STUDY TITLE

In Vitro Simulated Gastric Fluid Digestibility Study of Truncated Cry1F Delta-endotoxin Derived from *Pseudomonas fluorescens*

DATA REQUIREMENTS

NA

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry1F δ-endotoxin

Title: *In Vitro* Simulated Gastric Fluid Digestibility Study of Truncated Cry1F Delta-endotoxin Derived from *Pseudomonas fluorescens*

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

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*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

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This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

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FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
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At the time this study was conducted, it was not subject to Good Laboratory Practice Standards, but was, nevertheless, conducted in accordance with existing and proposed standards.

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ABBREVIATIONS

AI	active ingredient
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BME	2-mercaptoethanol
BSA	bovine serum albumin
CAPS	3-cyclohexylamino-1-propane sulfonic acid
CBB	coomassie brilliant blue
kDa	kiloDalton
MW	molecular weight
PAb	polyclonal antibody
PBST	phosphate buffered saline (10mM phosphate buffer, 138 mM NaCl, 2.7 mM KCl) with 0.05% Tween 20, pH 7.4
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
TSN	Dow AgroSciences Test Substance Number

In Vitro Simulated Gastric Fluid Digestibility Study of Truncated Cry1F Delta-endotoxin Derived from *Pseudomonas fluorescens*

ABSTRACT

Corn plants have been genetically modified through the introduction of a synthetic gene which encodes for a truncated version of an insecticidal protein (Cry1Fa2, commonly referred to as Cry1F) isolated from *Bacillus thuringiensis* var. *aizawai* strain PS811. This protein when expressed in corn cultivars provides crop resistance against lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*). The purpose of this study was to evaluate the digestibility of the truncated Cry1F protein in a simulated gastric fluid (SGF) model. The test and control substances were incubated with SGF for specific time intervals and then analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

INTRODUCTION

The purpose of this study was to evaluate the digestibility of truncated Cry1F protein in simulated gastric fluid. The protein used as a test substance in this study was produced with a bacterium *Pseudomonas fluorescens* (*Pf*). The microbially derived Cry1F was shown to be biochemically and insecticidally equivalent to the Cry1F protein produced in transgenic TC1507 corn plants (2, 3). Bovine serum albumin (BSA) and β-lactoglobulin were included in the experimental design of this study for comparative purposes. BSA was used as a positive control for the experiment since it is known to degrade readily in SGF, and β-lactoglobulin was used as a poorly digestible control since it is known to persist in SGF. The test and control proteins were incubated with SGF for specific time intervals and were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The biochemical and immunological methods employed in this study are standard techniques for protein analysis. SDS-PAGE separates proteins based on the apparent molecular weight (mass). Western blotting of proteins to a nitrocellulose membrane following SDS-PAGE and immunodetection with a protein specific antibody is widely used to identify the authenticity of a molecule in a crude preparation.

EXPERIMENTAL

Test Substances

The test substance used in this study is listed in the following table:

Test Substance	ID Number	% AI (w/w)	Reference
Cry1F	TSN101788	13.7%	BIOT 013056 (1)

AI: active ingredient

The recombinant truncated Cry1F protein was produced in *P. fluorescens* strain MR872, which was prepared at the Dow AgroSciences facility in San Diego, California. The Cry1F protein was expressed in the bacterial cells as inclusion bodies and after fermentation the inclusion bodies were extracted and washed. The truncated Cry1F was prepared by trypsinolysis of the full length Cry1F protein. The Dow AgroSciences (DAS) test substance number for this lot of the truncated Cry1F protein is TSN 101788. The Cry1F protein was 13.7% of the total solid weight of the lyophilized powder (1). It should be noted that in this lyophilized preparation, the purity of the Cry1F protein was quite high (>80%) in terms of the percentage of Cry1F protein to total protein as determined by SDS-PAGE analysis (1, 2). In the lyophilized powder, besides proteins (Cry1F, its low molecular weight fragments and other proteins), there were also non-proteinaceous substances such as buffering chemicals and salts that were present in the liquid formulation before freeze-drying. The non-proteinaceous substances account for major portion of the weight in the lyophilized powder. This microbially derived Cry1F protein has been demonstrated to be equivalent to the transgenic corn-produced Cry1F in other studies (2, 3).

