF followed by SIF digestion) min. After electrophoresis, proteins were visualized by staining the gels with Colloidal Brilliant Blue G (Sigma, St. Louis, MO).

The Colloidal Brilliant Blue G staining procedure was selected because it is an effective method for detecting nanogram quantities of a protein in a gel (Neuhoff et al., 1988). After separation of the proteins, the gels were fixed in a solution containing 7% (v/v) acetic acid and 40% (v/v) methanol for 30 min and stained for ~ 16 h in 1× Brilliant Blue G-Colloidal stain solution containing 20% (v/v) methanol. The gels were destained for 30 s in 10% (v/v) acetic acid, 25% (v/v) methanol and then completely destained for ~4 h (SGF) and ~ 5 h (SGF followed by SIF) in a 25% (v/v) methanol solution. Images were captured using a Bio-Rad GS-800 densitometer (BioRad, Hercules, CA). The results of the *in vitro* digestibility of Cry1Ac in SGF and SGF followed by SIF were determined by visual examination of the stained gels.

The LOD of the Cry1Ac protein was visually estimated from the Colloidal Brilliant Blue G stained SDS-PAGE data. Various dilutions of the SGF T0 specimen were loaded onto a gel and separated by SDS-PAGE. The SDS-PAGE gel that was used to estimate the LOD was run concurrently with the gel used to assess Cry1Ac protein digestibility in SGF. The dilutions of the SGF T0 specimen were loaded at volumes that represented approximately 0.8, 0.4, 0.1, 0.05, 0.02, 0.01, 0.005, 0.0025, 0.001, and 0.0005 µg total protein per lane.

8.4 Western Blot Analysis

Specimens from the SGF, SGF followed by SIF, and SIF digestions of the Cry1Ac protein were separated by SDS-PAGE using pre-cast tricine 10-20% polyacrylamide gradient mini-gels with tricine running buffer. The protein amount loaded in each lane was based on pre-digestion concentrations of the Cry1Ac protein. The digestion

samples were diluted with 1× LB to a concentration of ~2 ng/µl, and ~10 ng of the Cry1Ac protein digestion specimens were loaded in each lane. The experimental controls were loaded in as amounts equal to the digestion specimens. All specimens from the SGF, SIF, and SGF followed by SIF digestions were heated to 95.9, 95.7 and 95.4 °C for 3, 3, and 5 min respectively, prior to loading on the gels. Electrophoresis was performed at a constant voltage of 125 V for 90, 90, and 75 min on specimens from SGF, SIF and SGF followed by SIF digestions, respectively. After electrophoresis, the proteins were electrotransferred onto nitrocellulose membranes with a pore size of 0.45 µm (Invitrogen, Carlsbad, CA) for 60 min at a constant current of 300 mA. Prestained molecular weight markers (Precision Plus Dual color Protein Standards, Bio-Rad, Hercules, CA) were used to verify electrotransfer of the proteins to the membranes.

Proteins transferred to nitrocellulose membranes were analyzed by western blot. The membranes were blocked for ~16 h at ~4 ºC with 5% (w/v) non-fat dry milk (NFDM) in a phosphate buffered saline - Tween® 20 (PBST) buffer. All subsequent incubations were performed at room temperature. Goat anti–Cry1Ac affinity purified antibody (lot 10000963) was incubated with the membranes for 60 min at a dilution of 1:500 in 1% (w/v) NFDM in PBST. Excess antibody was removed by three 5 min washes with PBST. The membranes were incubated with HRP–conjugated rabbit anti-goat IgG (lot G819862-B) at a dilution of 1:10,000 in 1% (w/v) NFDM in PBST for 90 min (only 60 min for the SGF followed by SIF digestion), and washed three times for 5 min (10 min for the SGF followed by SIF digestion) with PBST. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ) and exposed to Hyperfilm ECL high performance chemiluminescence film (GE Healthcare, Piscataway, NJ). Films were developed using a Konica SRX101A automated film processor (Konica, Tokyo, Japan). The films were scanned using a Bio-Rad GS-800 densitometer to produce electronic images to be used as figures for reporting purposes.

The approximate molecular weights of the proteins observed on the western blot were visually estimated relative to the positions of the molecular weight markers.

The LOD for the western blot analysis procedure was estimated for the Cry1Ac protein by loading various dilutions of the SGF and SIF zero time point (SGF T0 and SIF T0, respectively) digestion specimens on separate gels. These gels were run concurrently with the SGF and SIF digestion western blot gels, respectively, and subjected to the same western blot procedure as described above. The following approximate total protein loadings (based upon the pre-digestion Cry1Ac concentration) of the SGF T0 and SIF T0 specimens were used for the western blot LOD analysis: 10, 5, 2.5, 1, 0.5, 0.2, 0.1, 0.05, and 0.025 ng per lane.

