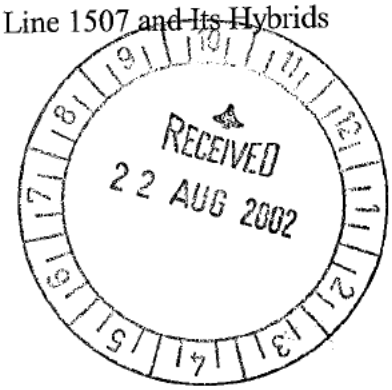
[redacted]

SUMMARY

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

STUDY TITLE

Polymerase Chain Reaction (PCR) Analysis of *B.t.* Cry1F Maize Line 1507 and Its Hybrids



DATA REQUIREMENTS

N/A

AUTHOR(S)

[redacted]

STUDY COMPLETED ON

January 31, 2002

PERFORMING LABORATORY

Regulatory Laboratories—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

GH-C 5371

Polymerase Chain Reaction (PCR) Analysis of *B.t.* Cry1F Maize Line 1507 and Its Hybrids

SUMMARY

Corn plants have been genetically modified through the introduction of a synthetic gene which encodes for a truncated version of an insecticidal protein, Cry1F, isolated from *Bacillus thuringiensis* var. *aizawai* strain PS811. When expressed in corn cultivars, the Cry1F protein confers crop resistance to lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*). The Cry1F corn plants also contain an herbicide-resistant selectable marker gene that expresses the protein phosphinothricin acetyltransferase (PAT). The PAT protein imparts tolerance to glufosinate-ammonium, the active ingredient in Liberty herbicide.

Polymerase chain reaction (PCR) was used to demonstrate the presence of the *cry*1F gene, *pat* gene, and an event specific sequence in transgenic *B.t.* Cry1F maize line 1507 and its hybrid derivatives. The test material consisted of 5 corn seed sources, T1S1 seed of *B.t.* Cry1F maize line 1507, two independent hybrids derived from event 1507 (Mycogen 2722 Cry1F and Pioneer 33P66 Cry1F) and the related non-transgenic hybrids of each (Mycogen 2722 and Pioneer 33P66 Control). Genomic DNA from leaf tissue was used for PCR amplification.

The results of PCR amplification indicated that the full-length coding sequences of *cry*1F and *pat*, and the event specific sequence located at the 3' end insert junction of *B.t.* Cry1F maize line 1507, were present in all samples of event 1507 and its hybrid derivatives, but not in the related corn controls. Based on these results, it is concluded that the Cry1F hybrids tested in this study, Mycogen 2722 Cry1F and Pioneer 33P66 Cry1F, were derived directly from the unique transgenic event, *B.t.* Cry1F maize line 1507.

STUDY TITLE

Polymerase Chain Reaction (PCR) Analysis of *B.t.* Cry1F Maize Line 1507 and Its Hybrids

DATA REQUIREMENTS

N/A

AUTHOR(S)

STUDY COMPLETED ON

January 31, 2002

PERFORMING LABORATORY

Regulatory Laboratories—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

GH-C 5371

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Genomic DNA from *B.t.*Cry1F maize line 1507 and its hybrids

Title: Polymerase Chain Reaction (PCR) Analysis of *B.t.* Cry1F Maize Line 1507 and Its Hybrids

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: [redacted]

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: Polymerase Chain Reaction (PCR) Analysis of *B.t.* Cry1F Maize Line 1507 and Its Hybrids

Study Initiation Date:	8/24/2001	Study Completion Date:	
Experimental Start Date:	8/24/2001	Experiment Termination Date:	12/22/2001

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

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[redacted] Date

Sponsor
Dow AgroSciences LLC

[redacted] Date

Submitter
Dow AgroSciences LLC

[redacted] Date

Study Director/Author
Dow AgroSciences LLC

QUALITY ASSURANCE STATEMENT

Compound: Genomic DNA from *B.t.*Cry1F maize line 1507 and its hybrids

Title: Polymerase Chain Reaction (PCR) Analysis of *B.t.* Cry1F Maize Line 1507 and Its Hybrids

Study Initiation Date: 8/24/2001 Study Completion Date: 1/31/2002

NON-GLP STUDY

SIGNATURE PAGE

[redacted] Date

Author
Dow AgroSciences LLC

[redacted] Date

Co-author
Dow AgroSciences LLC

[redacted] Date

Co-author
Dow AgroSciences LLC

[redacted] Date

Global Leader, Biotechnology Regulatory Science
Dow AgroSciences LLC

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Polymerase Chain Reaction (PCR) Analysis of *B.t.* Cry1F Maize Line 1507 and Its Hybrids

ABSTRACT

Corn plants have been genetically modified through the introduction of a synthetic gene that encodes for a truncated version of an insecticidal protein, Cry1F, isolated from *Bacillus thuringiensis* var. *aizawai* strain PS811. When expressed in corn cultivars, the Cry1F protein confers crop resistance to lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*). The Cry1F corn plants also contain an herbicide-resistant selectable marker gene that expresses the protein phosphinothricin acetyltransferase (PAT). The PAT protein imparts tolerance to glufosinate-ammonium, the active ingredient in Liberty herbicide.

Polymerase chain reaction (PCR) was used to demonstrate the presence of the *cry*1F gene, *pat* gene, and an event specific sequence in transgenic *B.t.* Cry1F maize line 1507 and its hybrid derivatives. The test material consisted of 5 corn seed sources, T1S1 seed of *B.t.* Cry1F maize line 1507, two independent hybrids derived from event 1507 (Mycogen 2722 Cry1F and Pioneer 33P66 Cry1F) and the related non-transgenic hybrids of each (Mycogen 2722 and Pioneer 33P66 Control). Genomic DNA from leaf tissue was used for PCR amplification.

The results of PCR amplification indicated that the full-length coding sequences of *cry*1F and *pat*, and the event specific sequence located at the 3' end insert junction of *B.t.* Cry1F maize line 1507, were present in all samples of event 1507 and its hybrid derivatives, but not in the related corn controls. Based on these results, it is concluded that the Cry1F hybrids tested in this study, Mycogen 2722 Cry1F and Pioneer 33P66 Cry1F, were derived directly from the unique transgenic event, *B.t.* Cry1F maize line 1507.

INTRODUCTION

B.t Cry1F maize line 1507 was generated by insertion of a 6235 bp DNA fragment (PHI8999A) isolated from *Pme* I digestion of plasmid PHP8999 into maize genome through microprojectile bombardment transformation (1). The 6235 bp DNA fragment (transgene) included two expression cassettes. One cassette contained the maize *ubi*ZM1 promoter (2), a *Bacillus thuringiensis* (*B.t.*) gene encoding an insecticidal crystal protein Cry1F, and an ORF25 PolyA terminator (3). The second cassette contained the CaMV 35S promoter (4), a coding sequence of phosphinothricin N-transferase (*pat*) from *Streptomyces viridochromogenes* (5) and CaMV 35S terminator. The Cry1F protein confers crop resistance to lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*) and the PAT protein confers resistance to glufosinate-ammonium herbicide and was used as a selectable marker in the process of transgenic maize regeneration. Through conventional plant breeding, this transgene has been introgressed into the hybrid corn lines Pioneer 33P66 Cry1F and Mycogen 2722 Cry1F.

The objective of this study was to demonstrate that the same *cry*1F gene, *pat* gene, and an event specific sequence located at the 3' end insert junction of *B.t.* Cry1F maize line 1507 were present in the transgenic *B.t.* Cry1F maize line 1507 and its hybrid derivatives.

LABORATORY PHASE

Test Substance

The test substance used in this study was the genomic DNA separately extracted from plants of *B.t.* Cry1F maize line 1507 and its hybrids. Two individual plants of three separated lines were tested. The lines consisted of the T1S1 generation of *B.t.* Cry1F maize line 1507 and two independent hybrids derived from the T1S1, Mycogen 2722 Cry1F and Pioneer 33P66 Cry1F.

