

ADVISORY COMMITTEE ON NOVEL FOODS AND PROCESSES

FOOD USE OF GM MAIZE LINE 1507: ADDITIONAL INFORMATION

Issue

This paper presents additional information provided by the applicant regarding the proteins present in GM maize line 1507, in response to a request from the Committee. Members are invited to consider whether they have any outstanding concerns on this aspect of the dossier.

Background

1. At the March meeting the Committee considered the response of the applicant to specific questions raised by the Committee regarding the food use of the GM maize line 1507 (ACNFP/71/8). Although the Committee were otherwise satisfied by the applicant's response, Members requested sight of a document (Evans, 1998) referred to by the applicant in relation to the size of the Cry1F protein doublet on the Western blot. The document mentioned is an internal report concerning the equivalency of microbial and maize expressed Cry1F protein.

2. Relevant extracts of the report containing data relating to the molecular weight of the maize derived protein is attached as Annex 1. The full report, which is mainly concerned with demonstrating the equivalence of the plant and microbial derived forms of Cry1F and their suitability for biochemical and toxicological studies, is available on request (Annex 2). The applicant has highlighted pages 26, 87, 94 and 95 (Figs. 2 and 3) of the report regarding the expected size of the protein.

Note: Appendix 1 of the report entitled: "Qualitative analysis and comparison of Cry1F from maize and bacterial origin", refers to the Cry1F protein from 1507 maize by a more specific nomenclature, Cry1Fa2

3. As part of a separate study to compare the recombinant Cry1F proteins of microbial and transgenic maize origin (Annex 3) the applicant has used matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI - TOF MS) to look at peptide mass fingerprints of the two proteins. After immunoaffinity purification the positive fractions were separated by SDS-PAGE and the respective bands excised and subjected to in gel digestion by trypsin (Annex 3, p14). The resulting peptide mixtures were then analysed by MALDI -TOF MS and the mass of the detected peptides compared to those deduced from the potential cleavage sites

in the Cry1F protein sequence. The detected peptide fragments from maize derived Cry1F were matched with peptides from residues 32 to 546 of the microbial protein (Annex 3, p15).

4. The applicant has also carried out N-terminal sequence analysis of the plant derived Cry1F protein (Annex 1, pages 27, 88, 89 and 98 – Fig. 5) and obtained a 5 amino acid sequence that corresponds to the expected N-terminus of proteolytically cleaved Cry1F. This is reported to match the expected N-terminus following cleavage by a trypsin like activity at residue 28 in the microbial Cry1F protein (page 27).

5. The applicant has also undertaken an immunoblot analysis to determine if there was any apparent post-translational modification of maize derived Cry1F protein involving carbohydrate residues (Annex 1, pages 27, 87, 88 and 97 - Figure 4B). Such residues can alter the apparent molecular weight of a protein in SDS-PAGE. The results suggest that the Cry1F protein is not glycosylated.

Committee Action Required

6. The Committee is asked to consider the information provided by the applicant and to advise whether they have any outstanding concerns over this aspect of the 1507 maize dossier.

Secretariat

April 2005

Annexes attached:

Annex 1 – Extracts from the report by Evans (1998) comprising: title page (p 1), table of contents (p 7-8), summary (p 10-12), objectives (p 14-17), methods (p 21-25) and the relevant results (p 26-27 and Appendix I, p 79-98).

Annex 3 – Study entitled: Characterisation of the Recombinant Cry1F protein Derived from *Pseudomonas fluorescens* and Transgenic Maize (Schafer and Schwedler, 2001)

Annexes available on request:

Annex 2 – Final Report of study entitled: Equivalency of Microbial and Maize Expressed Cry1F Protein; Characterisation of Test Substances for Biochemical and Toxicological Studies (Evans, 1998)

ADVISORY COMMITTEE ON NOVEL FOODS AND PROCESSES

Extracts from the report by Evans (1998) comprising: title page (p 1), table of contents (p 7-8), summary (p 10-12), objectives (p 14-17), methods (p 21-25) and the relevant results (p 26-27 and Appendix I, p 79-98).

This document is available on request from the ACNFP Secretariat:

acnfp@foodstandards.gsi.gov.uk

**Secretariat
May 2005**

ADVISORY COMMITTEE ON NOVEL FOODS AND PROCESSES

Study entitled: Characterisation of the Recombinant Cry1F protein Derived from *Pseudomonas fluorescens* and Transgenic Maize (Schafer and Schwedler, 2001).