8.5 N-Terminal Sequencing

N-terminal sequencing by Edman degradation was used to determine the N-terminal sequence of the transiently stable fragment from the SGF digestion with apparent molecular weight of ~4 kDa.

8.5.1 Protein Blot for N-Terminal Sequence Analysis

The specimen SGF T1 was used to further characterize the transiently stable fragment of ~4 kDa. This specimen corresponds to the 30 s digestion of the Cry1Ac protein which provided a sufficient amount of the fragment for sequencing.

The SGF T1 specimen was heated at 95.6 °C for 3 min, and loaded in triplicate at 3.6 µg per lane onto a tricine 10-20% polyacrylamide gradient 10-well gel. Precision Plus pre-stained molecular weight markers were loaded in parallel to verify electrotransfer of the protein to the membrane and estimate the size of the stained bands observed. Electrophoresis was performed at a constant voltage of 125 V for 90 min. Electrotransfer to a 0.45 µm PVDF membrane (Invitrogen, Carlsbad, CA) was performed for 60 min at a constant current of 300 mA. The blot was stained with Coomassie Blue R-250 stain (Bio-Rad, Hercules, CA) and then destained for $\geq$ 5 min with Coomassie Blue R-250 destain (Bio-Rad, Hercules, CA) to visualize the markers and the stable fragments. The blot was scanned using a Bio-Rad GS-800 densitometer to produce an electronic image.

8.5.2 N-Terminal Sequencing

The band corresponding to the transiently stable fragment of ~4 kDa was excised from the blot. N-terminal sequence analysis was performed according to the current version of BR-EQ-0265 for 15 cycles using automated Edman degradation chemistry (Hunkapillar et al., 1983). An Applied Biosystems 494 Procise Sequencing System with 140C Microgradient system and 785 Programmable Absorbance Detector and Procise™ Control Software (version 1.1a) were used. Chromatographic data were collected using Atlas^{99} software (version 3.59a, LabSystems, Altrincham, Cheshire, England). A PTH-amino acid standard mixture (Applied Biosystems, Foster City, CA) was used to chromatographically calibrate the instrument for the analysis. This mixture served to verify system suitability criteria such as percent peak resolution and relative amino acid chromatographic retention times. A control protein (10 picomole β-lactoglobulin, Applied Biosystems, Foster City, CA) was analyzed before and after the analysis of the two protein bands to verify that the sequencer met performance criteria for repetitive yield and sequence identity. Identity of the fragment was established by comparing identified amino acids to the expected Cry1Ac protein sequence (Arackal et al., 2008).

9.0 Control of Bias

Measures taken to control bias in this study were the inclusion of experiments that tested the stability of the Cry1Ac without proteolytic enzymes and that tested the stability of test systems (i.e. proteolytic enzymes) to account for any effects due to the digestibility model in the absence of the pepsin and pancreatin enzymes or the absence of the test substance. Digestion specimens and LOD samples were analyzed concurrently to eliminate run-to-run variation.

10.0 Rejected Data

Two sets of western blots, which included Cry1Ac protein digestion in SGF and its LOD estimation as well as digestion in SIF and its LOD estimation, were rejected because the detected signal was too weak to accurately estimate an LOD. The protocol requires that the "digestion specimens and lower limit of detection samples will be analyzed concurrently to eliminate run-to-run variation" (Protocol Section 8.0, Control of Bias), therefore the Cry1Ac SGF and SIF digestion western blots were also rejected.

11.0 Results and Discussion

11.1 Pepsin Activities in SGF

To assess the suitability of the SGF test system used in this study, pepsin activity was evaluated before conducting the digestions of the Cry1Ac protein in SGF and in SGF followed by SIF. The experimentally observed pepsin activity was 2773 units activity per mg of pepsin powder. The SGF preparation was within the acceptable interval of pepsin activity, 2280 to 4280 units per mg pepsin powder. Therefore, the test system was shown to be suitable for use in this study.

11.2 Pancreatin Activity in SIF

To assess the suitability of the SIF test system used in this study, pancreatin activities in SIF were evaluated before use. An SIF preparation was made each day for digestion of the Cry1Ac protein in SIF alone and digestion of the Cry1Ac in SGF followed by SIF. The experimentally observed pancreatin activities were 10,500 and 11,800 U/ml for the SIF and SGF followed by SIF, respectively, which were within the acceptable interval of SIF activity (8,000 to 14,000 U/ml of SIF). Therefore, the test systems were shown to be suitable for use in this study.