Control Substance

The control substance used in this study was the genomic DNA separately extracted from plants of non-transgenic hybrid isolines of Mycogen 2722 Cry1F and Pioneer 33P66 Cry1F. Two individual plants of each of the two control lines were sampled.

Reference Substances

A DNA marker (Life Technology, catalog # 10787-026) containing a mixture of DNA fragments with different sizes served as reference substance to estimate the sizes of PCR amplified products.

Plasmid DNA of PHP8999, the transformation vector used to create *B.t.* Cry1F maize line 1507, was used as a positive control for the PCR reactions.

Test System

Seeds from *B.t* Cry1F maize line 1507 T1S1, its hybrid derivatives, Mycogen 2722 Cry1F and Pioneer TC1507PP, and their related non-transgenic controls were planted in the greenhouse of Dow AgroSciences (Indianapolis, Indiana, USA). All plants were uniquely identified by labeled stakes and grown under typical greenhouse conditions for corn.

Confirmation of Cry1F Protein Expression

An immunoassay was performed prior to extraction of genomic DNA from the plants in the greenhouse. Cry1F Lateral Flow Immunodiagnosis Test Strips (Strategic Diagnostics Inc., Lot # 7903500) specific for Cry1F protein were used to confirm that the transgenic plants produced the protein (positive) and non-transgenic control plants were negative.

Genomic DNA Extraction and Quantification

A DNAeasy Plant Mini Kit from Qiagen (Catalog # 69106) was used to extract genomic DNA from plant leaf tissue samples according to the protocol suggested by the manufacturer. DNA samples were dissolved in DNase free water. The concentration of DNA was determined by measuring the absorbency of the solution at 260 nm on Molecular Devices Spectra Max 190 spectrophotometer.

PCR Primers

The four sets of primers that were used in this study are listed in the Confidential Appendix and their positions are diagrammed in Figure 1. Construct specific primers covering the full-length coding region of *cry*1F and *pat* genes were used to verify the presence of the transgenes. A set of primers based on the maize alcohol dehydrogenase 1 gene (*adh*-1, Genbank accession # X04050) were used for the positive control. These 3 sets of primers were synthesized by Integrated DNA Technologies, Inc. The fourth set of primers designed to amplify an event specific sequence located in the 3' insert junction region of *B.t* Cry1F maize line 1507 were validated by GenScan Europe AG (6) and synthesized by Sigma Genosys.

PCR Amplification

HotStar *Taq* PCR kit from Qiagen (Catalog # 203445) and Ampli *Taq* Gold PCR kit from Applied BioSystem (catalog # 4306894) were used for PCR amplification according to manufacturers' suggested protocols. The concentration of each primer was 0.4 μM in a 50-μL PCR reaction volume. For the amplification of the full-length coding region of *cry*1F and *pat* genes, 300 ng of genomic DNA was used as a template. For the amplification of 1507 event specific sequence and *adh*-1 gene, 200 ng of genomic DNA was used as a template.

PCR conditions for different sequence targets are listed in Table 1. PCR amplification was performed with a Gene Amp PCR 9700 system manufactured by PE Applied BioSystem.

PCR Product Detection

PCR products were viewed by electrophoresis in 1% agarose gel containing 1X TAE buffer (40 mM Tris-acetic and 1 mM EDTA) and ethidium bromide (~0.5 µg/mL), followed by image recording under UV light.

RESULTS

Results of the PCR amplifications are summarized in Table 2.

*cry*1F Gene Detection

The *cry*1F amplification was used to detect the full length of *cry1F* coding sequence, resulting in an amplicon size of 1816 bp (Figure 1, Table 2). The expected amplicon was present in the plasmid PHP8999, and all the samples of *B.t* Cry1F maize line 1507 and its hybrid derivatives (Figure 2, lane 2, lanes 5-8, and 11-12). No PCR product was amplified from the non-transgenic hybrid controls as shown in lanes 3-4 and 9-10 of Figure 2.

pat Gene Detection

The *pat* amplification was used to detect the full length of the *pat* coding sequence, resulting in an amplicon size of 525 bp (Figure 1, Table 2). The expected amplicon was present in the plasmid PHP8999, and all the samples of *B.t* Cry1F maize line 1507 and its hybrid derivatives (Figure 3, lane 2, lanes 5-8, 11-12). No PCR product was amplified from the non-transgenic hybrid controls as shown in lanes 3-4 and 9-10 of Figure 3.

1507 Event Specific Sequence Detection

The 1507 amplification was used to detect the presence of the specific sequence located at the 3' end insert junction of *B.t.* Cry1F maize line 1507, resulting in an amplicon of 194 bp (Figure 1, Table 2). The expected amplicon was present in all the samples of *B.t* Cry1F maize line 1507 and its hybrid derivatives (Figure 4, lanes 5-8, and 11-12). The PCR product was not present in the plasmid PHP8999 (Figure 4, lane 2), or in the non-transgenic hybrid controls as shown in lanes 3-4 and 9-10 of Figure 4.

adh-1 Gene Detection

The *adh*-1 amplification was used as a PCR positive control. A set of primers based on the maize alcohol dehydrogenase 1 gene (*adh*-1, GenBank accession # X04050) was used to amplify a DNA fragment from bp 1088 to1594 of the *adh*-1 gene (Table 2). Since this gene is a native corn gene, the expected amplicon of 507 bp was present in all the samples including *B.t* Cry1F maize line 1507, the 1507 hybrid derivatives, and the non-transgenic hybrid controls (Figure 5, lanes 3-12).

CONCLUSIONS

PCR amplification indicated that the full-length coding sequences of *cry*1F and *pat*, and the event specific sequence located at the 3' end insert junction of *B.t.* Cry1F maize line 1507, were present in the *B.t.*Cry1F maize line 1507 and its hybrid derivatives, but not in the related hybrid controls. Based on these results, it is concluded that the Cry1F hybrids tested in this study, Mycogen 2722 Cry1F and Pioneer 33P66 Cry1F, were derived directly from the unique transgenic event, *B.t.* Cry1F maize line 1507.

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Table 1. PCR Conditions for Different Sequence Targets

Target Sequence	**PCR Kit**	**Pre-denature (°C/min)**	**Denature (°C/sec.)**	**Annealing (°C/sec.)**	**Extension (°C/min:sec)**	**Final Extension (°C/min)**
Cry1F gene	Qiagen HotStar *Taq*	95/15	94/30	57/30	72/2:30	72/7
			30 cycles			
Pat gene	Qiagen HotStar *Taq*	95/15	94/30	56/30	72/1:00	72/7
			30 cycles			
1507 event specific sequence	Applied Biosystem Ampli*Taq* Gold	95/5	94/30	60/30	72/0:45	72/7
			35 cycles			
adh-1 gene	Qiagen HotStar *Taq*	95/15	94/30	57/30	72/1:00	72/7
			30 cycles			

Table 2. PCR Amplification of *cry1F*, *pat*, and *adh*-1 from *B.t.* Cry1F Maize Line 1507, its Hybrid Derivatives, and Non-Transgenic Maize Controls

PCR Target Sequence	**Primer Set**	**Amplicon Size (bp)**	**Location in the insert and border region of 1507**	**Amplicon presence in *B.t.*Cry1F maize line 1507 and hybrid derivatives**	**Amplicon presence in non-transgenic maize control**
Cry1F gene	1507_1/1507_2	1880	4578-6367	YES	NO
Pat gene	1507_3/1507_4	5527	766-8317	YES	NO
1507 specific sequence	Cry1F_f/Cry1F_r	194	8576-8770	YES	NO
Maize *adh*-1 gene	ZmADH-1/ZmADH-2	507	*adh*-1 gene Base 1088-1594	YES	YES