**Secretariat
May 2005**

SUMMARY

(In accordance with 40 CFR part 152, this summary is available
for public release after registration)

STUDY TITLE

Characterization of the Recombinant Cry1F protein Derived from *Pseudomonas fluorescens* and
Transgenic Maize

DATA REQUIREMENTS

None

AUTHOR(S)

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STUDY COMPLETED ON

September 18, 2001

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GH-C 5294

Characterization of the Recombinant Cry1F protein Derived from *Pseudomonas fluorescens* and Transgenic Maize

SUMMARY

Corn plants have been genetically modified by 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 aizawai* strain PS811. This protein when expressed in corn cultivars confers the crop resistance to lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*). Because it would be difficult to isolate sufficient amounts of biologically active Cry1F protein from corn tissue to perform toxicological studies, the proteins were produced with a bacterium *Pseudomonas fluorescens* (*Pf*) through recombinant DNA technology. The microbial derived Cry1F protein was used in various toxicology and ecotoxicology studies. It was therefore important to characterize the biochemical properties of both the plant and microbial derived proteins.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (stained with coomassie blue and glycoprotein detection methods), Western blot, and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were used to characterize the biochemical properties of the proteins. The Cry1F protein from *Pf* and transgenic maize (event TC1507) were shown to be equivalent with respect to immunoreactivity, peptide mass fingerprints and the lack of post-translational glycosylation. There was a slight difference in the apparent molecular weight of the two proteins but this could be accounted for by additional truncation of the maize derived protein during purification. The current study revealed that the biochemical identity of *P. fluorescens* produced Cry1F protein was equivalent or comparable to that produced in transgenic TC1507 corn plants. This data supports the use of the microbial protein for registration of transgenic corn expressing Cry1F.

STUDY TITLE

Characterization of the Recombinant Cry1F protein Derived from *Pseudomonas fluorescens* and Transgenic Maize

DATA REQUIREMENTS

None

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GH-C 5294

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry1F δ -endotoxin

Title: Characterization of the Recombinant Cry1F protein Derived from *Pseudomonas fluorescens* and Transgenic Maize

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).*

Company: Dow AgroSciences LLC

Company Agent: P. L. Hunst

Title: Regulatory Manager

Signature: _____

Date: _____

*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

Title: Characterization of the Recombinant Cry1F protein Derived from *Pseudomonas fluorescens* and Transgenic Maize

Study Initiation Date: August 2, 2001 Study Completion Date:
Experimental Start Date: August 2, 2001 Experiment Termination Date: August 24, 2001

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

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ISBN 92-64-12367-9, Paris 1982

At the time this study was conducted, it was not subject to the Good Laboratory Practice Standards and was, therefore, not monitored by the quality assurance unit.

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QUALITY ASSURANCE STATEMENT

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NON-GLP STUDY

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ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl phosphate
BME	2-mercaptoethanol
CBB	coomassie brilliant blue
CV	column volumes
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
IAC	immunoaffinity chromatography
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MW	molecular weight
NBT	<i>p</i> -nitroblue tetrazolium
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
PMSF	phenylmethylsulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
TSN	Dow AgroSciences Test Substance Number

Characterization of the Recombinant Cry1F protein Derived from *Pseudomonas fluorescens* and Transgenic Maize

ABSTRACT

Corn plants have been genetically modified by 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 aizawai* strain PS811. This protein when expressed in corn cultivars confers the crop resistance to lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*). Because it would be difficult to isolate sufficient amounts of biologically active Cry1F protein from corn tissue to perform toxicological studies, the proteins were produced with a bacterium *Pseudomonas fluorescens* (*Pf*) through recombinant DNA technology. The microbial derived Cry1F protein was used in various toxicology and ecotoxicology studies. It was therefore important to characterize the biochemical properties of both the plant and microbial derived proteins.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (stained with coomassie blue and glycoprotein detection methods), Western blot, and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were used to characterize the biochemical properties of the proteins. The Cry1F protein from *Pf* and transgenic maize (event TC1507) were shown to be equivalent with respect to immunoreactivity, peptide mass fingerprints and the lack of post-translational glycosylation. There was a slight difference in the apparent molecular weight of the two proteins but this could be accounted for by additional truncation of the maize derived protein during purification. The current study revealed that the biochemical identity of *P. fluorescens* produced Cry1F protein was equivalent or comparable to that produced in transgenic TC1507 corn plants. This data supports the use of the microbial protein for registration of transgenic corn expressing Cry1F.

INTRODUCTION

Corn plants have been genetically modified by 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 (approximately 66 kDa) when expressed in corn cultivars confers resistance to lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*). Because it would be difficult to isolate sufficient amounts of biologically active Cry1F protein from corn tissue to perform toxicological studies, the proteins were produced with a bacterium *Pseudomonas fluorescens* (*Pf*) through recombinant DNA technology. The microbial derived Cry1F protein was used in various toxicology and eco-toxicology studies. It was, therefore, important to characterize the biochemical properties of both the plant and microbial derived proteins.