11.3 Digestibility of the Cry1Ac Protein in SGF

11.3.1 Assessment of the Cry1Ac Protein Digestibility in SGF by Colloidal Brilliant Blue G Gel Staining of SDS-PAGE

The digestibility of the Cry1Ac protein in SGF was assessed by SDS-PAGE separation of the specimens and visual analysis of Colloidal Brilliant Blue G stained SDS-PAGE data(Figure 1). The SDS-PAGE for the digestibility assessment (Figure 1, panel A) was run concurrently with a separate SDS-PAGE to estimate the LOD of the Cry1Ac protein (Figure 1, panel B). The LOD of the

full-length (131.7 kDa) Cry1Ac protein was visually estimated to be 0.0025 µg. The LOD estimated for the Cry1Ac protein was used to calculate the maximum amount of Cry1Ac protein that could remain visually undetected after digestion, which corresponded to approximately 0.31% of the total protein loaded:

$$\frac{Estimated\ LOD \times 100\%}{Protein\ loaded\ \ per\ lane} \cong \%\ of\ \ protein\ remaining$$

$$\frac{0.0025\,\mu g \times 100\%}{0.8\,\mu g} \cong 0.31\%$$

The gel used to assess the digestibility of the Cry1Ac protein in SGF (Figure 1, panel A) was loaded with ~0.8 µg of total protein (based on pre-digestion protein concentrations) for each of the digestion time specimens. Visual examination of SDS-PAGE data showed that the full-length Cry1Ac protein was digested within 30 s of incubation in SGF (Figure 1A, lane 5). Therefore, based on the LOD, more than 99.69% (100% - 0.31% = 99.69%) of the full-length Cry1Ac protein was digested within 30 s of incubation in SGF. A protein fragment of ~4 kDa was observed throughout the digestion (Figure 1A, lanes 5-11), but appears to degrade to a smaller ~3.5 kDa fragment visible at the 30 min and 60 min time points on the raw data.

No change in the full-length Cry1Ac protein band intensity was observed in the absence of pepsin in the controls SGF P0 and SGF P7 (Figure 1A, lanes 3 and 12) indicating that the digestion of the Cry1Ac protein was due to the proteolytic activity of pepsin present in SGF and not due to instability of the protein while incubated at pH ~1.2 at ~37 °C for 60 min.

The controls SGF N0 and SGF N7 (Figure 1A, lanes 2 and 13), which evaluate the stability of the pepsin in the test system (SGF) lacking the Cry1Ac protein demonstrated that the pepsin was observed as a stained protein band at ~38 kDa throughout the experimental phase.

11.3.2 Assessment of the Cry1Ac Protein Digestibility in SGF by Western Blot Analysis

The digestibility of the Cry1Ac protein in SGF was also evaluated by western blotting (Figure 2). The western blot used to assess the stability of the Cry1Ac protein to pepsin digestion (Figure 2, panel A) was run concurrently with a western blot to estimate the LOD of the Cry1Ac protein (Figure 2, panel B). The LOD of the full-length Cry1Ac protein was visually estimated to be 0.5 ng. The LOD estimated for the Cry1Ac protein was used to calculate the maximum amount of Cry1Ac protein that could remain visually undetected after digestion, which corresponded to approximately 5% of the total protein loaded:

$$\frac{Estimated\ LOD \times 100\%}{Protein\ loaded\ \ per\ lane} \cong \%\ of\ \ protein\ remaining$$

$$\frac{0.5\,ng \times 100\%}{10\,ng} = 5\%$$

The gel used to assess the Cry1Ac protein *in vitro* digestibility by western blot was loaded with 10 ng per lane of total protein (based on pre-digestion concentrations) for each of the digestion time points. Western blot analysis demonstrated that the Cry1Ac protein was digested below the LOD within 30 s of incubation in SGF (Figure 2A, lane 5). Based on the western blot LOD for the Cry1Ac protein, the conclusion was that more than 95% $(100\% - 5\% = 95\%)$ of the Cry1Ac protein was digested within 30 s. No bands were detected in the lanes corresponding to the different digestion points (Figure 2A, lanes 5-11) indicating that the Cry1Ac-specific antibody does not recognize the ~4 kDa fragment observed in the stained gel.

No change in the full-length Cry1Ac protein band intensity was observed in the absence of pepsin in the controls SGF P0 and P7 (Figure 2A, lanes 3 and 12). This result reaffirms that the test substance was stable in the test system without pepsin.

No immunoreactive bands were observed in controls SGF N0 and SGF N7 (Figure 2, lanes 2 and 13). This result indicates that non-specific interactions between the test system components and the Cry1Ac-specific antibody did not occur under these experimental conditions.