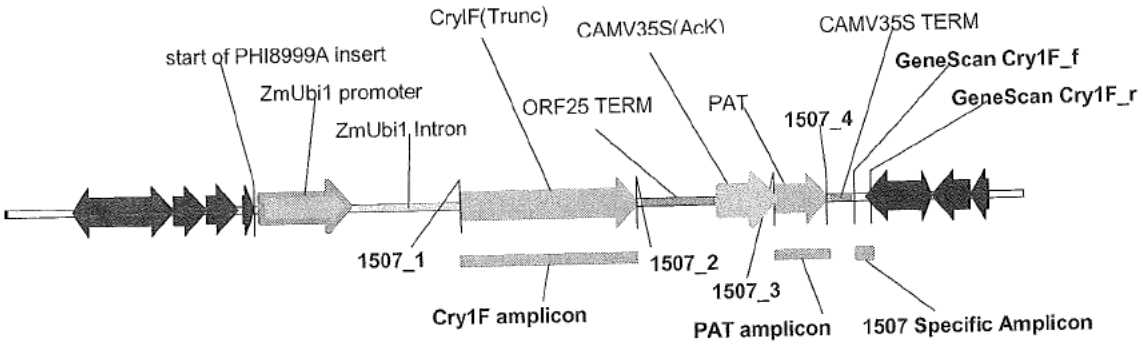

Figure 1. Diagram of the Insert of *B.t.* Cry1F Maize Line 1507

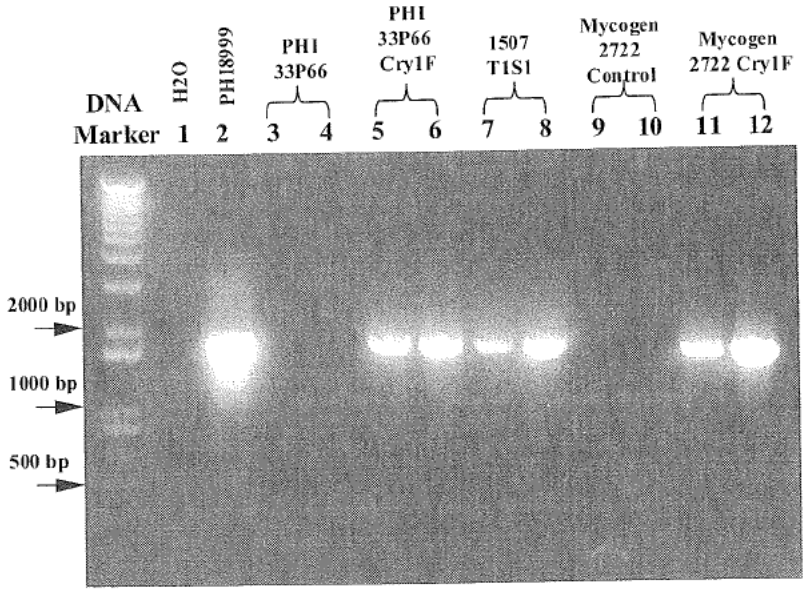

Figure 2. PCR Amplification of *cry1F* Gene in *B.t.* Cry1F Maize Line 1507 and its Hybrids

[redacted]

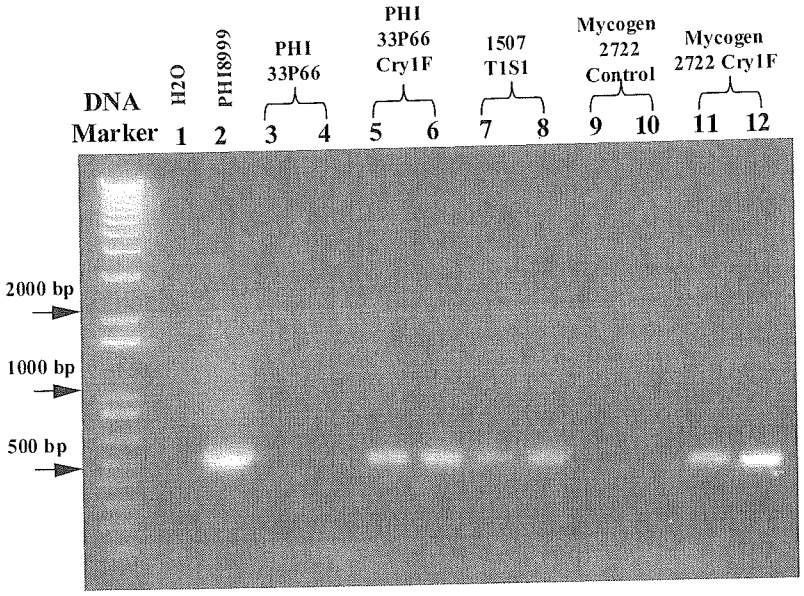

Figure 3. PCR Amplification of *pat* Gene in *B.t.* Cry1F Maize Line 1507 and its Hybrids

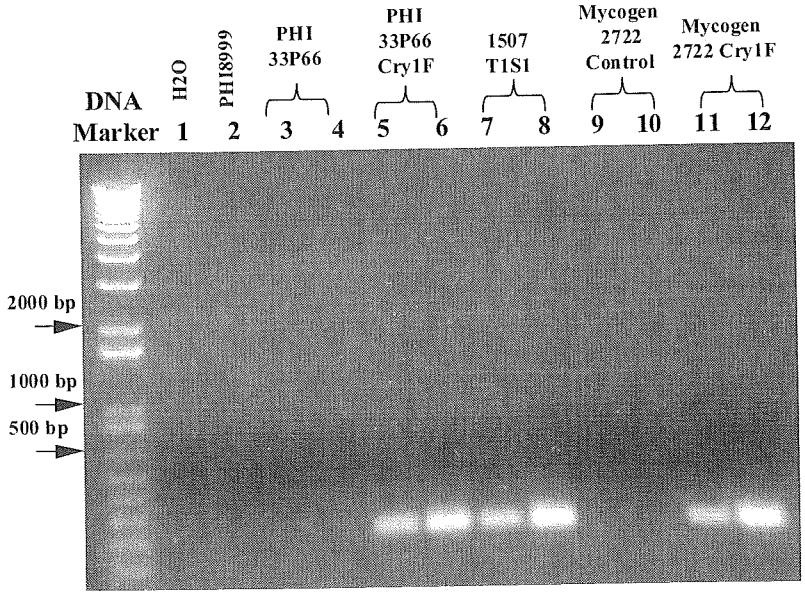

Figure 4. PCR Amplification of an Event Specific Sequence in *B.t.* Cry1F Maize Line 1507 and its Hybrids

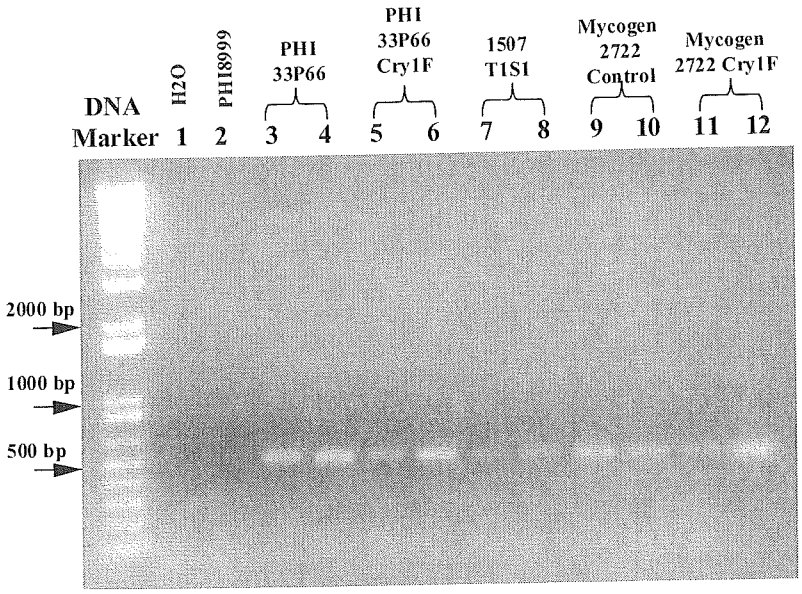

Figure 5. PCR Amplification of *Adh*-1 Gene in *B.t.* Cry1F Maize Line 1507 and its Hybrids