Control Substances

The positive and negative control substances used in this study are listed in the following table:

Control Substance	Purity	Reference	ID Number
Bovine serum albumin (BSA)	≥ 96%	Sigma Catalog #A9418	Lot 118H0595
β-lactoglobulin	≥ 90%	Sigma Catalog #L7880	Lot 70K7049

Reference Substances

The reference substances used in this study are listed in the following table:

Reference Substance	Product Name	Lot Number	Assay	Reference
Molecular Weight Markers	Bio-Rad Kaleidoscope Prestained Standards	90032	Biochemical/ Western Blotting	Bio-Rad Cat# 161-0324 MW Markers of 213, 128, 85, 42.6, 31.2, 18, and 8.5 kDa
Molecular Weight Markers	BenchMark Protein Ladder	1097741	Biochemical/ SDS-PAGE	GibcoBRL Cat# 10747-012, MW Markers of 220, 160, 120, 100, 90, 80, 70, 60, 50, 40, 30, 25, 20, 15, and 10 kDa

Reference substances were chosen by the procedure used.

Test Methods

Equimolar (~0.074 mM) solutions of the test and control substances were prepared as follows. TSN101788 (Cry1F) was reconstituted by weighing 35.6 mg of lyophilized powder in a 4-mL test tube and adding 1 mL of 10 mM CAPS, pH 11.0. BSA was reconstituted by weighing 24.8 mg of powder in a 15-mL centrifuge tube and adding 5 mL of 3.0 mM NaCl, pH 1.2. β-lactoglobulin was reconstituted by weighing 13.6 mg of powder and adding 5 mL of 3.0 mM NaCl, pH 1.2. Simulated gastric fluid (SGF) (pH ~1.2) containing approximately 0.3% (w/v) pepsin (Sigma Aldrich, St. Louis, MO) was prepared as described in the United States Pharmacopeia (4).

The digestions were performed for time intervals of 0, 15 and 30 seconds, and for 1, 2, 5, 10 and 15 minutes in a water bath set to 37 °C. The three proteins, Cry1F, BSA, and β-lactoglobulin were digested as follows. Three 1.5-mL microcentrifuge tubes, each containing a 285-µL aliquot of SGF, were placed in the 37 °C water bath. After 5 minutes, 73.2 µg (15 µL) of Cry1F, 74.4 µg (15 µL) of BSA, and 40.8 µg (15 µL) of β-lactoglobulin, respectively, were added to the tubes and a timer was set. After each specified incubation interval, 20 µL of the reaction mixture was removed and added to tubes containing stop solution (8 µL sodium carbonate). The stopped reactions were then placed on ice until all of the time points were sampled for the three proteins. An SGF reagent blank was prepared by substituting water for the sample protein and incubating for 0 and 15 minutes at 37 °C. For each of the proteins above, a zero time point (undigested control) was prepared as follows. First, an aliquot of SGF was stopped with sodium carbonate and the respective protein was added to the solution. The digested proteins and their undigested 'controls' were stored in a freezer overnight.

The samples (each protein at each time point) were mixed with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), containing freshly added β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA). Single 10-20% polyacrylamide gels (Zaxis Inc., Hudson, OH) of BSA and β-lactoglobulin and duplicate gels of Cry1F were set up and loaded as described in the following table.

Protein	Volume of sample loaded per lane for SDS-PAGE analysis	Amount of protein (before digestion) loaded per lane for SDS-PAGE analysis	Volume of sample loaded per lane for Western blot analysis	Amount of protein (before digestion) loaded per lane for Western blot analysis
BSA	25 µL	~1.77 µg	N/A	N/A
β-lactoglobulin	25 µL	~0.97 µg	N/A	N/A
Cry1F	10 µL	~0.58 µg	10 µL	~ 0.12 µg

The samples were then electrophoresed at a constant amperage of 30 mA per gel for 60 minutes. The electrophoresis buffer was Tris/Glycine/SDS buffer from Bio-Rad. After separation, three of the gels were stained with Pierce Gel Code Blue Stain. Proteins on the remaining Cry1F gel

were electro-blotted to a nitrocellulose membrane using a Bio-Rad Criterion Blotter under a constant voltage of 100 v. Following protein transfer, the membrane was blocked with PBST and 5% powdered milk. Polyclonal antibody specific to Cry1F (Strategic Diagnostics Incorporated, Newark, NJ, lot #200.310-4) was then added to the blot to allow for protein detection. A conjugate of goat anti-rabbit IgG (H+L)- alkaline phosphatase (Pierce, Rockford, IL) was used as the secondary antibody. A substrate solution containing 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 0.025% 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and 0.05% p-nitroblue tetrazolium chloride (NBT) was used for colorimetric development of the immunoreactive protein bands.