The biochemical and immunological methods employed in study are among those that have been well established 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. In addition, staining for carbohydrate moieties linked to polypeptides (following electrophoresis) is a standard test to detect post-translational glycosylation of proteins. When treated with periodic acid, glycols present in glycoproteins are oxidized to aldehydes. The aldehydes are next exposed to a proprietary stain (Pierce Chemical Co.) and the glycols of the glycoprotein are visualized. Peptide mass fingerprinting by MALDI-TOF MS following trypsinolysis is among the most powerful tools in examining the sequence equivalency between two proteins (Henzel and Stults, 1996).

MATERIALS AND METHODS

Generation and Purification of Test Substances

The recombinant microbial derived Cry1F protein was expressed by *P. fluorescens* strain MR872 and purified as described in Appendix C of Project ID: MYCO98-001 (Evans 1998). The solubilized, truncated Cry1F, TSN101788 (Collins 2001) was aliquoted and stored at $-80\text{ }^{\circ}\text{C}$ until use. Transgenic maize TC1507 leaf tissue was harvested fresh at the Mycogen field research station in Windfall, IN. The leaves from the entire corn plant were removed, placed in plastic bags, stored on ice and transported to the Dow AgroSciences Indianapolis laboratory. Upon receipt in the lab, the leaves were stored frozen at $-20\text{ }^{\circ}\text{C}$ until use. Corn leaf powder was prepared by grinding the tissue (mid-vein removed) in liquid nitrogen using a mortar and pestle. The proteins were extracted with PBST (Sigma Chemical, Catalog#: P-3563), 1 mM EDTA, 2 mM DTT, 1 mM PMSF, pH 7.4. Large pieces of the leaf tissue were removed by filtering through several layers of cheese cloth followed by centrifugation at 17000xg for 30 minutes. The proteins were fractionated by using 75% ammonium sulfate precipitation and centrifugation. The protein precipitate was resuspended in PBST and filtered through a 0.45 micron membrane. The clarified extract was applied by gravity flow to a 4.2-mL immunoaffinity column specific for Cry1F (Gao *et al.*, 2001) and non-specific proteins were removed by washing with 5 CV of PBST. The bound Cry1F protein was eluted with approximately 25 mL of 50 mM pyridine/acetic acid buffer, pH 3.2 and dried under vacuum centrifugation (sixteen 1.5 mL fractions). The protein was resuspended with 20 mM Tris pH 8.0 buffer and used for the subsequent studies.

SDS-PAGE and Western Blot

SDS-PAGE was performed with Zaxis gels (Zaxis Catalog#: 110-0420T212) and an Integrated Separation Systems gel module. Protein samples were mixed with Laemmli sample buffer (Bio-Rad Catalog#:161-0737) containing 5% freshly added BME and boiled for 5 minutes at $100\text{ }^{\circ}\text{C}$.

The electrophoresis was conducted at a constant amperage of 40 mA per gel for 60 minutes using Tris/glycine/SDS buffer (Bio-Rad Catalog#: 161-0732). After separation, the gel was cut in half with a fresh razor blade and half was stained with coomassie brilliant blue and half was stained with GelCode Glycoprotein stain (Pierce Catalog#: 24562). In addition, a second gel was prepared and the proteins were electro-blotted to a nitrocellulose membrane (Bio-Rad Catalog#:162-0145) with a Mini Trans-Blot electrophoretic transfer cell for 60 minutes under a constant voltage of 100 volts. For immunodetection, a Cry1F specific polyclonal rabbit antibody (Strategic Diagnostics Inc., Lot#: 200.310-4) was used as the primary antibody. A conjugate of goat anti-rabbit IgG (H+L) and calf intestinal alkaline phosphatase (Pierce Chemical, Catalog#: 31340) was used as the secondary antibody. A substrate solution containing SIGMA *Fast* NBT/BCIP (Sigma Chemical, Catalog#: B5655) was used for colorimetric development and visualization of the immuno-reactive protein bands (DAS notebook #: E1213).

Detection of Post-translational Glycosylation

The *Pseudomonas* derived Cry1F and immunoaffinity purified maize Cry1F protein preparations were separated by SDS-PAGE. After electrophoresis, the gel was cut in half and stained with either CBB to visualize all protein bands, or with GelCode Glycoprotein Staining Kit (Pierce, catalog #24562) to visualize glycoproteins. The procedure for glycoprotein staining was briefly described as the following. After electrophoresis, the gel was fixed in 50% MeOH for 30 min and rinsed with 3% acetic acid. This was followed by an incubation period with the oxidation solution from the test kit for 15 min. The gel was once again rinsed with 3% acetic acid and incubated with GelCode glycoprotein staining reagent for 15 min. Finally, it was immersed in the reduction solution for 10 min, and then rinsed with 3% acetic acid. The glycoproteins were visualized as magenta bands on a light pink background.