11.4 N-Terminal Sequencing for Identification of the Transiently Stable SGF Fragment

N-terminal sequencing was performed to identify the transiently stable ~4 kDa fragment visible during the SGF digestion of the Cry1Ac protein. Two sequences were identified as matches to internal Cry1Ac fragments starting at amino acid positions 415 and 882 (Figure 3). This indicates that the transiently stable fragment was derived from the Cry1Ac protein.

11.5 Digestibility of the Cry1Ac Protein in SGF Followed by SIF

To better understand the fate of the Cry1Ac protein during gastrointestinal digestion, the protein was sequentially digested in SGF followed by digestion in SIF.

11.5.1 Assessment of the Cry1Ac Protein Digestibility in SGF Followed by SIF by Colloidal Brilliant Blue G Gel Staining of SDS-PAGE

The digestibility of Cry1Ac in SGF followed by SIF was assessed by visual analysis of Colloidal Brilliant Blue G stained SDS-PAGE and western blot data. The gel used to assess the stability of the Cry1Ac protein to pepsin followed by pancreatin was loaded with ~0.8 µg total Cry1Ac protein (based on pre-digestion concentrations) per specimen. After the SGF digestion phase, the stained SDS-PAGE data revealed, as expected, that the full-length Cry1Ac protein was undetectable at the 2 min time point, while the ~4 kDa fragment was visible (Figure 4A, lane 3). After subsequent exposure to SIF, the ~4 kDa fragment was not visible at the SEQ T1 (1 min) time point (Figure 4A, lane 7). This result clearly indicates that the ~4 kDa fragment, that was transiently stable in SGF, digested rapidly upon exposure to SIF.

No change in the ~4 kDa fragment intensity was observed in the absence of pancreatin in controls SEQ P0 and SEQ P7 (Figure 4A, lanes 5 and 14). This result indicates that digestion of the ~4 kDa fragment was due to the proteolytic activity of pancreatin present in SIF and not due to the instability of the fragment at ~37 °C over the course of the experiment.

The controls SEQ N0 and SEQ N7 (Figure 4A, lanes 4 and 15) demonstrated the range of pancreatin integrity in the test system over the course of the experiment. The intensity of some pancreatin bands decreased during the course of the experiment, most likely due to test system autodigestion. This is not expected to adversely impact the SIF results as the ~4 kDa transiently stable fragment was no longer visible by 1 minute, whereas the pancreatin in the SEQ N7 positive control sample had been exposed to the test system for two hours.

11.5.2 Assessment of the Cry1Ac Protein Digestibility in SGF Followed by SIF by Western Blot Analysis

The digestibility of Cry1Ac protein in SGF followed by SIF was also assessed by western blot (Figure 4B) using ~10 ng total protein (based on pre-digestion concentrations) for each of the incubation time points. Visual examination of the western blot showed that, as expected, the only band observed was the full-length Cry1Ac at the SEQ 0min time point in the SGF phase (Figure 4B, lane 2).

No proteolytic fragments were observed in controls SEQ P0 and SEQ P7 (Figure 4B, lanes 5 and 14). The controls, SEQ N0 and SEQ N7 (Figure 4B, lanes 4 and 15), demonstrated the absence of non-specific Cry1Ac antibody interactions with the SIF test system during the SIF digestion phase of the experiment.

11.6 Digestibility of the Cry1Ac protein in SIF

11.6.1 Assessment of Cry1Ac digestibility in SIF by Western Blot Analysis

The digestibility of the full-length Cry1Ac protein in SIF was assessed by western blot (Figure 5). The western blot used to assess the *in vitro* digestibility of the Cry1Ac protein in SIF (Figure 5, panel A) was run concurrently with the western blot used to estimate the LOD (Figure 5, panel B) of the Cry1Ac protein. The LOD was visually estimated to be 0.5 ng. The LOD estimated for the Cry1Ac protein was used to calculate the maximum amount of Cry1Ac protein that could remain visually undetected after digestion, which corresponded to approximately 5% of the total protein loaded:

$$\frac{Estimated\ LOD \times 100\%}{Protein\ loaded\ \ per\ lane} \cong \%\ of\ \ protein\ remaining$$

$$\frac{0.5\ ng \times 100\%}{10\ ng} = 5\%$$

The gel used to assess digestibility of the Cry1Ac protein *in vitro* by western blot was loaded with 10 ng total protein (based on pre-digestion Cry1Ac concentrations) for each of the incubation time points. Western blot analysis demonstrated that a band corresponding to the full-length Cry1Ac protein was digested below the LOD within 5 min of incubation in SIF (Figure 5A, lane 5). Therefore, based on the LOD, more than 95% (100% - 5% = 95%) of the full-length Cry1Ac protein was digested within 5 min. The Cry1Ac protein was digested to the trypsin-resistant core (~55 kDa), which was stable throughout the SIF digestion (Figure 5A, lanes 5-12).

