

**Assessment of the in vitro Digestibility of the Cry1A.105 proteins in
simulated Gastric Fluid**

**Study No.
MSL-19929**

Title

**Assessment of the *in vitro* Digestibility of the Cry1A.105 Protein in Simulated
Gastric Fluid**

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Study Completed On

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Performing Laboratory

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Quality Assurance Statement

Study Title: Assessment of the *in vitro* Digestibility of the Cry1A.105 Protein in Simulated Gastric Fluid

Study Number: 05-01-62-02

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

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

| Dates of Inspection / Audit | Phase | Date Reported To: | |
|--------------------------------|--------------------|-------------------|------------|
| | | Study Director | Management |
| 08/04/2005 | Digestive Fate | 08/04/2005 | 08/04/2005 |
| 07/27/2005 | Western Blot | 08/04/2005 | 08/04/2005 |
| 10/19/2005 | Raw Data Audit | 10/25/2005 | 10/25/2005 |
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Quality Assurance Unit
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November 2, 2005
Date

Study Information Page

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Study Certification Page

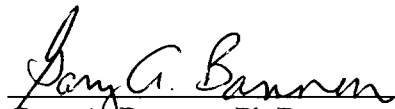
The results reported in this Final Report accurately reflect the data generated under study number 05-01-62-02.

Approved By:



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Abbreviations¹

| | |
|------------------|---|
| CEW | Corn earworm |
| CFR | Code of Federal Regulations |
| DF | Dilution factor |
| EC ₅₀ | Effective protein concentration to inhibit the growth of the target insect by 50% |
| ECL | Enhanced chemiluminescence |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| EPA | Environmental Protection Agency |
| FIFRA | Federal Insecticide, Fungicide, and Rodenticide Act |
| HRP | Horseradish peroxidase |
| IgG | Immunoglobulin G |
| LOD | Limit of Detection |
| ILSI | International Life Science Institute |
| LB | Laemmli buffer |
| MALDI-TOF MS | Matrix assisted laser desorption ionization – time of flight mass spectrometry |
| NFDM | Non-fat dry milk |
| PBST | Phosphate buffered saline containing Tween-20 |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SGF | Simulated gastric fluid |
| SOP | Standard operating procedure |
| T | Time |
| TCA | Trichloroacetic acid |
| US | United States |
| 1 × 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 |

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 has developed plants that produce the Cry1A.105 insecticidal protein and are protected from feeding damage caused by European corn borer (*Ostrinia nubilalis*) and other lepidopteran insect pests. Cry1A.105 is a modified *Bacillus thuringiensis* Cry1A protein with 93.6 % overall amino acid sequence identity to the Cry1Ac protein.

The purpose of this study was to assess the *in vitro* digestibility of the Cry1A.105 protein in simulated gastric fluid (SGF) containing the proteolytic enzyme pepsin. The Cry1A.105 protein used in this study was produced in and purified from *E. coli*. The *E. coli*-produced Cry1A.105 protein was characterized prior to the digestibility study. Digestibility of the Cry1A.105 protein in SGF was assessed using stained SDS-polyacrylamide gel and western blot analysis.

The results of this study demonstrated that the full-length Cry1A.105 protein was rapidly digested during incubation in SGF. At least 99.3% of the full-length Cry1A.105 protein was digested within 30 seconds when analyzed using Colloidal Brilliant Blue G stained polyacrylamide gels. Greater than 95% of the Cry1A.105 protein was digested in SGF within 30 seconds when specimens were analyzed using western blot. A faint ~4.5 kDa band was observed between the 30-second and 20-minute digestion time points when analyzed using a Colloidal Brilliant Blue G stained polyacrylamide gel. Neither this band, nor any other immunoreactive band was detected using western blot analysis.

2.0 Introduction

Monsanto has developed plants that produce the Cry1A.105 insecticidal protein and are protected from feeding damage caused by European corn borer (*Ostrinia nubilalis*) and other lepidopteran insect pests. Cry1A.105 is a modified *Bacillus thuringiensis* Cry1A protein with 93.6 % overall amino acid sequence identity to the Cry1Ac protein.

