UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

SUBJECT: EPA Review of the Product Characterization and Human Health Data in Support of the Experimental Use Permit (EUP) Application for the Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton, Submitted by Dow AgroSciences [ID#: 068467-EUP-A; Submission: S614436; DP Barcode: D282687; Case: 071326; 23 Studies MRID#'s: 45607901-03; 45542301-14; 45542318-22, 45542324 and the Revised EUP Protocols, dated November 6, 2002].

TO: Leonard Cole (PM-90)
Regulatory Action Leader
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

FROM: Sharlene R. Matten, Ph.D., Biologist
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

THROUGH: John L. Kough, Ph.D., Senior Scientist
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

ACTION REQUESTED: To review product characterization, human health data, preliminary field efficacy data, and EUP Protocols submitted by Dow AgroSciences to support their application for an Experimental Use Permit for Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton

CONCLUSIONS

The product characterization, human health, insect resistance management plan, preliminary field efficacy data, and the Section G EUP protocols (with the exception of the containment conditions for the Puerto Rico sites) are adequate to support the granting of an Experimental Use Permit (EUP) for cotton expressing the Cry1F (synpro), Cry1Ac (synpro), and PAT proteins. Based on review of the data, there is a reasonable certainty of no harm to humans and animals based on minimal toxicity and exposure to these proteins under the conditions of the EUP.

Under the proposed EUP program, transgenic Cry1F/Cry1Ac will only be grown on 2826 acres spread across 14 states (AR, AL, AZ, CA, FL, GA, LA, MO, MS, NC, NM, SC, TN, and TX) and Puerto Rico. Under the conditions of the EUP, the use of Dow AgroScience's Cry1F + Cry1Ac transgenic cotton is expected to pose a minimal risk to human health and the environment. Minimal exposure to the Cry1F or Cry1Ac proteins is expected for humans, animals, and other non-target organisms and target pest susceptibility is expected to remain unchanged (i.e., limited selection pressure) due to the following reasons: limited exposure to either Cry1F or Cry1Ac because of limited total acreage (2826 A) and limited acreage at any one location for each protocol, containment provisions to mitigate gene flow, and limited duration (one year) of the EUP. Because of the reasons stated above, the likelihood of insect resistance evolution is extremely minimal to non-existent. Dow's IRM plan is acceptable for the EUP.

Based upon the human health data provided, there does not appear to be a significant risk for toxic and or allergenic effects to humans or animals due to exposure to the Cry1F (synpro), Cry1Ac (synpro), or PAT proteins. The Cry1F and Cry1Ac proteins are classified as Toxicity Category III: $LD_{50.}$> 700 mg/kg body weight for Cry1Ac, $LD_{50.}$> 600 mg/kg body weight for Cry1F and $LD_{50.}$> 375 mg Cry1F/kg body weight and LD_{50}>350 Cry1Ac mg/kg body weight for the stacked Cry1F/Cry1Ac proteins. Cry1Ac and Cry1F proteins are not stable to digestion in simulated gastric fluid (<1 min), nor do they share significant sequence similarity to known toxins or allergens. In addition, no Cry1 proteins have been implicated in toxic and/or allergenic reactions in humans or animals.

Product characterization data indicate that plant-produced and bacterially-produced Cry1F protein is equivalent. While adequate for an EUP additional data are needed for the biochemical equivalency comparisons for Cry1Ac. Southern blot data of restriction enzyme digests suggest that the Cry1Ac single gene event, Cry1F single gene event, and the stacked Cry1F/Cry1Ac cotton events contain a single, unique, insertion of the transgenic DNA from the appropriate plasmids. Based on the segregation analyses, transgenic cotton lines carrying *cry1F* (synpro) or *cry1Ac* (synpro) alone, or stacked *cry1F* (synpro)*/cry1Ac* (synpro) exhibited stable Mendelian inheritance of the insect resistance traits.

Preliminary expression data indicate the range of the mean values of Cry1F protein is: young leaves (4.52- 5.39 ng/mg tissue), terminal leaves (4.49-4.98 ng/mg tissue), squares (4.71-5.86 ng/mg tissue) and pollen (0.05-0.20 ng/mg tissue). Detection of Cry1F in pollen was sporadic. Cry1F was not detectable in nectar. The range of the mean values of Cry1Ac protein is: young leaves (0.93-1.58 ng/mg tissue), terminal leaves (0.53-1.08 ng/mg tissue), squares (0.51-0.78 ng/mg tissue) and pollen

(0.45-0.71 ng/mg tissue). Cry1Ac was not detected in nectar. Overall, Cry1Ac was expressed at much lower levels (approximately 4-5 times lower) than Cry1F in any of the plant tissues examined, except for pollen.