RESULTS

The positive and negative controls, BSA and β-lactoglobulin, respectively, responded as expected (Table 1). BSA was not detected at the 15 second time point when subjected to the simulated gastric environment (Figure 2, lane 3). β-lactoglobulin remained readily detectable for 15 minutes (the duration of the experiment) (Figure 1, lane 10). The test protein (Cry1F and its minor degradation fragments) were not detectable at 15 seconds as demonstrated by both SDS-PAGE (Figure 3, Panel B, lane 4) and Western blot analysis (Figure 3, Panel A, lane 4).

CONCLUSION

Truncated microbial Cry1F protein is readily digested by pepsin (<15 seconds) under simulated gastric conditions (pH 1.2) as demonstrated by both SDS-PAGE and Western blot analysis.

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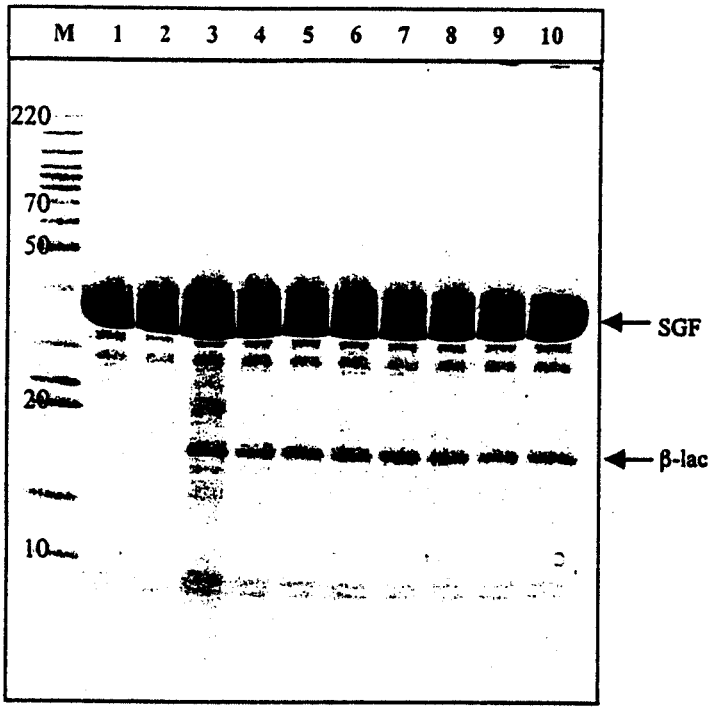
Table 1. Results of the *In Vitro* Digestibility Study of Truncated, Microbial Cry1F Protein in Simulated Gastric Fluid

Protein	Digestion time demonstrated by SDS-PAGE analysis	Digestion time demonstrated by Western blot analysis
BSA	<15 seconds	N/A
Cry1F	<15 seconds	<15 seconds
β-lactoglobulin	>15 minutes	N/A



Lane Assignments for β-lactoglobulin SDS-PAGE Gel

M - Benchmark Protein Ladder

1 - SGF Reagent Blank, 0 minute incubation

2 - SGF Reagent Blank, 15 minute incubation

3 - β-lactoglobulin (0.97 μg), 0 minute digestion

4 - 15 second digestion

5 - 30 second digestion

6 - 1 minute digestion

7 - 2 minute digestion

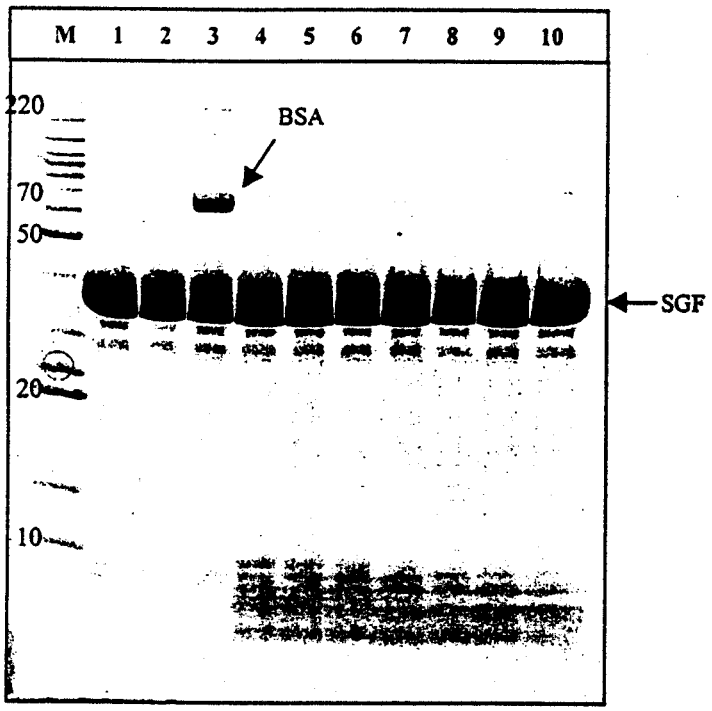
8 - 5 minute digestion

9 - 10 minute digestion

10 - 15 minute digestion

* Note MW markers are labeled in kDa.