Currently, proteins introduced into commercial food crops through the techniques of biotechnology are evaluated for safety, including an assessment of the potential to be allergenic. One aspect of this assessment includes analysis of the digestibility of the target protein in an SGF assay containing pepsin. The correlation between protein allergenicity and protein stability in an *in vitro* pepsin digestion assay has been previously established (Astwood et al., 1996). Proteins that are highly digestible are expected to be less likely to cause sensitization or allergic reaction when consumed. Recently, the International Life Science Institute (ILSI) standardized the pepsin digestibility assay protocol in a multi-laboratory evaluation (Thomas et al., 2004). The SGF formulation, time course, and experimental parameters used in this study followed the conditions used in the ILSI multi-laboratory evaluation.

3.0 Purpose

The purpose of this study was to assess the stability of the Cry1A.105 protein in simulated gastric fluid containing the proteolytic enzyme pepsin.

4.0 Materials

4.1 Test Substance

The test substance was the Cry1A.105 protein. The Cry1A.105 protein (Analytical Protein Standard lot 20-100073) was isolated from a fermentation batch of *E. coli* containing pMON96851 expression plasmid. This protein has been characterized and has a total protein concentration of 1.2 mg/ml and a purity of 92 %. Functional activity was confirmed using an insect bioassay with the larvae of a susceptible pest, corn earworm (CEW). The EC₅₀ value was 5.8 ng/ml of diet. Prior to its application to the test system, the test substance was stored in a -80 °C freezer in a test substance storage buffer containing 25 mM CAPS, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT, pH ~10.3.

4.2 Control Substance

There was no control substance for this study.

4.3 Reference Substance

There was no reference substance for this study. Analytical reference standards (e.g., molecular weight markers) used in this study were documented in the data and are described in this report.

4.4. Characterization of the Test Substance

The characterization of the physicochemical and functional properties of the test substance was performed under characterization plan 20-100073 and is described on the Certificate of Analysis. The following properties were established for the Cry1A.105 protein: identity (N-terminal sequencing, MALDI-TOF mass spectrometry (MS), immunodetection), concentration (amino acid analysis), purity (SDS-PAGE/densitometry), molecular weight (SDS-PAGE/densitometry, MALDI-TOF MS), stability (SDS-PAGE/densitometry) and activity (CEW bioassay).

5.0 Test System

The test system for this study was simulated gastric fluid (SGF) that contains the proteolytic enzyme pepsin. The SGF was prepared using a highly purified form of pepsin (Catalog number P-6887, Sigma Company, St. Louis, MO). The SGF was formulated so that ten units of pepsin activity per microgram of total protein from the test substance 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. One unit of activity is defined as a change in $A_{280\text{ nm}}$ of 0.001 per minute at 37 °C, measured as trichloroacetic acid (TCA) soluble products using hemoglobin as the substrate. The stock SGF solution was prepared by adding pepsin powder (26.6 mg) to 33.2 ml of an acidic sodium chloride solution (2 mg/ml NaCl, 10 mM HCl, pH 1.3). After the activity of pepsin in SGF was confirmed, the stock SGF solution was diluted to provide approximately 1500 units pepsin activity/ml of solution.

5.1 Justification for Selection of the Test System

In vitro digestion models are used widely to assess the nutritional value of ingested proteins based on their amino acid bioavailability. Also, the correlation between protein allergenicity and protein stability in an *in vitro* pepsin digestion assay has been previously established (Astwood et al., 1996). Recently the pepsin digestibility assay protocol was standardized by ILSI in a multi-laboratory evaluation test (Thomas et al., 2004). This multi-laboratory test showed that results of the *in vitro* pepsin digestion assay are reproducible when a common protocol is followed. The SGF formulation, time course, and experimental parameters used in this study followed the conditions used in the ILSI multi-laboratory evaluation test.

5.2 Specimens

Specimens were generated by incubating the test substance with the test system for the times specified in section 5.3. See Sections 6.0 through 7.0 for details on the preparation and analysis of specimens. Specimens will be retained in a -80 °C freezer one year, after which they will no longer afford analytical evaluation and may be discarded.