SUMMARY

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

STUDY TITLE

Generation of Corn Processed Products From Wet and Dry Milling Using Cry1F Corn Grain

DATA REQUIREMENTS

None

AUTHOR

[redacted]

STUDY COMPLETED ON

January 10, 2002

PERFORMING LABORATORY

Regulatory Laboratories—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

001044

Generation of Corn Processed Products From Wet and Dry Milling Using Cry1F Corn Grain

SUMMARY

Corn has been modified by the insertion of a gene from *Bacillus thuringiensis* subspecies *aizawai*. The *B. t.* gene encodes for an insecticidally active delta-endotoxin, Cry1F, which controls European corn borer (*Ostrinia nubialis*), a major pest of corn. In addition, the corn has been modified by the insertion of a gene which encodes for the herbicidally active protein phosphinothricin acetyltransferase (PAT) which inactivates the active ingredient in Liberty herbicide. This study was conducted to collect samples of common processed product fractions from wet and dry milling from control (non-transgenic) corn grain and transgenic Cry1F corn grain. The grain used was collected prior to this study. These samples were stored at approximately -80 °C and were made available for use in other studies. No analyses on these samples were performed in association with this study.

STUDY TITLE

Generation of Corn Processed Products From Wet and Dry Milling Using Cry1F Corn Grain

DATA REQUIREMENTS

None

AUTHOR

[redacted]

STUDY COMPLETED ON

January 10, 2002

PERFORMING LABORATORY

Regulatory Laboratories—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

001044

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry1F Corn

Title: Generation of Corn Processed Products From Wet and Dry Milling Using Cry1F Corn Grain

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: [redacted]

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: Generation of Corn Processed Products From Wet and Dry Milling Using Cry1F Corn Grain

Study Initiation Date:	07-Dec-2000	Study Completion Date:	
Experimental Start Date:	31-Jan-2001	Experiment Termination Date:	15-Feb-2001

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United States Environmental Protection Agency
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FEDERAL REGISTER, August 17, 1989

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

This study does not meet requirements of 40 CFR Part 160.

[redacted] _____ Date _____

Sponsor
Dow AgroSciences LLC

[redacted] _____ Date _____

Submitter
Dow AgroSciences LLC

[redacted] _____ Date _____

Study Director/Author
Dow AgroSciences LLC

QUALITY ASSURANCE STATEMENT

Compound: Cry1F Corn

Title: Generation of Corn Processed Products From Wet and Dry Milling Using Cry1F Corn Grain

Study Initiation Date: 07-Dec-2000 Study Completion Date: January 10, 2002

NON-GLP STUDY

SIGNATURE PAGE

[redacted]
Author
Dow AgroSciences LLC

Date

[redacted]
Reviewer
Dow AgroSciences LLC

Date

[redacted]
Reviewer
Dow AgroSciences LLC

Date

[redacted]
Global Leader, Biotechnology Regulatory Science
Dow AgroSciences LLC

Date

TABLE OF CONTENTS

Generation of Corn Processed Products From Wet and Dry Milling Using Cry1F Corn Grain

ABSTRACT

Corn has been modified by the insertion of a gene from *Bacillus thuringiensis* subspecies *aizawai*. The *B. t.* gene encodes for an insecticidally active delta-endotoxin, Cry1F, which controls European corn borer (*Ostrinia nubialis*), a major pest of corn. In addition, the corn has been modified by the insertion of a gene which encodes for the herbicidally active protein phosphinothricin acetyltransferase (PAT) which inactivates the active ingredient in Liberty herbicide. This study was conducted to collect samples of common processed product fractions from wet and dry milling.

Control (non-transgenic) corn grain and transgenic corn grain derived mainly from Cry1F transgenic event TC1507, collected prior to the initiation of this study, was processed by Texas A&M Food Protein Research and Development Center into wet and dry mill fractions using processing methods which simulated commercial processing. The wet mill fractions collected were whole corn (RAC), steepwater concentrate, germ, hull, gluten, starch, solvent extracted germ, soapstock, crude oil, refined oil, and bleached and deodorized oil. The dry mill fractions collected were whole corn (RAC), grits, meal, hull, flour, solvent extracted germ, soapstock, crude oil, refined oil, and bleached and deodorized oil. The fractions were stored at -80 °C at Dow AgroSciences and were made available for use in other studies. No analyses on these samples were performed in association with this study.

This study was conducted non-GLP.

INTRODUCTION

Corn has been modified by the insertion of a gene from *Bacillus thuringiensis* subspecies *aizawai*. The *B. t.* gene encoded for an insecticidally active delta-endotoxin, Cry1F, which controls European corn borer (*Ostrinia nubialis*), a major pest of corn. This grain contained the plant-optimized version of truncated Cry1F (po-*cry*1F). In addition to the po-*cry*1F gene, the corn grain also possessed a synthetic gene that encoded for phosphinothricin acetyltransferase (PAT) protein which inactivates the active ingredient in Liberty herbicide, glufosinate-ammonium by blocking the conversion of glutamate and ammonia into glutamine via glutamine synthetase.

The purpose of this study was to collect samples of typical processed products of wet and dry milling which may be consumed by humans or animals.

EXPERIMENTAL

Test Substance

The test substance (corn grain) was grown at the Mycogen Seeds Research Farm, Fjelland Unit, in Huxley, Iowa. Approximately 960 lbs. of grain was harvested on October 24, 2000, combined, and bagged without drying.

The grain contained the plant-optimized version of truncated Cry1F (po-*cry*1F), event TC1507. The grain used was from open pollinated plants, thus the grain may have contained the genes from other corn products in development. The corn consisted of a blend from a number of putative hybrids, primarily 2722, but lesser amounts of 2110, 2395, 2500, 2593, 2657, 2784, 2832, 2833, and 2853 which may have also been present.

The sample was identified as lot number Hux2000F. A test substance number (TSN) was not assigned to this sample. Details of the source experiment are located at VXNT01\HUXResearch\Rouse 00\2000 Data Book\Other Tests\619.xls.

Control Substance

The control substance (corn grain) was grown at the Mycogen Seeds Research Farm, Fjelland Unit, in Huxley, Iowa. Approximately 550 lbs. of grain was harvested dried, and shelled.

The grain was believed to be free of any Cry1F events, such as TC1507. The grain consisted of a bin-run #2 yellow dent corn of various unknown origins.

The sample was identified as lot number Hux2000C. A test substance number (TSN) was not assigned to this sample.

Test Substance and Control Substance Storage and Shipment

The grain was stored under low humidity at approximately 50 °F in Huxley, Iowa, prior to shipment to Texas A&M. The grain was shipped at ambient temperature from Huxley, Iowa, on December 1, 2000 (transgenic) and on December 19, 2000 (control) by Federal Express. The samples were received ambient at Texas A&M on December 5, 2000 (transgenic) and December 22, 2000 (control). The grain was stored frozen at below 10 °F at Texas A&M until processing. Shipping and receiving dates are summarized in Table 1.

PROCESSING

Processing Method

Samples were processed at Texas A&M Food Protein Research and Development Center using processing methods which simulate commercial processing. The milling of the corn was performed using the following Texas A&M standard operating procedures (SOPs): SOP 8.5, revision 12, small-scale wet milling of corn; SOP 8.6, revision 11, small-scale dry milling of corn; SOP 8.11, revision 07, laboratory bleaching of vegetable oil; and SOP 8.13, revision 07, laboratory deodorization of vegetable oil. Duplicate samples were taken in order to have smaller sample sizes per sample and as a precaution in case samples were lost during shipment.