MALDI-TOF MS Peptide Mass Fingerprinting of Plant and Microbial Derived Cry1F

MALDI-TOF MS was conducted at the Proteomics Lab of Dow AgroSciences LLC. *P. fluorescens* produced truncated Cry1F and immunoaffinity purified, immunoreactive proteins were further separated from other contaminants by SDS-PAGE. The respective bands were excised from the gel and placed into siliconized Eppendorf microcentrifuge tubes, and destained with 50% acetonitrile in 12.5 mM NH₄HCO₃. The gel pieces were dried using vacuum centrifugation, and digested with sequencing grade trypsin (Roche Diagnostics, Indianapolis, Indiana) overnight (approximately 16 hours) at 37 °C. The peptides were extracted with 50% acetonitrile in 0.5% TFA. After brief centrifugation to pellet the gel pieces, the supernatant containing the peptides were decanted and dried in a Savant Speed-Vac. The peptides were then suspended in 0.1% TFA and cleaned with ZipTip resin. The post-cleaning peptides were eluted into siliconized microcentrifuge tubes with 75% acetonitrile/0.1% TFA, dried using vacuum centrifugation, and stored at -20 °C freezer until MALDI-TOF MS analysis. The samples were re-dissolved in 6 µL of 0.1% TFA for MALDI analysis. The instrument used was a PerSeptive Biosystems (Framingham, MA) Voyager DE-STR MALDI-TOF mass spectrometer. The instrument utilizes a 337 nm nitrogen laser for the desorption/ionization event and a 3.0 meter reflector time-of-flight tube. On a stainless steel MALDI sample plate, 0.5 µL of the solubilized peptides was mixed with 0.5 µL of MALDI matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% TFA) and allowed to air dry. External calibration was performed by using a solution of angiotensin I, ACTH (clip 1-17), ACTH (clip 18-39), and ACTH (clip 7-38). Internal calibration was performed using the autolytic trypsin peak at *m/z* 2163.05. All mass spectra were collected in the positive ion reflector mode with delayed extraction.

RESULTS AND DISCUSSION

Purification of Cry1F Protein from Transgenic TC1507 Corn Extract

Immunoaffinity chromatography (IAC) was conducted on the TC1507 transgenic corn leaf extract. Fractions (1.5 mL each) were collected from the elution effluent and concentrated to dryness using vacuum centrifugation and resuspended in 40 μ L of Tris buffer (>30x concentration). Fractions (#3-15) were examined by SDS-PAGE and the results showed that in fractions #7-15, there was a major protein band of an approximate MW of 65 kDa (Data not shown, DAS Notebook E1213). Western blot analysis of fractions 10 and 11 demonstrated the major protein band to be immunoreactive to the specific anti-Cry1F polyclonal antibody (Figure 1, Panel A). It was also observed that below the 65 kDa band, there were several bands which were immunoreactive to the anti-Cry1F antibodies. Although the amounts of these proteins were too low to be seen on SDS-PAGE (Figure 1, Panel B) they were highly antigenic. These minor bands are most likely truncated products of Cry1F which were captured and subsequently purified by IAC in addition to the intact full length protein.

SDS-PAGE and Western Blot Analysis

In the tox lot preparation of *P. fluorescens* MR872 (TSN 101788) the major Cry1F band, as visualized on coomassie stained SDS-PAGE gels, was approximately 66 kDa. As expected (Figure 2), the corresponding maize derived Cry1F was slightly lower at approximately 65 kDa. (Figure 1, Panel B). This is consistent with the previous findings as discussed in the report prepared by Evans (Evans, 1998) where the two proteins were nearly indistinguishable. Predictably, the plant purified fractions contained a number of proteolytic products that were reactive with the anti-Cry1F PAb (Figure 1, Panel A) in addition to the intact protein. This could be accounted for by the need to highly concentrate the purification fractions for visualization on SDS-PAGE which may have contributed to additional protein degradation. As described above, the immunoaffinity column was prepared with anti-Cry1F polyclonal antibodies, and therefore

all available epitopes were most likely captured and purified with the intact Cry1F core protein. These truncation products, though highly immunoreactive, were minor components of the final purified preparation. This was confirmed by the CBB stained gel (Figure 1, Panel B).