5.3 Procedure for Identification of Specimens

Alphanumerical codes were used to distinguish incubation time points: (where T = time, P = protein only, and N = no protein):

| <u>Targeted Incubation Time Point</u> | <u>Designation(s)</u> |
|---------------------------------------|-----------------------|
| 0 min | T0, P0, N0 |
| 30 sec | T1 |
| 2 min | T2 |
| 5 min | T3 |
| 10 min | T4 |
| 20 min | T5 |
| 30 min | T6 |
| 1 h | T7, P7, N7 |

6.0 Experimental Design

6.1 Digestibility of the Test Substance in SGF

Digestion of the test substance in SGF was evaluated over time by analyzing specimens from all incubation time points. The target digestion temperature was 37 ± 2 °C.

The test substance protein was diluted to 0.6 mg total protein /ml by mixing 200 µl of the protein at 1.2 mg/ml and 200 µl of storage buffer (25 mM CAPS, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT, pH 10.3) and then heated at 44.5 °C for 10 minutes. Digestion samples were prepared by adding 200 µl of the diluted test substance to a tube containing 800 µl of SGF. The tube contents were vortex mixed and immediately placed in a 37.2 °C water bath and subsequently vortex mixed every 30 - 60 sec throughout the digestion experiment. Samples (100 µl) were removed at targeted times of 0.5, 2, 5, 10, 20, 30, and 60 min (specimens T1 to T7, respectively) and placed in a tube containing quenching mixture. Quenching mixture contained 35 µl of carbonate buffer [700 mM Na₂CO₃, pH 11.0], and 35 µl of 5× Laemmli buffer (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 zero incubation time point (T0) was prepared in a separate tube. SGF (80 µl) was quenched by adding 35 µl of carbonate buffer and 35 µl of 5× LB prior to the addition of 20 µl of the diluted test substance.

All quenched samples were heated to 75-100 °C for 5-10 min, frozen on dry ice, and stored in a -80 °C freezer until analyzed.

6.2 Experimental Controls

Experimental controls were prepared to determine the stability of the test substance in the test system buffer lacking pepsin [10 mM HCl, 2 mg/ml NaCl, pH 1.3]. These experimental controls were identified with the letter "P". The zero incubation time point (P0) was prepared in a separate tube. Test system buffer (80 µl) was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× LB prior to the addition of 20 µl of the diluted test substance. The 60 min incubation time point (P7) was prepared by adding 20 µl of the diluted test substance to test system buffer lacking pepsin (80 µl). The tube was vortex mixed and immediately placed in a 37.1 °C water bath. After 60 min of incubation, the sample was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× LB.

Additional experimental controls were prepared to evaluate the stability of the pepsin in the test system (SGF) lacking the test substance and to determine if non-specific interaction occurs between the test system components and the antibodies during western blot analysis of the specimens. These experimental controls contained an aliquot of the test system incubated with test substance storage buffer instead of the test substance and were identified with the letter "N". The zero incubation time point (N0) was in a separate tube. Test system (80 µl) was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× LB prior to addition of 20 µl of the storage buffer (25 mM CAPS, pH 10.3, 1 mM benzamidine-HCl, 0.1 mM EDTA, and 0.2 mM DTT). The 60 min incubation time point (N7) was prepared by adding 20 µl storage buffer to 80 µl of test system. The tube was vortex mixed and placed in a 37.1 °C water bath. After 60 min of incubation, the sample was quenched by addition of 35 µl of carbonate buffer and 35 µl of 5× SB.

All experimental controls were heated at 75-100 °C for 5-10 min, frozen on dry ice, and stored in a -80 °C freezer until analyzed.

7.0 Analytical Methods

Activity of the SGF was assessed using a pepsin activity assay. The digestibility of the Cry1A.105 protein in SGF was assessed using stained polyacrylamide gels and western blot analysis. The limit of detection (LOD) of the Cry1A.105 protein for these methods was determined concurrently.

7.1 SGF Activity Assay

The SGF activity assay was used to confirm the suitability of the test system before its use with the test substance. Acceptable activity was defined as a pepsin activity per mg of pepsin powder (0.03 mg of powder per ml of diluted SGF) equal to the activity of pepsin per mg of pepsin powder as determined by the manufacturer (± 1000 units/mg). One unit of pepsin activity in this assay is defined as the amount of pepsin that will produce a change in the absorbance at

280 nm of 0.001 per min at pH 1.2-2.0 at 37 ± 2 °C. The assay is used to estimate the amount of soluble peptides present in a TCA solution after pepsin digestion of 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 peptide is directly proportional to the amount of protease activity.