Wet mill fractions collected were whole corn (RAC), steepwater concentrate, germ, hull, gluten, starch, solvent extracted germ, soapstock, crude oil, refined oil, and bleached and deodorized oil. Dry mill fractions collected were whole corn (RAC), grits, meal, hull, flour, solvent extracted germ, soapstock, crude oil, refined oil, and bleached and deodorized oil. Sample amounts collected for the wet and dry milling are summarized in Tables 2 and 3, respectively.

Specific details of the wet and dry milling processing including the material balance sheets are located in the Texas A&M processing reports in Appendix A and B, respectively.

Process Fraction Storage and Shipment

Process fraction samples were stored frozen at or below 10 °F from the time of fraction generation until shipment to Dow AgroSciences. Samples were shipped to Dow AgroSciences by overnight Federal Express in insulated boxes with dry ice. Processing, shipping, and receiving dates are summarized in Table 1.

Sample Receipt, Storage, and Preparation

Unique Dow AgroSciences sample numbers were assigned to the samples upon receipt at Dow AgroSciences. These sample numbers were used to track the samples throughout receipt, and storage. Samples were identified in groups based upon type of milling, fraction, and replicate.

Upon receipt at Dow AgroSciences, the samples were inspected for physical condition and then logged into Dow AgroSciences Sample Tracking and Reporting (DSTAR) system. All samples were received frozen and in good condition. The samples were stored in temperature-monitored freezers at -20 °C or -80 °C.

ANALYTICAL

Sample Analysis

No analyses of the samples was performed in conjunction with this study.

Statistical Treatment of Data

No statistical treatment of the data was performed.

RESULTS, DISCUSSION AND CONCLUSIONS

Control and Cry1F corn grain was processed by Texas A&M Food Protein Research and Development Center into wet and dry mill fractions using processing methods which simulated commercial processing. Wet mill fraction samples collected were whole corn (RAC), steepwater concentrate, germ, hull, gluten, starch, solvent extracted germ, soapstock, crude oil, refined oil, and bleached and deodorized oil. Dry mill fraction samples collected were whole corn (RAC),

grits, meal, hull, flour, solvent extracted germ, soapstock, crude oil, refined oil, and bleached and deodorized oil. The samples were stored at -20 °C or -80 °C and are available for use in other studies.

ARCHIVING

Final report, processing reports, and all raw data (verified and signed copies) associated with this study are filed in the Dow AgroSciences facility archives, Indianapolis, Indiana.

Table 1. Summary of Sampling, Shipping, Receiving, and Processing Dates

DAS[a] Sample Group Number[b]	Sample Description	Shipped From Huxley, IA[c]	Received at Texas A&M[d]	Processing Date[e]	Shipped From Texas A&M[f]	Received at DAS[g]	DAS Moved To -80 °C Freezer
Hux2000C[h]	Control Corn Grain	19-Dec-00	21-Dec-00	NA[i]	NA	NA	NA
Hux2000F[h]	Cry1F Corn Grain	01-Dec-00	05-Dec-00	NA	NA	NA	NA
Wet Milling							
343196	Grain - RAC	NA	NA	31-Jan-01	19-Feb-01	20-Feb-01	22-Feb-01
343218	Steepwater	NA	NA	07-08 Feb01	19-Feb-01	20-Feb-01	20-Feb-01
343226	Germ	NA	NA	07-08 Feb01	19-Feb-01	20-Feb-01	20-Feb-01
343234	Hull	NA	NA	07-08 Feb01	19-Feb-01	20-Feb-01	20-Feb-01
343242	Gluten	NA	NA	07-08 Feb01	19-Feb-01	20-Feb-01	20-Feb-01
343250	Starch	NA	NA	07-08 Feb01	19-Feb-01	20-Feb-01	20-Feb-01
343269	Solvent Extracted Germ	NA	NA	09-Feb-01	19-Feb-01	20-Feb-01	20-Feb-01
343277	Soapstock	NA	NA	14-Feb-01	19-Feb-01	20-Feb-01	20-Feb-01
343285	Crude Oil	NA	NA	13-Feb-01	19-Feb-01	20-Feb-01	20-Feb-01
343293	Refined Oil	NA	NA	14-Feb-01	19-Feb-01	20-Feb-01	20-Feb-01
343307	Bleached/Deodorized Oil	NA	NA	15-Feb-01	19-Feb-01	20-Feb-01	20-Feb-01
Wet Milling - Duplicate Sample							
343315	Grain - RAC	NA	NA	31-Jan-01	19-Feb-01	20-Feb-01	NA
343323	Steepwater	NA	NA	07-08 Feb01	19-Feb-01	20-Feb-01	NA
343331	Germ	NA	NA	07-08 Feb01	19-Feb-01	20-Feb-01	NA
343358	Hull	NA	NA	07-08 Feb01	19-Feb-01	20-Feb-01	NA
343366	Gluten	NA	NA	07-08 Feb01	19-Feb-01	20-Feb-01	NA
343374	Starch	NA	NA	07-08 Feb01	19-Feb-01	20-Feb-01	NA
343382	Solvent Extracted Germ	NA	NA	09-Feb-01	19-Feb-01	20-Feb-01	NA
343390	Soapstock	NA	NA	14-Feb-01	19-Feb-01	20-Feb-01	NA
343404	Crude Oil	NA	NA	13-Feb-01	19-Feb-01	20-Feb-01	NA
343412	Refined Oil	NA	NA	14-Feb-01	19-Feb-01	20-Feb-01	NA
343420	Bleached/Deodorized Oil	NA	NA	15-Feb-01	19-Feb-01	20-Feb-01	NA

Table 1. (Cont.) Summary of Sampling, Shipping, Receiving, and Processing Dates

DAS[a] Sample Group Number[b]	Sample Description	Shipped From Huxley, IA[c]	Received at Texas A&M[d]	Processing Date[e]	Shipped From Texas A&M[f]	Received at DAS[g]	DAS Moved To -80 °C Freezer
Dry Milling							
342963	Grain - RAC	NA	NA	31-Jan-01	12-Feb-01	13-Feb-01	14-Feb-01
342971	Composite Grits	NA	NA	05-Feb-01	12-Feb-01	13-Feb-01	14-Feb-01
342998	Composite Meal	NA	NA	05-Feb-01	12-Feb-01	13-Feb-01	14-Feb-01
343005	Hull	NA	NA	05-Feb-01	12-Feb-01	13-Feb-01	14-Feb-01
343013	Flour	NA	NA	05-Feb-01	12-Feb-01	13-Feb-01	14-Feb-01
343021	Solvent Extracted Germ	NA	NA	06-Feb-01	12-Feb-01	13-Feb-01	14-Feb-01
343048	Soapstock	NA	NA	08-Feb-01	12-Feb-01	13-Feb-01	14-Feb-01
343056	Crude Oil	NA	NA	07-Feb-01	12-Feb-01	13-Feb-01	14-Feb-01
343064	Refined Oil	NA	NA	08-Feb-01	12-Feb-01	13-Feb-01	14-Feb-01
343072	Bleached/Deodorized Oil	NA	NA	09-Feb-01	12-Feb-01	13-Feb-01	14-Feb-01
Dry Milling - Duplicate Sample							
343080	Grain - RAC	NA	NA	31-Jan-01	12-Feb-01	13-Feb-01	16-Feb-01
343099	Composite Grits	NA	NA	05-Feb-01	12-Feb-01	13-Feb-01	16-Feb-01
343102	Composite Meal	NA	NA	05-Feb-01	12-Feb-01	13-Feb-01	16-Feb-01
343110	Hull	NA	NA	05-Feb-01	12-Feb-01	13-Feb-01	16-Feb-01
343129	Flour	NA	NA	05-Feb-01	12-Feb-01	13-Feb-01	16-Feb-01
343137	Solvent Extracted Germ	NA	NA	06-Feb-01	12-Feb-01	13-Feb-01	16-Feb-01
343145	Soapstock	NA	NA	08-Feb-01	12-Feb-01	13-Feb-01	16-Feb-01
343153	Crude Oil	NA	NA	07-Feb-01	12-Feb-01	13-Feb-01	16-Feb-01
343161	Refined Oil	NA	NA	08-Feb-01	12-Feb-01	13-Feb-01	16-Feb-01
343188	Bleached/Deodorized Oil	NA	NA	09-Feb-01	12-Feb-01	13-Feb-01	16-Feb-01

[a] DAS = Dow AgroSciences

[b] For the milled samples, the control sample is sample 01 within the sample group number (SGN) while the transgenic sample is 02 within the SGN.