Detection of Glycosylation

Detection of carbohydrates possibly covalently linked to Cry1F (microbial and maize derived) was assessed by the GelCode Glycoprotein Staining Kit from Pierce. *P. fluorescens* derived and the immunoaffinity-purified transgenic corn derived Cry1F proteins were electrophoresed simultaneously. A glycoprotein, horseradish peroxidase, was loaded as a positive indicator for glycosylation and a non-glycoprotein, soybean trypsin inhibitor, was employed as a negative control. The results showed that both the corn- and *P. fluorescens*-derived Cry1F proteins had no detectable carbohydrates (Figure 1, Panel C).

Tryptic Peptide Mass Fingerprints

The Cry1F proteins derived from both *P. fluorescens* and transgenic corn TC1507 were separated by SDS-PAGE (Figure 1, Panel B), and the respective bands were excised and subjected to in-gel digestion by trypsin. The resulting peptide mixture was analyzed by MALDI-TOF MS to determine the peptide mass fingerprint coverage. The masses of the detected peptides were compared to those deduced based on potential trypsin cleavage sites on the sequence of Cry1F. Figure 2 illustrates the theoretical cleavage which was generated in silico using PAWS (Protein Analysis Worksheet) freeware from Proteometrics LLC. The predicted amino acid digest (and MW) of the trypsin resistant toxin core of *P. fluorescens* and transgenic corn derived Cry1F proteins are also described in Figure 2. This core, when denatured, is readily digested by trypsin and will generate numerous peptide peaks.

In the trypsin digest of *P. fluorescens*-derived truncated Cry1F protein, 19 peptides were identified matching the theoretical deduced peptide masses of Cry1F (Table 1). The peptide fragments detected were between residues 32 and 546 of Cry1F. In the trypsin digest of the

transgenic corn derived Cry1F protein, 20 peptides were identified matching the theoretical deduced peptide masses (Table 1). The peptide fragments detected were also between residues 32 and 546 of Cry1F. Only the plant Cry1F had an additional match with residues 522-529. The peptide coverage of the protease resistant toxin core for both the microbial and maize derived Cry1F was excellent. There were several unidentified peptides detected in the MALDI-TOF-MS spectrum (Figures 3 and 4). Many factors could contribute to the formation of these non-match peptides, such as over digestion (which resulted in non-specific cleavage), self-digestion products of trypsin, and random breakage of peptides during ionization.

CONCLUSIONS

The current study revealed that the biochemical identity of *P. fluorescens* produced Cry1F protein was equivalent or comparable to that produced in transgenic TC1507 corn plants. As expected, the Cry1F produced in transgenic plants was subject to protease cleavage, resulting in truncated forms and breakdown products. The core toxin of an apparent MW of 65 kDa was predominant in the transgenic corn leaf extract and the core toxin was similar to the truncated Cry1F generated by treating the *P. fluorescens* produced full length Cry1F with trypsin (66 kDa). The Cry1F protein from both expression sources lacked detectable post-translational glycosylation. Tryptic peptide mass fingerprints by in-gel digestion and MALDI-TOF MS, provided direct supporting evidence that the Cry1F produced by *P. fluorescens* and the transgenic corn are equivalent molecules.

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Table 1. Tryptic Peptide Mass Data (m/z [M+H]⁺) of Cry 1F Proteins Obtained by MALDI-TOF MS

Full Length Cry1Fsynpro residue #	Theoretical mass (m/z)	<i>P. fluorescens</i> trypsinized Cry1F [M+H]	TC1507 Maize Cry1F [M+H]
32-42	1227.72	1227.70	1227.68
100-113	1612.81	1612.81	1612.79
114-125	1441.67	1441.66	1441.65
172-193	2434.15	2434.21	2434.16
194-200	878.55	878.51	878.50
204-217	1675.79	1675.75	1675.75
252-263	1394.72	1394.69	1394.68
264-286	2509.21	2509.24	2509.19
312-324	1413.71	1413.70	1413.68
358-366	1033.56	1033.52	1033.52
367-379	1386.71	1386.70	1386.69
380-392	1416.68	1416.67	1416.67
431-442	1376.62	1376.62	1376.59
452-463	1301.63	1301.60	1301.58
464-471	911.58	911.53	911.52
472-483	1269.68	1269.66	1269.65
484-494	1089.56	1089.53	1089.52
522-529	925.46	ND ^b	925.43
530-538	1007.54	1007.51	1007.50
539-546	924.48	924.44	924.43

Note:

^a Two digit decimals were used for mass data in this table although raw data obtained from the MALDI-TOF-MS spectrometer were shown in 4 digit decimals. A peptide was considered a match if its m/z is within m/z 0.1 error range of its theoretical m/z .

^b ND: not detected.