Briefly, the SGF 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 minutes prior to starting the reactions. Diluted SGF (1 ml) was added to each replicate of test samples and both test and blank samples were incubated at 37.0 °C for an additional 10 min. The reaction was stopped by addition of 10 ml of chilled 5% (v/v) TCA to the test and blank samples. Diluted SGF (1 ml) was then added to the blank samples. Samples were mixed and then incubated another 5-10 min at 37.4 °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. The activity of pepsin was calculated using the following equation:

$$\frac{MeanTest_{A280nm} - MeanBlank_{A280nm}}{0.001 \times 10 \text{ min} \times 1 \text{ ml}} \times DF ,$$

where 0.001 is the change in the absorbance at 280 nm per min at pH 1.2-2.0 and 37 ± 2 °C 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.

7.2 SDS-PAGE

Samples containing 1× LB from the SGF *in vitro* digestion of the Cry1A.105 protein were separated by SDS-PAGE using pre-cast tricine 10-20% polyacrylamide gradient mini-gels and tricine running buffer (Invitrogen, Carlsbad, CA). The protein loaded per lane was based on the pre-digestion total protein concentration of the Cry1A.105 protein. All experimental controls were loaded at the same volume as those containing Cry1A.105 protein so that all other components would be comparable. All samples were heated at 100.3 °C for 5 min prior to loading on the gels. Protein markers were used to estimate the relative molecular weight. Electrophoresis was performed at a constant voltage of 125 V for 85 minutes. After electrophoresis, proteins were either visualized by staining the gel with colloidal Brilliant Blue G (section 7.3), or the gel was subjected to electrotransfer of proteins to nitrocellulose membrane for western blot analysis (section 7.4).

7.3 Colloidal Brilliant Blue G Staining

The colloidal Brilliant Blue G staining method was selected because it is an effective method for detecting nanogram quantities of protein on a gel (Neuhoff et al., 1988). Mark12 molecular weight markers (Invitrogen, Carlsbad, CA) were used to estimate the relative molecular weight of visualized proteins and peptides. Based on pre-digestion concentrations, approximately 0.7 µg of total protein was loaded per lane. After separation of 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 approximately 20 h in 1× Brilliant Blue G-colloidal stain solution containing 20% (v/v) methanol. The gels were briefly destained for 30 s in a 10% (v/v) acetic acid, 25% (v/v) methanol solution and completely destained for ~5 h in a 25% (v/v) methanol solution. Images were captured using a Bio-Rad GS-800 densitometer. The results of the *in vitro* digestibility of Cry1A.105 protein were determined by visual examination of the stained gels.

The approximate molecular weights of the full-size protein and proteolytic fragment observed on the colloidal Brilliant Blue G stained gels were visually determined relative to the positions of the molecular weight markers.

The LOD of the Cry1A.105 protein using the colloidal Brilliant Blue G staining procedure was determined. Various dilutions of the zero time point (T0) digestion specimen were loaded onto a separate gel that was run concurrently with the gel used to assess digestibility. Aliquots of the T0 digestion sample representing approximately 700, 350, 100, 50, 20, 10, 5, and 2.5 ng total protein were used for the stained LOD gel.

7.4 Western Blot Analysis

Specimens from the SGF *in vitro* digestions were also analyzed using western blotting. Based on pre-digestion concentrations, approximately 20 ng of total protein were loaded per lane. Following electrophoresis, pre-stained molecular weight markers (Precision Plus Protein Standards, Bio-Rad, Hercules CA) were used to verify electrotransfer of proteins to the membrane. Proteins were electrotransferred to nitrocellulose membranes (0.45 µm pore size, Invitrogen) for 90 min at a constant voltage of 25 V.