[c] Date on which samples were shipped from Mycogen in Huxley, IA to Texas A&M for processing.

[d] Date on which samples were received at Texas A&M from Mycogen in Huxley, IA.

[e] Date fraction was generated and collected from Processing (RAC is for date of collection only).

[f] Date on which processed fraction samples were shipped from Texas A&M to Dow AgroSciences.

[g] Date on which processed fraction samples were received at Dow AgroSciences from Texas A&M.

[h] Mycogen Seed sample number.

[i] NA = Not applicable.

Table 2. Size of Samples From Wet Milling

Dow AgroSciences Sample Group Number	Sample Description	Sample Size	
		Control	Transgenic
343196	Grain - RAC	2.0 lbs	2.0 lbs
343218	Steepwater	1 qt	1 qt
343226	Germ	0.5 lbs	0.5 lbs
343234	Hull	2.5 lbs	2.5 lbs
343242	Gluten	2.0 lbs	2.0 lbs
343250	Starch	2.5 lbs	2.5 lbs
343269	Solvent Extracted Germ	1.6 lbs	2.5 lbs
343277	Soapstock	26.4 g	25.4 g
343285	Crude Oil	185.0 g	175.0 g
343293	Refined Oil	180.0 g	170.2 g
343307	Bleached/Deodorized Oil	193.1 g	182.5 g
Duplicate Samples			
343315	Grain - RAC	2.0 lbs	2.0 lbs
343323	Steepwater	1 qt	1 qt
343331	Germ	0.5 lbs	0.5 lbs
343358	Hull	2.5 lbs	2.5 lbs
343366	Gluten	2.0 lbs	2.0 lbs
343374	Starch	2.5 lbs	2.5 lbs
343382	Solvent Extracted Germ	1.6 lbs	2.5 lbs
343390	Soapstock	26.5 g	25.4 g
343404	Crude Oil	185.0 g	175.0 g
343412	Refined Oil	180.0 g	170.2 g
343420	Bleached/Deodorized Oil	193.1 g	182.4 g

Table 3. Size of Samples From Dry Milling

Dow AgroSciences Sample Group Number	Sample Description	Sample Size	
		Control	Transgenic
342963	Grain - RAC	2.0 lbs	2.0 lbs
342971	Composite Grits	2.0 lbs	2.0 lbs
342998	Composite Meal	2.0 lbs	2.0 lbs
343005	Hull	2.0 lbs	2.0 lbs
343013	Flour	0.8 lbs	2.0 lbs
343021	Solvent Extracted Germ	2.0 lbs	2.0 lbs
343048	Soapstock	36.1 g	53.0 g
343056	Crude Oil	125.0 g	200.0 g
343064	Refined Oil	100.2 g	200.1 g
343072	Bleached/Deodorized Oil	145.9 g	212.9 g
Duplicate Sample			
343080	Grain - RAC	2.0 lbs	2.0 lbs
343099	Composite Grits	2.0 lbs	2.0 lbs
343102	Composite Meal	2.0 lbs	2.0 lbs
343110	Hull	2.0 lbs	2.0 lbs
343129	Flour	0.8 lbs	2.0 lbs
343137	Solvent Extracted Germ	2.0 lbs	2.0 lbs
343145	Soapstock	36.2 g	53.1 g
343153	Crude Oil	125.0 g	200.0 g
343161	Refined Oil	100.0 g	200.1 g
343188	Bleached/Deodorized Oil	145.7 g	212.9 g

APPENDIX A—Texas A&M Processing Report - Generation of Corn Processed Products Using Cry1F Corn - Wet Milling

SUMMARY

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

STUDY TITLE

Detection of Cry1F Protein in Dry Milled and Wet Milled Processed Fractions Made from Cry1F Corn Grain

DATA REQUIREMENTS

none

AUTHOR(S)

[redacted]

STUDY COMPLETED ON

January 14, 2002

PERFORMING LABORATORY

Regulatory Laboratories—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

GH-C 5365

Detection of Cry1F Protein in Dry Milled and Wet Milled Processed Fractions Made from Cry1F Corn Grain

SUMMARY

Corn plants have been genetically modified through the introduction of a synthetic gene that encodes for a truncated version of an insecticidal protein, Cry1F, isolated from *Bacillus thuringiensis aizawai* strain PS811. When expressed in corn cultivars, the Cry1F protein confers crop resistance to lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*). The Cry1F corn plants also contain a herbicide-resistant selectable marker gene that expresses the protein phosphinothricin acetyltransferase (PAT). The PAT protein imparts tolerance to glufosinate-ammonium, the active ingredient in Liberty herbicide.

This study was conducted to determine whether the Cry1F protein could be detected by ELISA in wet-milled and dry-milled fractions of corn grain containing BT Cry1F maize line 1507. Corn grain samples were processed into commercially representative fractions (Robb, 2001). During the dry milling process, the grain was exposed to temperatures up to 79 °C during preparation of the grits, meal, hull, flour, and solvent extracted germ. Temperatures up to 91 °C were used in the production of steepwater, germ, hull, gluten, and starch fractions from wet milled grain. Processing of oil and soapstock fractions from dry and wet milling involves exposure to temperatures of 90-104 °C with chemicals such as hexane, NaOH, and bleach. Each of the processed fractions was analyzed using a Cry1F ELISA (manufactured by Strategic Diagnostics Inc., Newark, Delaware, USA) to determine if the Cry1F protein could be detected.

Cry1F protein was detected by ELISA in grain, grits, meal, hull, flour, germ, and gluten from wet and dry milling. Cry1F was not detected in the soapstock due to the destruction to Cry1F protein by temperature and chemicals used in the process, such as NaOH and bleach. These results for Cry1F are consistent with data reported for other transgenic proteins from corn (Chen et al., 2000) and soybean (Chen et al., 2001). In these studies, it was demonstrated that the

transgenic proteins (such as Cry1Ab and 5-enolpyruvylshikimate-3-phosphate synthase) present in MON810 corn and GTS 40-3-2 soybeans can be detected by ELISA and do not break down during the milling process.

STUDY TITLE

Detection of Cry1F Protein in Dry Milled and Wet Milled Processed Fractions Made from Cry1F Corn Grain

DATA REQUIREMENTS

none

AUTHOR(S)

[redacted]

STUDY COMPLETED ON

January 14, 2002

PERFORMING LABORATORY

Regulatory Laboratories—Indianapolis Lab
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, Indiana 46268-1054

LABORATORY STUDY ID

GH-C 5365

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

Compound: Cry1F corn

Title: Detection of Cry1F Protein in Dry Milled and Wet Milled Processed Fractions Made from Cry1F Corn Grain

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: [redacted]

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: Detection of Cry1F Protein in Dry Milled and Wet Milled Processed Fractions Made from Cry1F Corn Grain

Study Initiation Date:	March 1, 2001	Study Completion Date:	
Experimental Start Date:	March 15, 2001	Experiment Termination Date:	May 4, 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

This study does not meet requirements of 40 CFR Part 160.