Preliminary data on efficacy of Cry1Ac cotton (event 3006-210-23) and Cry1F+Cry1Ac cotton (event 281-24-236/3006-210-23) indicate virtually complete control of the tobacco budworm and the pink bollworm. However, the efficacy of the Cry1F event 281-24-236 showed complete control of tobacco budworm, but the control of pink bollworm did not differ from that of the control.

RECOMMENDATIONS

The Cry1Ac cotton-insect-pest susceptibility study (MRID 455423-07) is "supplemental, but not upgradeable," the study should not be redone. The field efficacy studies would indicate the susceptibility to both Cry1Ac and Cry1F proteins under actual field use conditions and these data are more reliable than the laboratory bioassays. Field efficacy studies are one component of the proposed EUP program. The preliminary field efficacy studies (MRID 455423-04) are acceptable.

There are four studies that are "supplemental, pending the final report." Final data reports are needed for these studies to be ungraded to acceptable. These four interim studies are:

455423-01	Characterization of DNA Inserted into Transgenic Cry1F Cotton Event 281-24-236 - Interim Report
455423-02	Molecular Characterization of Cry1F (synpro)/Cry1Ac (synpro) Stacked Transgenic Cotton Events 281-48-81 and 3006-210-23 - Interim Report
456079-03	Molecular Characterization of Cry1Ac (synpro) Transgenic Cotton Events 3006-48-81 and 3006-210-23 - Interim Report
455423-22	Field Expression of Cry1F (synpro), Cry1Ac (synpro), and Phosphinothricin Acetyltransferase (PAT) Proteins in Transgenic Cotton Plants, Cottonseed, and Cottonseed Processed Products - Interim Report

The biochemical equivalency of Cry1Ac study is classified as "supplemental, upgradeable with additional data." This means that additional biochemical equivalency data are needed to upgrade this study to acceptable prior to a regulatory decision for Section 3 full commercial registration. The study is:

455423-06	Biochemical Characterization of Cry1Ac derived from Transgenic Cotton and *Pseudomonas fluorescens;*

Additionally, heat stability (stability of the Cry1Ac, Cry1F, and PAT proteins under food processing

conditions) data are needed to support a Section 3 full commercialization registration. These data would support the conclusion that these proteins pose minimal allergenic potential.

Exemptions for the Requirement of a Tolerance, Cry1F, Cry1Ac, and PAT
An existing tolerance exemption, CFR 40 Section180.l155, exists for *Bacillus thuringiensis* subsp. *kurstaki* Cry1Ac and the genetic material necessary for its production in all plants. An existing tolerance exemption, CFR 40 Section180.1151, exists for phosphinothricin acetyltransferase (PAT) and the genetic material necessary for its production in all plants. Data provided by Dow AgroSciences are adequate to support these existing tolerance exemptions for Cry1Ac and PAT. A tolerance exemption, CFR 40 Section 180.1217, exists for *Bacillus thuriengiensis* Cry1F protein and the genetic material necessary for its production in corn. The data submitted would support extending the tolerance exemption to include cotton. Dow AgroSciences has provided data, including analytical method development (Lateral Flow Strips and ELISA) and ELISA validation, to support amending the existing tolerance exemption to include Cry1F protein and the genetical material necessary for its production in cotton. The analytical methods for Cry1Ac and Cry1F proteins are reviewed under separate cover.

EXPERIMENTAL USE PERMIT (REVISED SECTION G, NOVEMBER 6, 2002)

The proposed EUP program (Revised Section G, November 6, 2002) is acceptable (EPA No. 68467-EUP-A) with the exception of the containment and isolation provisions for Puerto Rico. In light of the paucity of data on the distribution of feral cotton in the U.S. Virgin Islands and Puerto Rico, the following mitigation measures should be taken to mitigate gene flow concerns during the EUP: test plots or breeding nurseries in Puerto Rico must be surrounded by at least 24 border rows of a suitable pollinator trap crop regardless of the plot size and must not be planted within 3 miles of feral cotton plants. Alternatively, these sites can be eliminated from the EUP protocols. For Florida, there should be no planting of transgenic Bt cotton south of route 60 (near Tampa) in Florida. The EUP program contains no sites in Florida south of route 60.