Membranes were blocked overnight in a 4 °C refrigerator with 5% (w/v) non-fat dry milk (NFDM) in phosphate buffered saline containing Tween-20 (PBST) buffer. All subsequent incubations (described below) were performed at room temperature. Membranes were incubated with rabbit anti-Cry1A.105 antibody (lot 070705JL) diluted 1:2,000 in PBST containing 1% (w/v) NFDM for 1 h. Excess serum was removed by three 10 min washes with PBST. The membrane was incubated with HRP-conjugated goat anti-rabbit IgG (Sigma) at a dilution of 1:10,000 in PBST containing 1% (w/v) NFDM for 1 h and again washed

(three 10 min washes) with PBST. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) and exposed (2, 5, and 10 minutes) to Hyperfilm ECL high performance chemiluminescence film (Amersham Biosciences). Films were developed using a Konica SRX101A automated film processor (Tokyo, Japan).

The approximate molecular weights of the full-size protein observed on the western blots were visually determined relative to the positions of the molecular weight markers.

The LOD for the Cry1A.105 protein using the western blot analysis procedure was determined. Various dilutions of the zero time point (T0) digestion specimen were loaded onto a separate gel that was run concurrently with the digestion western blot gel and subjected to the same western blot procedure as described above. Aliquots of the T0 digestion sample representing approximately 7, 3.5, 2, 1, 0.5, 0.2, 0.1, and 0.05 ng total protein were used for the western blot LOD analysis.

7.5 Statistical Methods

No statistical analysis was performed.

8.0 Control of Bias

Measures taken to control bias in this study were the inclusion of both stability and test system experimental controls to account for any effects due to the model in the absence of the pepsin enzyme and the absence of the test substance. Digestion specimens and lower limit of detection samples were analyzed concurrently to eliminate run-to-run variation.

9.0 Rejected Data

One set of data, which included determination of pepsin activity, *in vitro* digestibility of Cry1A.105 in SGF, and western blot analysis of Cry1A.105 SGF digestions was rejected because this set of data was generated before the protocol was amended to include dilution of the test substance and vortexing of the digestion tube throughout the duration of the digestion to improve exposure of the test protein to the test system. One set of SDS-PAGE gels was rejected because the band representing pepsin in the T7 specimen was not observed on the gel, which most probably was a result of a loading error. One set of western blots was rejected because a significant reduction in the amount of the full-length Cry1A.105 protein in the T0 specimen was observed. This was likely caused by a loading error.

10.0 Protocol Amendments

The protocol contained a few typographical errors, which were corrected by amendments. Section 6.1, describing digestibility of the test substance in SGF, was amended to address the possibility of test substance precipitation and/or aggregation at low pH. There was no negative impact on the study as a result of these changes.

11.0 Protocol Deviations

Throughout the experimental phase of the study, one protocol deviation occurred. The preparation of the T0 specimen and experimental controls P0, P7, N0, and N7 followed the amended procedure for the digestibility of the test substance, even though they were not specifically referenced in the amendment. This deviation improved the quality of the study data, because the T0 specimen and experimental controls were prepared in a similar manner to the study specimens.

12.0 Results and Discussion

12.1 Pepsin Activity in SGF

The pepsin activity in SGF was evaluated before conducting the digestion trials to assess the suitability of the test system used in this study. The experimentally observed activity was 2429 units per mg pepsin powder, which 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.

12.2 Assessment of the Extent of Digestion of the Cry1A.105 Protein by Pepsin using Colloidal Brilliant Blue G Gel Staining of SDS-PAGE

The extent of digestion of the Cry1A.105 protein was evaluated by visual analysis of colloidal Brilliant Blue G stained polyacrylamide gels (Figure 1). The SDS-PAGE for the digestibility assessment (Figure 1A) was run concurrently with a separate SDS-PAGE to determine the LOD of Cry1A.105 protein (Figure 1B). The limit of detection of the full-length (~130 kDa) Cry1A.105 protein was visually estimated to be 0.005 µg or approximately 1% of the total protein loaded:

$$\frac{0.005 \mu\text{g} \times 100\%}{0.7 \mu\text{g}} \cong 0.7\%$$

The gel used to assess the stability of the Cry1A.105 protein to pepsin (Figure 1A) was loaded with ~0.7 µg (based on pre-digestion concentrations) for each of the digestion time points. Visual examination of the stained gel showed that the full-length (~130 kDa) Cry1A.105 protein was digested below LOD within 30 seconds of digestion in SGF (Figure 1A, lane 5). Therefore, at least 99.3% (100% – 0.7% = 99.3%) of the full-length Cry1A.105 protein was digested within 30 seconds of incubation of Cry1A.105 protein in SGF based on the Colloidal Brilliant Blue G Gel stained SDS-PAGE analysis. A faint band with a molecular weight of approximately 4.5 kDa was observed at a very low level

between the 30-second and 20-minute digestion time points (Figure 1A, lanes 5-9). No protein band was visible at the 30-minute digestion time point (Figure 1A, lane 10).