[redacted] _____ Date _____

Sponsor
Dow AgroSciences LLC

[redacted] _____ Date _____

Submitter
Dow AgroSciences LLC

[redacted] _____ Date _____

Study Director/Author
Dow AgroSciences LLC

QUALITY ASSURANCE STATEMENT

Compound: Cry1F corn

Title: Detection of Cry1F Protein in Dry Milled and Wet Milled Processed Fractions Made from Cry1F Corn Grain

Study Initiation Date: March 1, 2001 Study Completion Date: January 14, 2002

NON-GLP STUDY

SIGNATURE PAGE

[redacted]
Author
Dow AgroSciences LLC

Date

[redacted]
Author
Dow AgroSciences LLC

Date

[redacted]
Author
Dow AgroSciences LLC

Date

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Detection of Cry1F Protein in Dry Milled and Wet Milled Processed Fractions Made from Cry1F Corn Grain

ABSTRACT

Corn plants have been genetically modified through the introduction of a synthetic gene that encodes for a truncated version of an insecticidal protein, Cry1F, isolated from *Bacillus thuringiensis aizawai* strain PS811. When expressed in corn cultivars, the Cry1F protein confers crop resistance to lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*). The Cry1F corn plants also contain a herbicide-resistant selectable marker gene that expresses the protein phosphinothricin acetyltransferase (PAT). The PAT protein imparts tolerance to glufosinate-ammonium, the active ingredient in Liberty herbicide.

This study was conducted to assay for the Cry1F protein in wet-milled and dry-milled fractions of corn grain containing Bt Cry1F maize line 1507. Corn grain samples were processed into commercially representative fractions (Robb, 2001). During the dry milling process, the grain was exposed to temperatures up to 79 °C during preparation of the grits, meal, hull, flour, and solvent extracted germ. Temperatures up to 91 °C were used in the production of steepwater, germ, hull, gluten, and starch fractions from wet milled grain. Processing of oil and soapstock fractions from dry and wet milling involves exposure to temperatures of 90-104 °C with chemicals such as hexane, NaOH, and bleach. Each of the processed fractions was analyzed using a Cry1F ELISA (manufactured by Strategic Diagnostics Inc., Newark, Delaware, USA) to determine if the Cry1F protein could be detected.

Cry1F protein was detected by ELISA in grain, grits, meal, hull, flour, germ, and gluten from wet and dry milling. Cry1F was not detected in the soapstock due to the destruction to Cry1F protein by temperature and chemicals used in the process, such as NaOH and bleach. These results for Cry1F are consistent with data reported for other transgenic proteins from corn (Chen et al., 2000) and soybean (Chen et al., 2001). In these studies, it was demonstrated that the transgenic proteins (such as Cry1Ab and 5-enolpyruvylshikimate-3-phosphate synthase) present

in MON810 corn and GTS 40-3-2 soybeans can be detected by ELISA and do not break down during the milling process.

INTRODUCTION

Corn plants have been genetically modified through the introduction of a synthetic gene that encodes for a truncated version of an insecticidal protein, Cry1F, isolated from *Bacillus thuringiensis aizawai* strain PS811. When expressed in corn cultivars, the Cry1F protein confers crop resistance to lepidopteran pests, including the European corn borer (*Ostrinia nubilalis*). The Cry1F corn plants also contain a herbicide-resistant selectable marker gene that expresses the protein phosphinothricin acetyltransferase (PAT). The PAT protein imparts tolerance to glufosinate-ammonium, the active ingredient in Liberty herbicide.

The purpose of this study was to determine whether the Cry1F protein could be detected by ELISA in wet-milled and dry-milled fractions of corn grain from Bt Cry1F maize line 1507. Each of these processed fractions were analyzed using a Cry1F ELISA manufactured by Strategic Diagnostics Inc., Newark, Delaware, USA.

EXPERIMENTAL

Test Substance

The test substance was processed fractions from dry and wet milling of grain from Bt Cry1F maize line 1507. The fractions collected and temperature exposures for each fraction are listed in Tables 1 and 2. Samples were stored at –80 °C prior to analysis.

Reference Substances

Standard	Lot Number	Protein Concentration, % dry weight	Analysis Date	Reference
Cry1F Truncated Microbial Protein	TSN101788	13.7%	February 2001	BIOT 013056

The Cry1F standard was obtained from the Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Building 304, Indianapolis, IN 46268-1054.

Bio-Rad Protein Assay Standard II Bovine Serum Albumin (Bio-Rad catalog No. 500-0007) was used as a reference substance for the total soluble protein analyses. Storage conditions and stability were according to the manufacturer's instructions.

Generation of Bt Cry1F Maize Line 1507 Processed Products by Dry Milling and Wet Milling

Processing of the whole corn was conducted at the Food Protein Research and Development Center of Texas A&M University in Bryan, Texas, USA. Samples were handled in a manner that minimizes the possibility of contamination with other corn. The dry milled fractions collected were whole corn (RAC), grits, meal, hull, flour, solvent extracted germ, soapstock, crude oil, refined oil, and bleached and deodorized oil. Wet milled corn samples collected were whole corn (RAC), steepwater concentrate, germ, hull, gluten, starch, solvent extracted germ, soapstock, crude oil, refined oil, and bleached and deodorized oil. The fractions were shipped to the Dow AgroSciences facility in Indianapolis, Indiana, USA, and stored at –80 °C (Robb, 2001)

Sample Preparation and Extraction

The sample extraction and preparation was conducted according to the procedure described in Dow AgroSciences analytical method GRM 00.31 (Young, D. L. and Walsh, C. M., 2000). In brief, dry samples were ground in a mortar and pestle or in a Wiley Mill grinder with a 40-mesh sieve. The ground fractions were stored at –80 °C prior to analysis. Liquid samples, such as oil or steepwater, underwent no further processing and were stored at –80 °C. Five milligram samples of the fractions were weighed in a sample tube and a metal bead was added to each tube. The samples were extracted by adding 0.750 mL of PBST (phosphate buffer saline with Tween 20, Sigma catalog No. P3563) to the tubes and placing the tubes in the Geno/Grinder automatic shaker/grinder for at least 5 minutes. The tubes were then centrifuged and the supernatant was transferred to a separate tube and aliquoted for analysis.

All collected fractions were analyzed. Dry milling fractions included whole corn (RAC), grits, meal, hull, flour, solvent extracted germ, soapstock, crude oil, refined oil, and bleached and deodorized oil. Wet milled fractions included whole corn (RAC), steepwater concentrate, germ, hull, gluten, starch, solvent extracted germ, soapstock, crude oil, refined oil, and bleached and deodorized oil.

ELISA Procedure

All samples were analyzed with Dow AgroSciences analytical method GRM 00.31 (Young, D. L. and Walsh, C. M., 2000) using the Cry1F Microtiter Plate ELISA Assay test kit (Part No. 7020000) available from Strategic Diagnostics, Inc. The assay uses a double antibody sandwich ELISA format. A 0.10-mL aliquot of the diluted sample is incubated with 0.10 mL of enzyme-conjugated anti-Cry1F protein monoclonal antibody in the wells of anti-Cry1F polyclonal antibody coated ELISA plate in a sandwich format. Both antibodies in the sandwich pair capture the truncated Cry1F protein in the sample. After an incubation period, the plate is washed to remove unbound reagents and sample. A second incubation is performed with a Color Reagent (TMB), the enzyme reaction is stopped and the color intensity of the samples is measured using a

microplate reader. The concentration of Cry1F protein in each sample is calculated from a standard curve generated using Cry1F truncated microbial protein.

The ELISA that was used for this analysis is highly specific to the active form of the Cry1F protein. In a study by Gao and Collins (2001), the ELISA was shown to detect native Cry1F protein, but not heat-treated, denatured Cry1F protein. In addition, lack of cross-reactivity with other Bt proteins has been demonstrated (see Appendix A).

Total Soluble Protein

The total extractable protein (TEP) concentration of the supernatant was determined by the Bradford method (Bradford, 1976) using the microtiter plate application of the Bio-Rad Protein Assay reagent (Bio-Rad catalog No. 500-0006). Bio Rad's Protein Assay Standard II was used as the protein standard.