Under the proposed EUP program, transgenic Cry1F/Cry1Ac will only be grown on 2826 acres spread across 14 states (AR, AL, AZ, CA, FL, GA, LA, MO, MS, NC, NM, SC, TN, and TX) and Puerto Rico. Under the conditions of the EUP, the use of Dow AgroScience's Cry1F + Cry1Ac transgenic cotton is expected to pose a minimal risk to human health and the environment. Minimal exposure to the Cry1F or Cry1Ac proteins is expected for humans, animals, and other non-target organisms and target pest susceptibility is expected to remain unchanged (i.e., limited selection pressure) due to the following reasons: limited exposure to either Cry1F or Cry1Ac because of limited total acreage (2826 A) and limited acreage at any one location for each protocol, containment provisions to mitigate gene flow, and limited duration (one year) of the EUP. Because of the reasons stated above, Dow's IRM plan is acceptable for the EUP.

CLASSIFICATION: The EUP program is classified as supplemental pending revision of

containment and isolation provisions for Puerto Rico or elimination of those sites.

DATA REVIEW RECORD FOR PRODUCT CHARACTERIZATION AND HUMAN HEALTH DATA

Active Ingredient(s): Cry1Ac (synpro) Protein (*Bacillus thuringiensis* subsp. *kurstaki* HD-73) and Cry1F (synpro) Protein (*Bacillus thuringiensis* var. *aizawai* strain PS811 (NRRL B-18484)) as Expressed in Cotton and the DNA which encodes for these Proteins

Product Name: Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton

Company Name: Dow AgroSciences, Indianapolis, IN

ID No: 068467-EUP-A

Chemical Number: 071326

Submission: S614436

DP Barcode: D282687

MRID No:

Product Characterization and Identity (885.1100)

455423-22	Field Expression of Cry1F (synpro), Cry1Ac (synpro), and PAT Proteins in Transgenic Cotton Plants, Cottonseed, and Cottonseed Processed Products
456079-02	Product Characterization of Cry1F (synpro) Protein
456079-01	Product Characterization of Cry1Ac (synpro) Protein
455423-01	Molecular Characterization of Cry1F (synpro) Transgenic Cotton Event 281-24-236
456079-03	Molecular Characterization of Cry1Ac (synpro) Transgenic Cotton Events 3006-48-81 and 3006-210-23
455423-02	Molecular Characterization of Cry1F (synpro)/Cry1Ac (synpro) Stacked Transgenic Cotton Events 281-24-236/3006-48-81 and 281-24-236/3006-210-23
455423-03	Biological Equivalency of Transgenic Cotton- and *Pseudomonas*-expressed Cry1F Proteins
455423-04	Biological Equivalency of Transgenic Cotton- and *Pseudomonas*-expressed Cry1Ac Proteins
455423-05	Biochemical Characterization of Cry1F derived from Transgenic Cotton and *Pseudomonas fluorescens*
455423-06	Biochemical Characterization of Cry1Ac derived from Transgenic Cotton and *Pseudomonas fluorescens*

455423-07 Cotton-Insect-Pest Susceptibility Study - Microbial B.t. Cry1F (synpro) Protein

455423-08 Cotton-Insect-Pest Susceptibility Study - Microbial B.t. Cry1Ac (synpro) Protein

455423-24 Field Efficacy of Cry1F (synpro), Cry1Ac (synpro) and Cry1F + Cry1Ac Stack Insecticidal Crystalline proteins from *Bacillus thuringiensis* var. *aizawai* strain PS811 and *Bacillus thuringiensis* subspecies *kurstaki* strain HD73 Cotton Events Against Tobacco Budworm and Pink Bollworm

Human Toxicity and Allergenicity

455423-12 Acute Oral Toxicity: Cry1F (synpro)Bacterial Protein (885.3050)

455423-13 Acute Oral Toxicity: Cry1Ac (synpro) Bacterial Protein (885.3050)

455423-14 Acute Oral Toxicity: Cry1F (synpro) + Cry1Ac (synpro) Bacterial Proteins (885.3050)