No change in the full-length Cry1Ac protein band intensity was observed in controls SIF P0 and SIF P9 which represents the test system without pancreatin (Figure 5A, lanes 3 and 13). This result indicates that the Cry1Ac protein was stable in the test system without pancreatin at ~37 ºC over the course of the experiment.

No immunoreactive bands were observed in controls SIF N0 and SIF N9, which represent the SIF test system without Cry1Ac protein (Figure 5A, lanes 2 and 14). This result demonstrates the absence of non-specific antibody interactions with the SIF test system during the digestion.

The digestion of Cry1Ac protein in SIF was not evaluated using stained gel because the complexity of the pancreatin test system makes it difficult to resolve proteolytic fragments. The Cry1Ac trypsin-resistant core was shown by western blot analysis (Figure 5A) to be stable throughout digestion in SIF. Additionally,

the digestion of Cry1Ac in SGF followed by SIF demonstrated the transiently stable fragment of ~ 4 kDa was completely digested within 1 min of incubation in SIF.

12.0 Conclusions

Digestibility of the Cry1Ac protein was evaluated in SGF and SIF. The results of the study demonstrate that greater than 99% of the full-length Cry1Ac protein was digested in SGF within 30 s when the Colloidal Brilliant Blue G stained SDS-PAGE data was visually analyzed and at least 95% of the full-length Cry1Ac protein was digested within 30 s when analyzed by western blot with using a Cry1Ac specific antibody. At least 95% of the full-length Cry1Ac protein was digested, as expected, to the trypsin-resistant core (~55 kDa) within 5 min during incubation in SIF alone. A transiently stable protein fragment migrating at ~4 kDa was observed at the 30 s time point in SGF when analyzed by a Colloidal Brilliant Blue G stained polyacrylamide gel, but neither this fragment nor any other immunoreactive peptides were detected using western blot analysis. The identity of the ~4 kDa fragment was determined by N-terminal sequencing to be a mixture of two degradation peptides from the Cry1Ac protein. The identified peptides matched Cry1Ac sequence starting at amino acid positions 415 and 882. When the Cry1Ac protein was subjected to the sequential enzymatic digestion, i.e. digestion in SGF followed by short exposure to SIF, the fragment that was stable during SGF digestion was digested in less than 1 min by SIF.

These results show that the full-length Cry1Ac protein in MON 87701 is rapidly digested in the model gastro-intestinal digestive system similar to the digestive fate of other well-characterized *Bt* Cry1 proteins. Rapid digestion of the full-length Cry1Ac protein in SGF and SIF, together with rapid degradation of the small transiently stable Cry1Ac fragments in SIF indicates that the Cry1Ac protein and its fragments are highly unlikely to pose an allergenic risk to human health.

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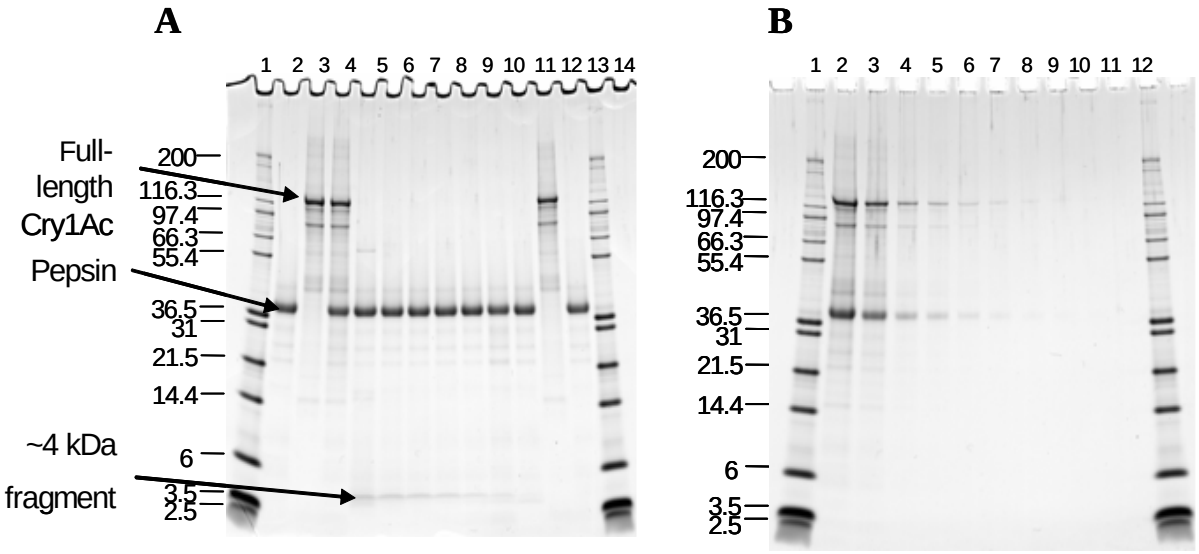