No change in the full-length Cry1A.105 protein band intensity was observed in the absence of pepsin in the experimental controls P0 and P7 (Figure 1A, lanes 3 and 12). This indicates that digestion of the Cry1A.105 protein was due to the proteolytic activity of pepsin present in SGF and not due to the instability of the test substance at pH 1.3 and 37°C.

The experimental controls evaluating the stability of the pepsin in the test system (SGF) lacking the test substance demonstrated that pepsin was observed as the stained protein band at ~38 kDa throughout the experimental phase (Figure 1A, lanes 2 and 13). The amount of pepsin slightly decreased between 30 and 60 min of the digestion, most probably due to enzyme auto-digestion.

12.3 Assessment of the Extent of Digestion of the Cry1A.105 Protein by Pepsin using Western Blot Analysis

The extent of digestion of the Cry1A.105 protein was also evaluated by a western blot method (Figure 2). The western blot used to assess the stability of the Cry1A.105 protein to pepsin digestion (Figure 2A) was run concurrently with a western blot to determine the LOD of Cry1A.105 protein (Figure 2B). The LOD of full-length (~130 kDa) Cry1A.105 protein was visually estimated to be 1 ng or 5 % of the total protein loaded:

$$\frac{1\text{ ng} \times 100\%}{20\text{ ng}} = 5\%$$

The gel used to assess the Cry1A.105 protein *in vitro* digestibility by western blot was loaded with 20 ng total protein of the test substance (based on pre-digestion concentrations) for each of the digestion time points. Western blot analysis demonstrated that the Cry1A.105 protein was digested below the LOD within 30 seconds of incubation in SGF (Figure 2A, lane 5). Based on the western blot LOD for the Cry1A.105 protein in SGF and the observation that no full-length protein or immunoreactive bands were observed on the western blot at the 30-second digestion time point, it was concluded that at least 95% (100% – 5% = 95%) of the full-length Cry1A.105 protein was digested within 30 seconds.

No change in the full-length Cry1A.105 protein band intensity was observed in the absence of pepsin in the experimental controls P0 and P7 (Figure 2A, lanes 3 and 12). This indicates that the test substance was stable in the test system without pepsin at pH 1.3 and ~37 °C over the course of the experiment.

No immunoreactive bands were observed in specimens N0 and N7 that represent test system experimental controls (Figure 2A, lanes 2 and 13). This indicates that

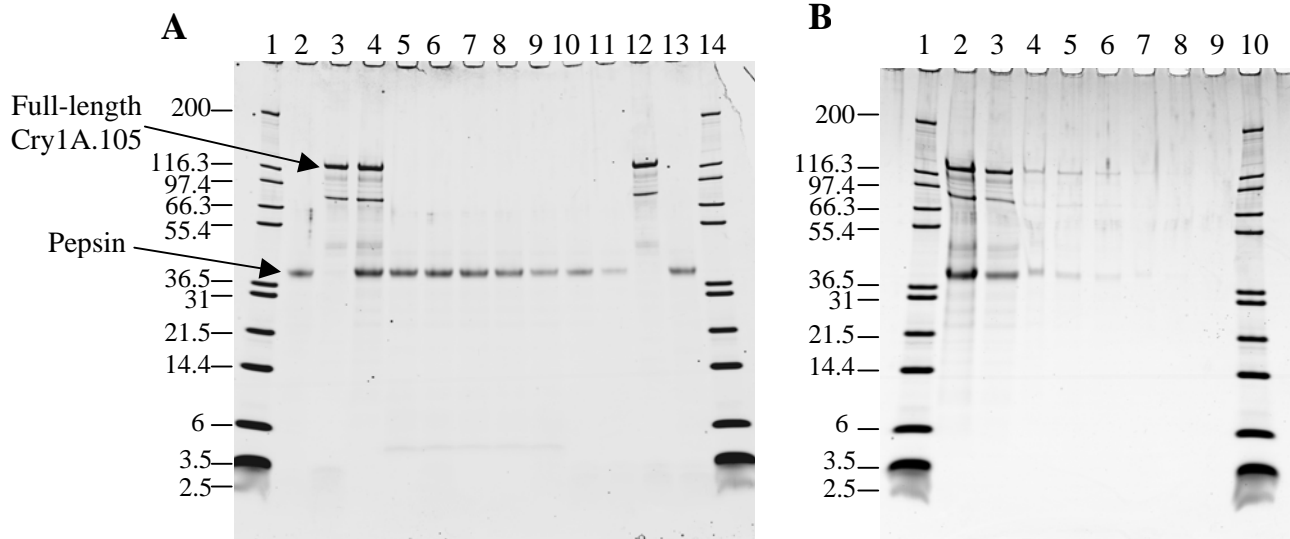
non-specific interactions between the test system components and the antibodies were not observed under these experimental conditions.