Figure 4. SDS-PAGE Analysis of β-lactoglobulin Protein Subjected to Digestion in Simulated Gastric Fluid



Lane Assignments for Bovine Serum Albumin SDS-PAGE Gel

M - Benchmark Protein Ladder

1 - SGF Reagent Blank, 0 minute incubation

2 - SGF Reagent Blank, 15 minute incubation

3 - Bovine Serum Albumin (1.77 μg), 0 minute digestion

4 - 15 second digestion

5 - 30 second digestion

6 - 1 minute digestion

7 - 2 minute digestion

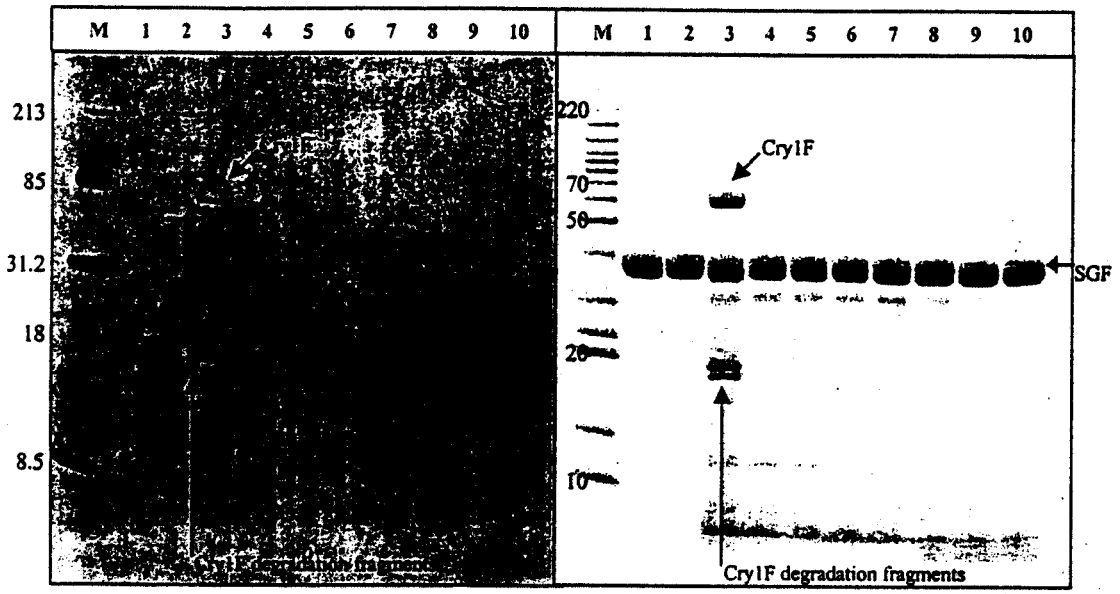
8 - 5 minute digestion

9 - 10 minute digestion

10 - 15 minute digestion

* Note MW markers are labeled in kDa.

Figure 5. SDS-PAGE Analysis of Bovine Serum Albumin Protein Subjected to Digestion in Simulated Gastric Fluid



Panel A: Cry1F Western Blot

Lane Assignments

M - Bio-Rad Prestained Standards

1 - SGF Reagent Blank, 0 minute incubation

2 - SGF Reagent Blank, 15 minute incubation

3 - Cry1F (0.12 μg), 0 minute digestion

4 - 15 second digestion

5 - 30 second digestion

6 - 1 minute digestion

7 - 2 minute digestion

8 - 5 minute digestion

9 - 10 minute digestion

10 - 15 minute digestion

Panel B: Cry1F SDS-PAGE Gel

Lane Assignments

M - Benchmark Protein Ladder

1 - SGF Reagent Blank, 0 minute incubation

2 - SGF Reagent Blank, 15 minute incubation

3 - Cry1F (0.58 μg), 0 minute digestion

4 - 15 second digestion

5 - 30 second digestion

6 - 1 minute digestion

7 - 2 minute digestion

8 - 5 minute digestion

9 - 10 minute digestion

10 - 15 minute digestion

* Note MW markers are labeled in kDa.

Figure 6. Western Blot and SDS-PAGE Analysis of Truncated, Microbial Cry1F Protein Subjected to Digestion in Simulated Gastric Fluid