Figure 1. SDS-PAGE Analysis of the Digestion of Cry1Ac Protein in SGF

Colloidal Brilliant Blue G stained SDS-PAGE data were used to assess the digestibility of Cry1Ac in SGF. **Panel A** corresponds to Cry1Ac protein digestion in SGF. Based on pre-digestion protein concentrations, 0.8 µg of total protein was loaded in each lane containing Cry1Ac protein. **Panel B** corresponds to the various amounts of SGF T0 loaded to estimate the LOD of the Cry1Ac protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, Cry1Ac protein migrated to approximately 131.7 kDa and pepsin to approximately 38 kDa (indicated by arrows on the left). Blank and empty lanes were cropped and lanes renumbered.

A

Lane	Sample	Incubation Time (min)
1	Mark 12 MWM	—
2	SGF N0	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Mark 12 MWM	—

B

Lane	Sample	Amount (µg)
1	Mark 12 MWM	—
2	T0, protein+SGF	0.8
3	T0, protein+SGF	0.4
4	T0, protein+SGF	0.1
5	T0, protein+SGF	0.05
6	T0, protein+SGF	0.02
7	T0, protein+SGF	0.01
8	T0, protein+SGF	0.005
9	T0, protein+SGF	0.0025
10	T0, protein+SGF	0.001
11	T0, protein+SGF	0.0005
12	Mark 12 MWM	—

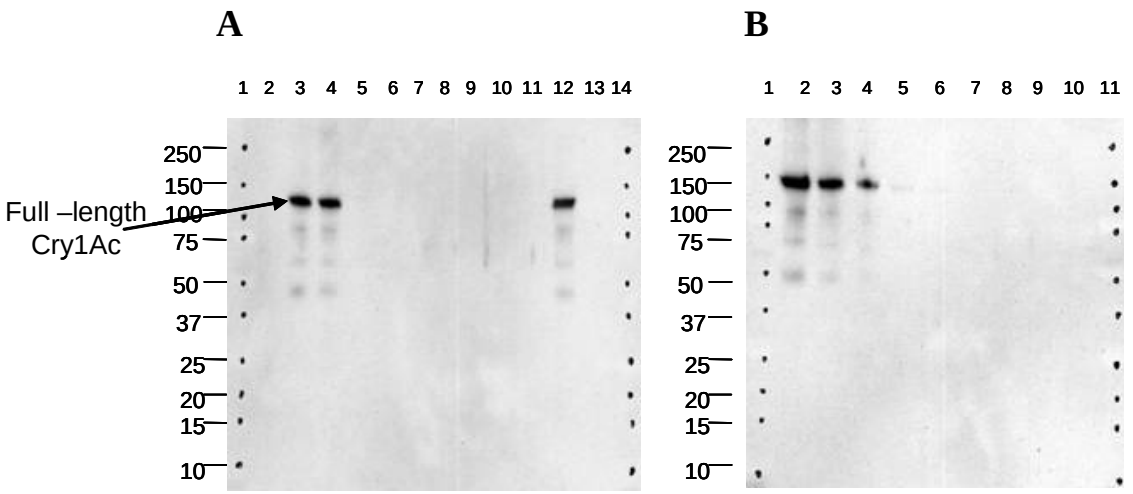

Figure 2. Western Blot Analysis of the Digestion of Cry1Ac protein in SGF

Panel A corresponds to a western blot of the Cry1Ac protein digested in SGF. Based on pre-digestion protein concentrations, 10 ng (total protein) was loaded in the lanes containing Cry1Ac protein. **Panel B** corresponds to the various amounts of SGF T0 loaded to estimate the LOD of the Cry1Ac protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, Cry1Ac protein migrated to approximately 131.7 kDa. A 5 min exposure is shown. Blank or empty lanes are cropped and lanes re-numbered.

A

Lane	Sample	Incubation Time (min)
1	Precision Plus MWM	—
2	SGF N0	0
3	SGF P0	0
4	SGF T0	0
5	SGF T1	0.5
6	SGF T2	2
7	SGF T3	5
8	SGF T4	10
9	SGF T5	20
10	SGF T6	30
11	SGF T7	60
12	SGF P7	60
13	SGF N7	60
14	Precision Plus MWM	—

B

Lane	Sample	Amount (ng)
1	Precision Plus MWM	—
2	T0, protein+SGF	10
3	T0, protein+SGF	5
4	T0, protein+SGF	2.5
5	T0, protein+SGF	1
6	T0, protein+SGF	0.5
7	T0, protein+SGF	0.2
8	T0, protein+SGF	0.1
9	T0, protein+SGF	0.05
10	T0, protein+SGF	0.025
11	Precision Plus MWM	—