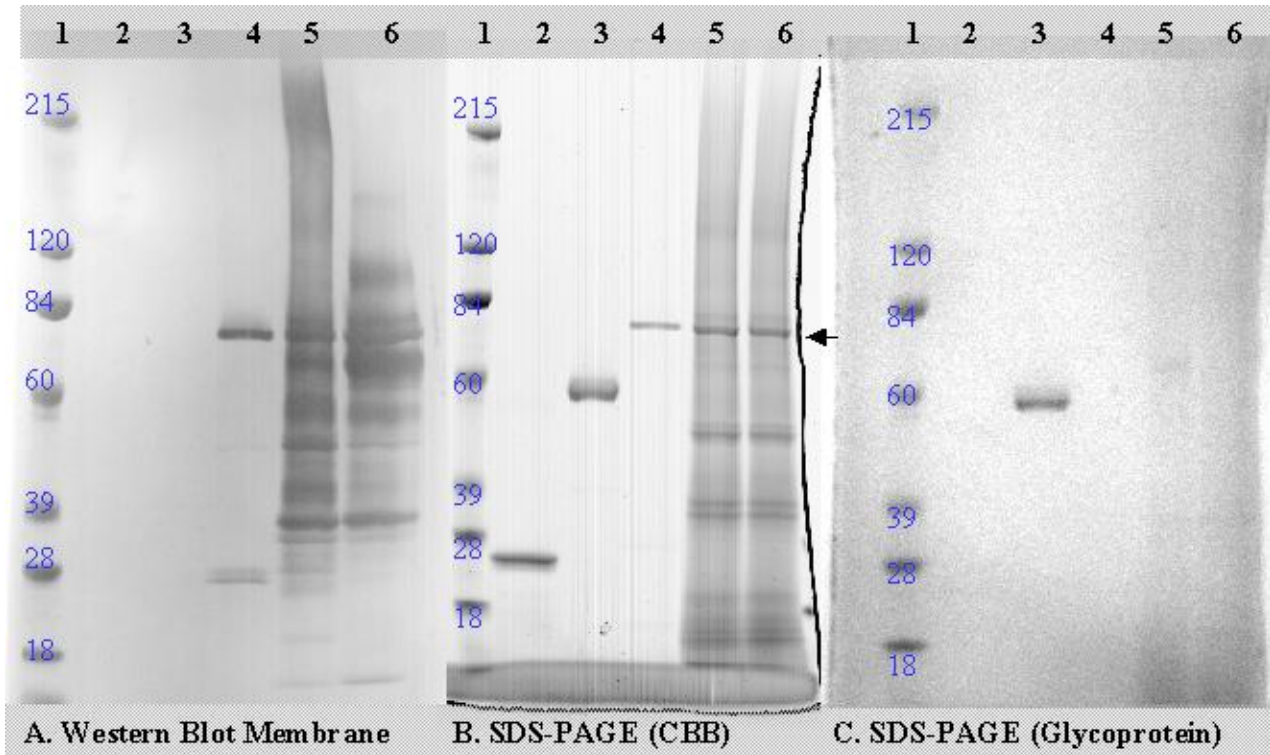


Figure 1. SDS-PAGE and Western Blot Analysis of Microbial and Maize Derived Cry1F Proteins

Panel A: Western Blot detected with rabbit anti-Cry1F polyclonal antibody.

Panel B: SDS-PAGE, 4-20% gradient gel, Coomassie brilliant blue stained.

Panel C: SDS-PAGE, 4-20% gradient gel, GelCode glycoprotein stained

Lane 1: Pre-stained molecular weight markers (Pierce Chemical, BlueRanger)

Lane 2: Soybean Trypsin Inhibitor (MW: 20.1 kDa), 1.25 $\mu\text{g}/\text{lane}$

Lane 3: Horseradish Peroxidase (MW: 44 kDa), 1.25 $\mu\text{g}/\text{lane}$

Lane 4: *P. fluorescens* MR872 derived Cry1F, 0.14 $\mu\text{g}/\text{lane}$

Lane 5: Maize derived, IAC purified Cry1F (Fraction 10)

Lane 6: Maize derived, IAC purified Cry1F (Fraction 11)