455423-09 Comparison of Amino Acid Sequence with Known Allergens: Cry1F (synpro) Protein as Expressed in Cotton

455423-10 Comparison of Amino Acid Sequence with Known Allergens: Cry1F (synpro) Protein as Expressed in Cotton

455423-11 Comparison of Amino Acid Sequence with Known Allergens: PAT Protein as Expressed in Cotton

455423-18 *In vitro* Digestibility of Bacterially-derived Cry1F (synpro)

455423-19 *In vitro* Digestibility of Bacterially-derived Cry1Ac (synpro)

455423-20 Thermolability of Cry1F (synpro) Delta-Endotoxin

455423-21 Thermolability of Cry1Ac (synpro) Delta-Endotoxin

BACKGROUND:

Dow AgroSciences has submitted the above product characterization, human health, and field efficacy data to support their application for an Experimental Use Permit for Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton.

Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281-3006 Cotton expresses both the Cry1F (synpro) and Cry1Ac (synpro) insect control proteins. These transgenic cotton plants also express phosphinothricin acetyl transferase (PAT) that confers tolerance to the herbicide glufosinate ammonium. A synthetic *cry1F* (synpro) transgene optimized for plant codon usage was transformed into cotton. The expressed Cry1F (synpro) protein effectively controls certain lepidopteran pests, e.g., *Heliothis virescens* (tobacco budworm). Similarly, a plant optimized *cry1Ac* (synpro) transgene was transformed into cotton. The expressed Cry1Ac (synpro) protein effectively controls certain lepidopteran pests, e.g., *Heliothis virescens* (tobacco budworm) and cotton bollworm (*Helicoverpa zea*), and pink bollworm (*Pectinophora gossypiella*. Cotton lines carrying the single events of Cry1F

and Cry1Ac were developed through a series of backcrosses and self-pollinations. Cotton lines containing both the Cry1F and Cry1Ac traits (i.e., stack) were developed by crossing a backcrossed BC3F1 line containing the Cry1F event to a backcrossed BC3F1 line containing one of the Cry1Ac events. The stacked event to be evaluated under the EUP is 281-24-236/3006-210-23

SUMMARY OF DATA SUBMITTED:

Summaries and discussion of each review supporting the product characterization, field efficacy, and human health safety of these products are provided below.

Product Characterization (885.1100)

455423-22 Field Expression of Cry1F (synpro), Cry1Ac (synpro), and PAT Proteins in Transgenic Cotton Plants, Cottonseed, and Cottonseed Processed Products

The purpose of this study is to provide quantitative data on the amount of Cry1F and Cry1Ac proteins in several different transgenic cotton events. This interim report provides quantitative data on the amount of Cry1F and Cry1Ac proteins in several different transgenic cotton events. Single gene events consisted of: events 281-24-236 (Cry1F alone), events 3006-210-23 (Cry1Ac alone), and events 3006-48-81(Cry1Ac alone). The stacked events consisted of: events 281-24-236 (Cry1F)/3006-210-23 (Cry1Ac) and events 281-24-236 (Cry1F)/3006-48-81 (Cry1Ac). The interim report contains data regarding the expression of Cry1F and Cry1Ac proteins in young leaves, terminal leaves, squares, pollen, and nectar.

The range of the mean values of Cry1F protein in these three events is: young leaves (4.52- 5.39 ng/mg tissue), terminal leaves (4.49-4.98 ng/mg tissue), squares (4.71-5.86 ng/mg tissue) and pollen (0.05-0.20 ng/mg tissue). Detection of Cry1F in pollen was sporadic. Cry1F was not detectable in nectar. The range of the mean values of Cry1Ac protein in these four events is: young leaves (0.93-1.58 ng/mg tissue), terminal leaves (0.53-1.08 ng/mg tissue), squares (0.51-0.78 ng/mg tissue) and pollen (0.45-0.71 ng/mg tissue). Cry1Ac was not detected in nectar. As expected, the Cry1F and Cry1Ac proteins were not detected in leaf, square, pollen or nectar samples from non-transgenic (null) cotton plants grown in the control plots. The absence of any detectable Cry1F or Cry1Ac protein in young leaves, terminal leaves, squares, pollen, and nectar in the control plants indicates the integrity of the samples was maintained from field to laboratory and that the serologically based assay was specific for the target protein.