A

Amino acid residue # from the N-terminus →	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Predicted Cry1Ac Sequence[1] (position 882) →	A	R	V	K	R	A	E	K	K	W	R	D	K	R	E
	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Observed Sequence→	A	R	V	K	R	A	E	K	K	(W)	R	D	K	X	E

B

Amino acid residue # from the N-terminus →	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Predicted Cry1Ac Sequence[1] (position 415) →	D	E	I	P	P	Q	N	N	N	V
	|	|	|	|	|	|	|	|	|	|
Observed Sequence→	D	E	I	P	P	Q	N	N	N	(V)

Figure 3. N-Terminal Sequencing of ~4 kDa Fragment

Two sequences were obtained and compared to the expected sequence of Cry1Ac. **Panel A** corresponds to the first sequence obtained (position 882) from the SGF T1 specimen. **Panel B** corresponds to the second sequence obtained (postion 415). The predicted amino acid sequence of the Cry1Ac protein was deduced from the coding region of the full-length *Cry1Ac* gene (Arackal et al., 2008). The "X" refers to an undesignated amino acid and ( ) denotes a tenuously designated amino acid. [1]The single letter IUPAC-IUB amino acid codes are: A, alanine; R, arginine; V, valine; E, glutamic acid; K, lysine; W, tryptophan; N, asparagine; D, aspartic acid; I, isoleucine; P, proline; and Q, glutamine.

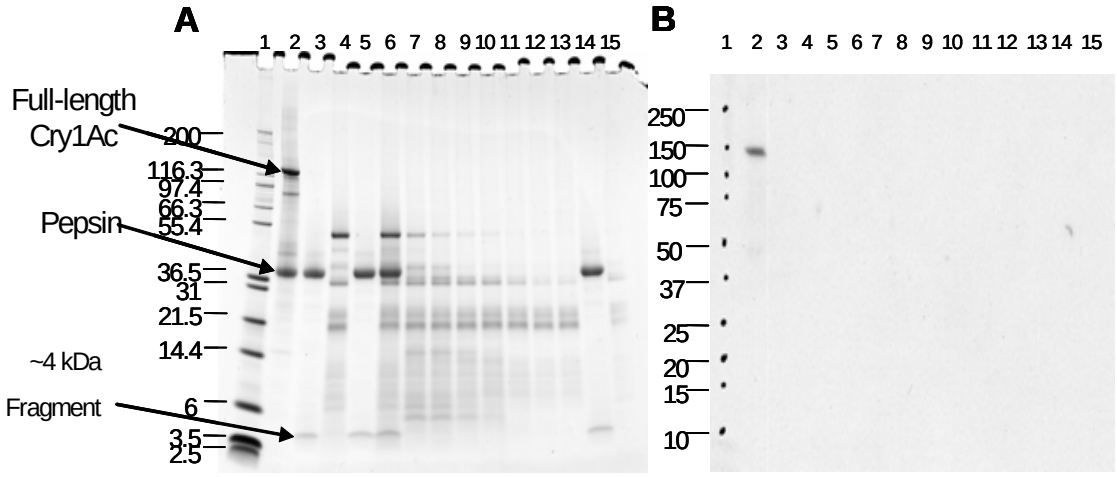

Figure 4. SDS-PAGE and Western Blot Analysis of the Digestion of Cry1Ac Protein in SGF Followed by SIF

Panel A corresponds to the colloidal stained SDS-PAGE data of the Cry1Ac protein digested in SGF followed by SIF. Based on pre-digestion protein concentrations, 0.8 µg of total protein was loaded per lane containing Cry1Ac protein. **Panel B** corresponds to the western blot of the Cry1Ac protein digested in SGF followed by SIF. Based on pre-digestion Cry1Ac protein concentrations, 10 ng of total protein was loaded per lane containing Cry1Ac protein. In both gels, Cry1Ac protein migrated to approximately 131.7 kDa. A 5 min exposure is shown.