*Arrow Denotes Cry1F protein

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1  M E N N I Q N Q C V P Y N C L N N P E V E I L N E E R s t g 30
31  r L P L D I S L S L T R f l l s e f v p g v g v a f g l f d 60
61  l i w g f i t p s d w s l f l l q i e q l i e q r I E T L E 90
91  R n r A I T T L R g l a d s y e i y i e a l r E W E A N P N 120
121 N A Q L R e d v r I R f a n t d d a l i t a i n n f t l t s 150
151 f e i p l l s v y v q a a n l h l s l l r D A V S F G Q G W 180
181 G L D I A T V N N H Y N R l i n l i h r Y T K h c l d t y n 210
211 q g l e n l r G T N T R q w a r F N Q F R r D L T L T V L D 240
241 I V A L F P N Y D V R t y p i q t s s q l t r E I Y T S S V 270
271 I E D S P V S A N I P N G F N R a e f g v r p p h l m d f m 300
301 n s l f v t a e t v r S Q T V W G G H L V S S R n t a g n r 330
331 I N F P S Y G V F N P G G A I W I A D E D P R P F Y R t l s 360
361 d p v f v r G G F G N P H Y V L G L R g v a f q q t g t n h 390
391 t r T F R n s g t i d s l d e i p p q d n s g a p w n d y s 420
421 h v l n h v t f v r W P G E I S G S D S W R a p m f s w t h 450
451 r S A T P T N T I D P E R i t q i p l v k A H T L Q S G T T 480
481 V V R g p g f t g g d i l r R t s g g p f a y t i v n i n g 510
511 q l p q r Y R a r I R y a s t t n l r I Y V T V A G E R i f 540
541 a g q f n k T M D T G D P L T F Q S F S Y A T I N T A F T F 570
571 P M S Q S S F T V G A D T F S S G N E V Y I D R f e l i p v 600
601 t a t l e a e s d l e r A Q K a v n a l f t s s n q i g l k 630
631 T D V T D Y H I D R v s n l v e c l s d e f c l d e k K e l 660
661 s e k V K h a k R l s d e r N L L Q D P N F R g i n r Q L D 690
691 R g w r G S T D I T I Q G G D D V F K e n y v t l l g t f d 720
721 e c y p t y l y q k I D E S K l k A Y T R y q l r G Y I E D 750
751 S Q D L E I Y L I R y n a k H E T V N V P G T G S L W P L S 780
781 A P S P I G K c a h h s h h f s l d i d v g c t d l n e d l 810
811 g v w v i f k I K t q d g h a r L G N L E F L E E K P L V G 840
841 E A L A R v k R a e k K w r D K r E K l e w e t n i v y k E 870
871 A K e s v d a l f v n s q y d r L Q A D T N I A M I H A A D 900
901 K r V H S I R e a y l p e l s v i p g v n a a i f e e l e g 930
931 r I F T A F S L Y D A R n h r S V L V V P E W E A E V S Q E V R v 960
961 g h v d v e e q n n h r S V L V V P E W E A E V S Q E V R v 990
991 c p g r G Y I L R v t a y k E G Y G E G C V T I H E I E N N 1020
1021 T D E L K f s n c v e e e v y p n n t v t c n d y t a t q e 1050
1051 e y e g t y t s r N R g y d g a y e s n s s v p a d y a s a 1080
1081 y e e k A Y T D G R r D N P C E S N R g y g d y t p l p a g 1110
1111 y v t k E L E Y F P E T D K v w i e i g e t e g t f i v d s 1140
1141 v e l l l m e e 1148

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Note: The molecular weight of the protease resistant Cry1F core is predicted to be 65.9 kDa for the microbial protein (amino acids 28-612) as compared to 65.0 kDa for the maize protein (amino acids 28-605, residue 604 changed to F from L). One amino acid difference exists between the gene expression products and the synthetic maize gene (I shown in blue). In the synthetic maize gene (amino acids 1-605) a conservative amino acid substitution of leucine (L) to phenylalanine (F) was introduced for cloning purposes (added restriction site). Alternating blocks of upper and lower case letters within the amino acid sequence are used to differentiate the potential peptides after trypsin digestion. The numbers on the left and right sides indicate the amino acid residue numbers.