Calculations

The absorbance of the standard calibration solutions was measured at 450 nm and a standard curve was generated from a quadratic regression.

Statistical Treatment of Data

Statistical treatment of data included the calculation of means, standard deviations, relative standard deviations, and quadratic regression correlation coefficients.

RESULTS AND DISCUSSION

Cry1F protein was detected by ELISA in grain, grits, flour, germ, meal, and hull from dry milled fractions (Table 1), and grain, germ, hull, gluten, and solvent extracted germ in the wet milled fractions (Table 2). Soapstock fractions from both wet and dry milled processing showed no detectable Cry1F protein, although significant total extractable protein (TEP) remained. Additionally, little TEP and no Cry1F protein were detected in the steepwater, starch, or oil fractions.

During dry milling, the corn grain was heated to a maximum of 71 °C for 30 minutes. For processing of the solvent extracted germ fraction, an additional 10 minute treatment at a temperature range of 71-79 °C was used. Those temperature treatments did not destroy immunoreactivity of the Cry1F protein in the ELISA (Gao, 2002). However, the addition of NaOH at temperatures from 73-90 °C for the production of the soapstock fraction, did eliminate all Cry1F protein detectable by ELISA, although a significant amount of total protein remained in the fraction. During wet milling, the corn grain was steeped at temperatures up to 54 °C for 22 to 48 hours. The germ and hull were additionally heated at temperatures ranging from 74 to 91 °C for a variable length of time during drying. The fractions subjected to those temperature treatments contained Cry1F protein that was detectable by ELISA. Treatment with NaOH at 73-90°C to produce the soapstock fraction eliminated Cry1F protein detection, but TEP level remained high. These results indicate that although the Cry1F protein polypeptide chain did not break down during the wet and dry milling processes, Cry1F protein was inactivated or broken down when the corn was processed with the more severe conditions (higher temperature and alkaline conditions) used to make soapstock.

In other studies (Young and Herman 1999, Mayes 1999) more stringent heating conditions were applied to grain from Event 1507. During fish food preparation, Cry1F containing corn meal was heated at approximately 104 °C for 15-30 min with a one time heating step up to 177 °C. The Cry1F protein could not be detected in the fish food by ELISA, nor the insecticidal activity by an insect bioassay, indicating that the functional three-dimensional structure of Cry1F was

destroyed during the preparation of the fish food. Additionally, a heat lability study with Cry1F protein demonstrated that the Cry1F protein lost biological activity following exposure for 30 minutes at temperatures greater than or equal to 75 °C (Herman 2000).

Like many other Bt endotoxins, Cry1F is a heat-labile protein. Our studies have shown consistent results with both recombinant (microbial) Cry1F protein and Cry1F transgenic corn grain and grain products. It was demonstrated that microbial Cry1F protein was stable to treatment of 60 °C for 30 min (as evidenced by biological activity data) but the protein was deactivated and lost biological activity after heat treatment at 75 °C for 30 min (Herman 2000). This result indicates the destruction and breakdown of the tertiary and secondary structure of the Cry1F protein occurs with at least a 30 minute exposure to temperatures 75 °C or greater. The wet and dry milled processed fractions were generally treated at temperatures under 75 °C and they also demonstrated stability of the Cry1F protein under those conditions.

The results for Cry1F are consistent with data reported for other transgenic proteins from corn (Chen et al., 2000) and soybean (Chen et al., 2001). In these studies, it was demonstrated that the transgenic proteins (such as Cry1Ab and 5-enolpyruvylshikimate-3-phosphate synthase) present in MON810 corn and GTS 40-3-2 soybeans can be detected by ELISA and do not break down during the milling process.

CONCLUSIONS

Cry1F protein was detected by ELISA in grain, grits, meal, hull, flour, germ, and gluten from wet and dry milling of event 1507 Cry1F corn grain. Cry1F was not detected in the soapstock due to the destruction to Cry1F protein by temperature and chemicals used in the process, such as NaOH and bleach. These results are consistent with data reported for other transgenic proteins from corn and soybean.

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8. Young, D. L. and Herman, R. A., "Characterization of Expressed Cry1F Protein in Maize Tissue (Pollen, Grain, Grain-Containing Feed, and Purified Maize-Expressed Cry1F Protein) and Microbial expressed Cry1F Delta Endotoxin by Biological and Biochemical Procedures", 1999, Study ID 990027, unpublished report of Dow AgroSciences LLC.

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Table 1. Cry1F and Total Extractable Protein Levels for Dry Milled Fractions, Including Temperature Exposures for Each Fraction

Sample ID	Process Fraction	Temperature (°C)	Duration (minutes)	Cry1F[a] (ng/mg)[b]	Total Protein[a] (µg/mg)[b]
34296302	Grain	na[c]	na	3.85	78.8
34297102	Grits	54-71	30	4.13	49.1
34299802	Meal	54-71	30	3.72	38.8
34300502	Hull	54-71	30	1.89	9.7
34301302	Flour	54-71	30	1.99	16.2
34302102	Solvent Ext.Germ	71-79(in hexane)	10	8.25	137.0
34304802	Soapstock	73-90 (in NaOH)	variable	0.00	60.9
34305602	Crude Oil	na	na	0.00	0.0
34306402	Refined Oil	na	na	0.00	0.0
34307202	Deodorized Oil	na	na	0.00	0.0

[a] Values represent mean value of 2 – 3 replicate samples.
[b] Fresh weight.
[c] na = not applicable.

Table 2. Cry1F and Total Extractable Protein Levels for Wet Milling Fractions, Including Temperature Exposures for Each Fraction

Sample ID	Process Fraction	Temperature (°C)	Duration (hours)	Cry1F[a] (ng/mg)[b]	Total Protein[a] (µg/mg)[b]
34319602	Grain	na[c]	na	3.15	75.0
34321802	Steepwater	49-54	22-48	0.00	8.6
34324202	Gluten	49-54	22-48	0.99	10.3
34325002	Starch	49-54	22-48	0.00	5.2
34322602	Germ	74-91	variable	6.77	133.0
34323402	Hull	74-91	variable	0.46	10.8
34326902	Solvent Ext.Germ	49-60 (in hexane)	1	2.12	50.7
34327702	Soapstock	73-90 (in NaOH)	variable	0.00	53.5
34328502	Crude Oil	na	na	0.00	0.0
34329302	Refined Oil	na	na	0.00	0.0
34330702	Deodorized Oil	na	na	0.00	0.0

[a] Values represent mean value of 2 – 3 replicate samples.
[b] Fresh weight.
[c] na = not applicable.

Appendix A—Cross-reactivity of Cry1F ELISA with other Bt proteins

Table 1. Absorbance readings at 650 nm for Cry1F and other Bt proteins across a range of concentrations using the Cry1F Microtiter Plate ELISA Assay test kit (Part No. 7020000, Strategic Diagnostics, Inc.)

conc (ng/mL)	Cry1F	Cry1Ab	Cry1Ac	Cry1Bd	Cry9A	Cry9B/ 1Ac	Cry9C	Cry9D	Cry9E
10000.000	1.339	0.115	0.136	0.121	0.112	0.247	0.084	0.367	0.172
5000.000	0.878	0.109	0.123	0.190	0.097	0.160	0.074	0.213	0.110
2500.000	0.508	0.099	0.105	0.112	0.101	0.118	0.074	0.155	0.107
1250.000	0.308	0.121	0.100	0.096	0.102	0.092	0.069	0.094	0.087
625.000	0.207	0.123	0.173	0.078	0.080	0.078	0.078	0.089	0.119
312.500	0.151	0.112	0.173	0.077	0.110	0.086	0.086	0.080	0.173
156.250	0.141	0.140	0.101	0.095	0.079	0.075	0.077	0.088	0.136
78.125	---	0.127	0.111	0.111	0.094	0.079	0.073	0.080	0.113

Unpublished data provided by Strategic Diagnostics, Inc.