13.0 Conclusions

The results of this study demonstrated that the full-length Cry1A.105 protein was rapidly digested after incubation in SGF. At least 99.3% of the full-length Cry1A.105 protein was digested within 30 seconds when analyzed using Colloidal Brilliant Blue G stained polyacrylamide gels. Greater than 95% of the Cry1A.105 protein was digested in SGF within 30 seconds when specimens were analyzed using western blot analysis. A faint ~4.5 kDa band was observed between the 30-second and 20-minute digestion time points when analyzed using Colloidal Brilliant Blue G stained polyacrylamide gels. Neither this band, nor any other immunoreactive band was detected when using western blot analysis.

14.0 References

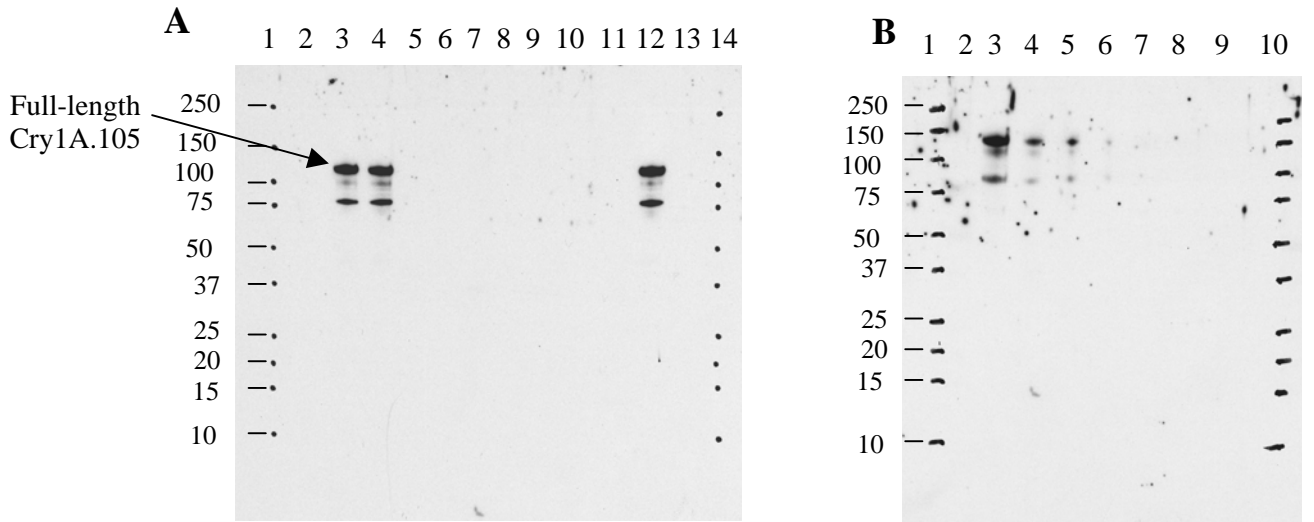
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| Lane | Sample | Incubation Time (min) | Lane | Sample | Amount (µg) |
|------|-------------------------|-----------------------|------|-------------------------|-------------|
| 1 | Molecular weight marker | — | 1 | Molecular weight marker | — |
| 2 | N0, SGF only | 0 | 2 | T0, protein+SGF | 0.7 |
| 3 | P0, protein only | 0 | 3 | T0, protein+SGF | 0.35 |
| 4 | T0, protein+SGF | 0 | 4 | T0, protein+SGF | 0.1 |
| 5 | T1, protein+SGF | 0.5 | 5 | T0, protein+SGF | 0.05 |
| 6 | T2, protein+SGF | 2 | 6 | T0, protein+SGF | 0.02 |
| 7 | T3, protein+SGF | 5 | 7 | T0, protein+SGF | 0.01 |
| 8 | T4, protein+SGF | 10 | 8 | T0, protein+SGF | 0.005 |
| 9 | T5, protein+SGF | 20 | 9 | T0, protein+SGF | 0.0025 |
| 10 | T6, protein+SGF | 30 | 10 | Molecular weight marker | — |
| 11 | T7, protein+SGF | 60 | | | |
| 12 | P7, protein only | 60 | | | |
| 13 | N7, SGF only | 60 | | | |
| 14 | Molecular weight marker | — | | | |