Figure 2. Amino Acid Sequence of Full Length Microbial MR872 Cry1F

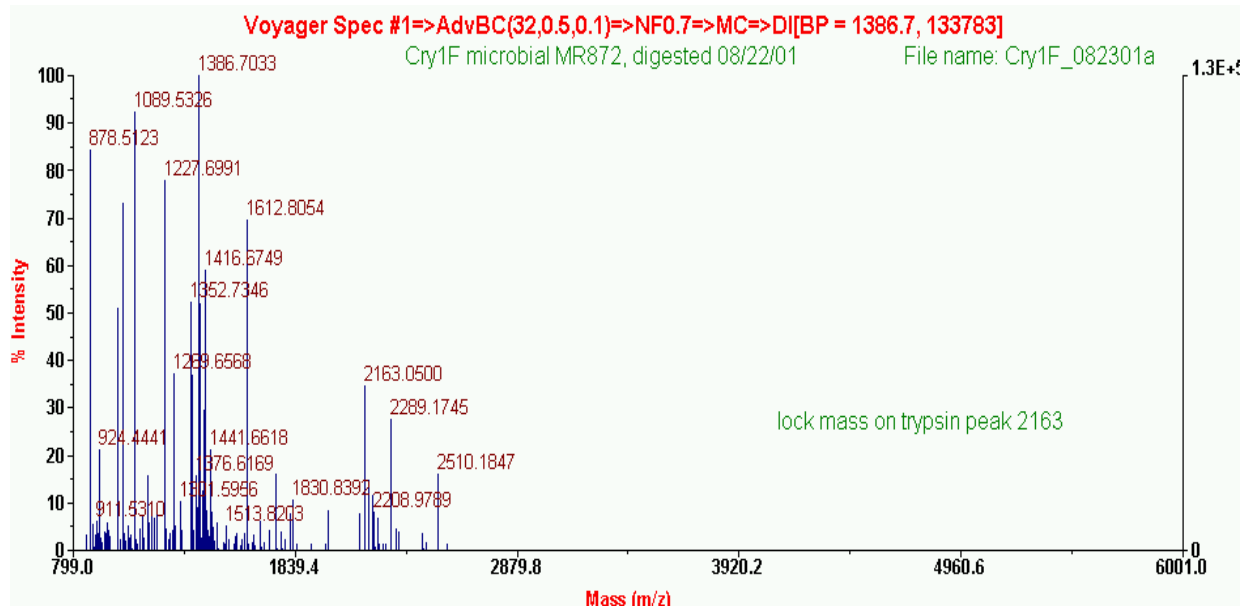


Figure 3. MALDI-TOF Mass Spectrum (deisotoped) of the Trypsin Digest of the Truncated Cry1F Derived from *P. fluorescens* MR872. Note: not every peak was labeled.

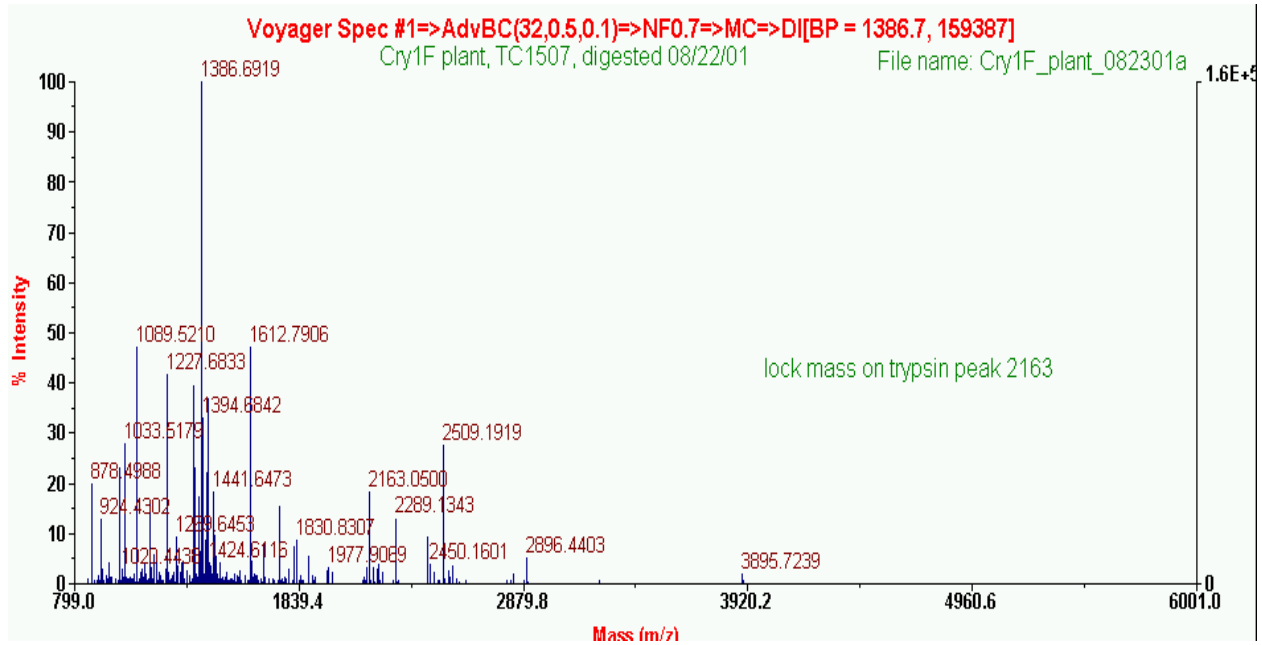


Figure 4. MALDI-TOF Mass Spectrum (deisotoped) of the Trypsin Digest of the Truncated Cry1F Derived from Transgenic Corn Event TC1507. Note: not every peak was labeled.