A

Lane	Sample	Incubation Time
1	Mark 12 MWM	—
2	SEQ 0min	0
3	SEQ 2min	2 min
4	SEQ N0	0
5	SEQ P0	0
6	SEQ T0	0
7	SEQ T1	1min
8	SEQ T2	2 min
9	SEQ T3	5 min
10	SEQ T4	10 min
11	SEQ T5	30 min
12	SEQ T6	1 h
13	SEQ T7	2 h
14	SEQ P7	2 h
15	SEQ N7	2 h

B

Lane	Sample	Incubation Time
1	Mark 12 MWM	—
2	SEQ 0min	0
3	SEQ 2min	2 min
4	SEQ N0	0
5	SEQ P0	0
6	SEQ T0	0
7	SEQ T1	1 min
8	SEQ T2	2 min
9	SEQ T3	5 min
10	SEQ T4	10 min
11	SEQ T5	30 min
12	SEQ T6	1 h
13	SEQ T7	2 h
14	SEQ P7	2 h
15	SEQ N7	2 h

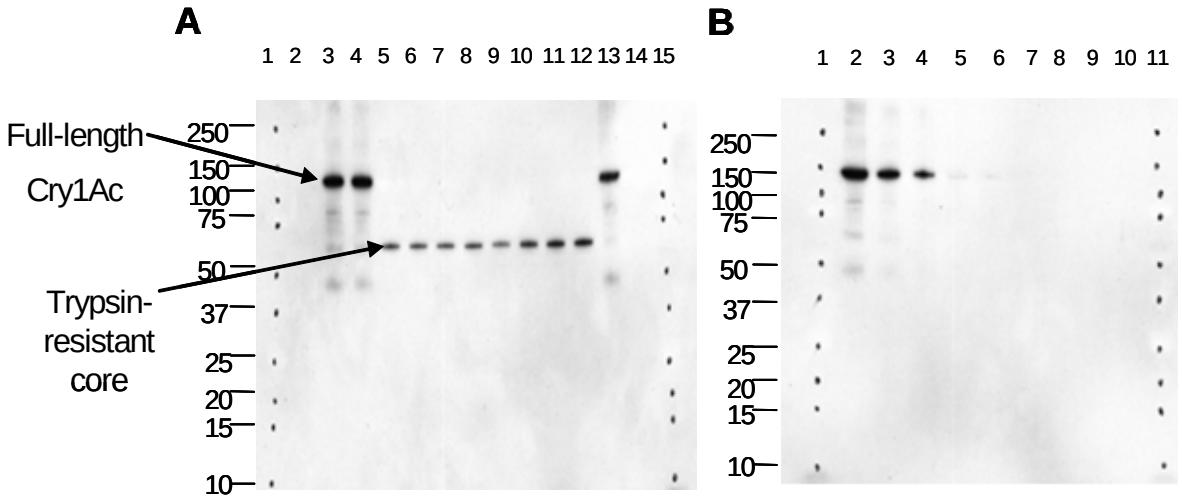

Figure 5. Western Blot Analysis of the Digestion of Cry1Ac protein in SIF

Panel A corresponds to the western blot analysis of the Cry1Ac protein digested in SIF. Based on pre-digestion protein concentrations, 10 ng (total protein) was loaded in the lanes containing Cry1Ac protein. **Panel B** corresponds to the various amounts of SIF T0 loaded to estimate the LOD of the Cry1Ac protein.corresponds to the limit of detection of Cry1Ac protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, Cry1Ac protein migrated to approximately 131.7 kDa. A 5 min exposure is shown. Blank and empty lanes are cropped and lanes re-numbered.

A

Lane	Sample	Incubation Time
1	Precision Plus MWM	—
2	SIF N0	0
3	SIF P0	0
4	SIF T0	0
5	SIF T1	5 min
6	SIF T2	15 min
7	SIF T3	30 min
8	SIF T4	1 h
9	SIF T5	2 h
10	SIF T6	4 h
11	SIF T7	8 h
12	SIF T8	24 h
13	SIF P8	24 h
14	SIF N8	24 h
15	Precision Plus MWM	—

B

Lane	Sample	Amount (ng)
1	Precision Plus MWM	—
2	T0, protein+SIF	10
3	T0, protein+SIF	5
4	T0, protein+SIF	2.5
5	T0, protein+SIF	1
6	T0, protein+SIF	0.5
7	T0, protein+SIF	0.2
8	T0, protein+SIF	0.1
9	T0, protein+SIF	0.05
10	T0, protein+SIF	0.025
11	Precision Plus MWM	—

Appendix 1. List of Applicable SOPs

SOP Number	Title
BR-ME-0460-02	Preparation of Simulated Gastric Fluid and Assay of the Proteolytic Activity
BR-ME-0461-03	Preparation of Simulated Intestinal Fluid and Assay of the Proteolytic Activity
BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-ME-0924-01	Electrotransfer of Proteins to Membranes
BR-ME-0392-01	Western Blot Analysis (Immunoblotting)
BR-EQ-0935-01	Konica SRX X-Ray Film Processors
BR-ME-0926-01	Staining of Proteins on Blot Membranes
BR-EQ-0265-02	Applied Biosystems 494 Procise™ Protein Sequencing System
BR-EQ-0599-04	Bio-rad GS-800 Densitometer System