Figure 1. Colloidal Brilliant Blue G stained SDS-polyacrylamide gels

Panel A corresponds to Cry1A.105 protein digestion in SGF. Based on pre-digestion protein concentrations, ~0.7 µg (total protein) was loaded in lanes containing Cry1A.105 protein. The incubation times are indicated. Panel B corresponds to the limit of detection of Cry1A.105 protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, Cry1A.105 protein migrated to approximately 130 kDa and pepsin to approximately 38 kDa (indicated by the arrows on the left). Blank or empty lanes were cropped and lanes renumbered.



| Lane | Sample | Incubation Time (min) | Lane | Sample | Amount (ng) |
|------|-------------------------|-----------------------|------|-------------------------|-------------|
| 1 | Molecular weight marker | — | 1 | Molecular weight marker | — |
| 2 | N0, SGF only | 0 | 2 | T0, protein+SGF | 7 |
| 3 | P0, protein only | 0 | 3 | T0, protein+SGF | 3.5 |
| 4 | T0, protein+SGF | 0 | 4 | T0, protein+SGF | 2 |
| 5 | T1, protein+SGF | 0.5 | 5 | T0, protein+SGF | 1 |
| 6 | T2, protein+SGF | 2 | 6 | T0, protein+SGF | 0.5 |
| 7 | T3, protein+SGF | 5 | 7 | T0, protein+SGF | 0.2 |
| 8 | T4, protein+SGF | 10 | 8 | T0, protein+SGF | 0.1 |
| 9 | T5, protein+SGF | 20 | 9 | T0, protein+SGF | 0.05 |
| 10 | T6, protein+SGF | 30 | 10 | Molecular weight marker | — |
| 11 | T7, protein+SGF | 60 | | | |
| 12 | P7, protein only | 60 | | | |
| 13 | N7, SGF only | 60 | | | |
| 14 | Molecular weight marker | — | | | |

Figure 2. Western blot analysis

Panel **A** corresponds to Cry1A.105 protein digestion in SGF. Based on pre-digestion protein concentrations, 20 ng (total protein) was loaded in lanes containing Cry1A.105 protein. The incubation times are indicated. Panel **B** corresponds to the limit of detection of the Cry1A.105 protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in each gel. In both gels, Cry1A.105 migrated to approximately 130 kDa. A 10 min exposure is shown. Blank or empty lanes were cropped and lanes renumbered.

Appendix. List of Applicable SOPs

| | |
|---------------|---|
| BR-ME-0388-02 | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| BR-ME-0392-01 | Western Blot Analysis (Immunoblotting) |
| BR-ME-0460-02 | Assay for Pepsin Activity in Simulated Gastric Fluid |
| BR-ME-0527-01 | Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels |
| BR-ME-0924-01 | Electrotransfer of Proteins to Membranes |
| BR-ME-0973-01 | Drying of Polyacrylamide Mini Gels Using Invitrogen Gel Drying System (Adaptation of Invitrogen Gel Drying Procedure) |
| BR-EQ-0599-02 | Bio-Rad GS-710 and GS-800 Densitometers |
| BR-EQ-0857-01 | Beckman Coulter DU-650 Spectrophotometer |