Cry1F and Cry1Ac proteins were expressed in young leaves, terminal leaves, squares, and pollen. Cry1F was expressed in much higher levels in young leaf, terminal leaf, and square samples than either pollen (about 50 X lower) or nectar (not detectable). In some pollen samples, Cry1F was not detected. Cry1Ac was expressed at much lower levels (approximately 4-5 times lower) than Cry1F in any of the plant tissues examined, except for pollen. While lower than Cry1F, levels of Cry1Ac were found to be similar in terminal leaves, squares, and pollen. Somewhat higher levels of Cry1Ac were

found in young leaves than the other plant tissues. Cry1Ac and Cry1F proteins were not detectable in nectar. Total protein levels per tissue were comparable in control and transgenic cotton lines. Pollen contained the greatest amount of protein followed by young leaves and squares. Terminal leaves, nectar, seed, or final products were not analyzed for total protein for this interim report.

CLASSIFICATION: SUPPLEMENTAL, PENDING FINAL REPORT

456079-02 Product Characterization of Cry1F (synpro) Protein

The purpose of this study is to characterize the transgenes (*cry1F* and *pat*). Data on all genetic elements used in the transformation, the method of transformation, and the stability of inheritance are discussed.

A synthetic *cry1F* (synpro) transgene optimized for plant codon usage was transformed into cotton using *Agrobacterium tumefaciens* strain LBA440 for plant transformation. Cry1F (synthetic protoxin = synpro) is a chimeric, full-length delta-endotoxin comprised of the first 604 amino acids of the insecticidal protein of Cry1Fa2 from *Bacillus thuringiensis* var. *aizawai* strain PS811 (NRRL B-18484) and 544 amino acids from the nontoxic portion of Cry1Ca3 (residues 605-640) and Cry1Ab1 (residues 641-1148) proteins. The Cry1Ca3 and Cry1Ab1 portions comprise the chimeric C-terminal domain which are removed by alkaline proteases in the insect gut during formation of the active Cry1Fa2 core insecticidal protein. The transgenic Cry1F (synpro) insecticidal protoxin is identical in amino acid sequence to the chimeric Cry1F/Cry1Ab protoxin produced by the recombinant *Pseudomonas fluorescens* strain MR872 used in the equivalency studies with the exception of 4 amino acid residues. The native *pat* gene was also modified and optimized. The expressed Cry1F (synpro) protein effectively controls certain lepidopteran pests, e.g., *Heliothis virescens* (tobacco budworm). The presence of the transgenes (*cry1F* and *PAT*) was verified by Southern blot analysis. Segregation analysis was performed using glufosinate to identify the presence of absence of the PAT selectable marker protein and qualitative ELISA strips for the Cry1Ac and Cry1F proteins. Single gene Events consisted of: Events 281-24-236 (Cry1F), Events 3006-210-23 (Cry1Ac), and Events 3006-48-81(Cry1Ac). The stacked Events consisted of: Events 281-24-236/3006-210-23 and Events 281-24-236/3006-48-81. Based on the segregation analyses, transgenic cotton lines carrying *cry1F* (synpro) alone or stacked with *cry1Ac* (synpro) exhibited stable Mendelian inheritance of the insect resistance traits.

CLASSIFICATION: ACCEPTABLE

456079-01 Product Characterization of Cry1Ac (synpro) Protein

The purpose of this study is to characterize the transgenes (*cry1Ac* and *pat*). Data on all genetic elements used in the transformation, the method of transformation, and the stability of inheritance are discussed.

A synthetic *cry1Ac* (synpro) transgene optimized for plant codon usage was transformed into cotton using *Agrobacterium tumefaciens* strain LBA440 for plant transformation. Cry1Ac (synthetic protoxin = synpro) is a chimeric, full-length delta-endotoxin comprised of the first 613 amino acids of the core insecticidal protein of Cry1Ac1 from *Bacillus thuringiensis* var. *kurstaki* strain HD73 and 543 amino acids from the nontoxic portions of Cry1Ca3 (residues 614-648) and Cry1Ab1 (residues 649-1156) proteins. The Cry1Ca3 and Cry1Ab1 portions comprise the chimeric C-terminal domain. These portions are removed by alkaline proteases in the insect gut during formation of the active Cry1Ac1 core insecticidal protein. The transgenic Cry1Ac (synpro) insecticidal protoxin has 1156 amino acids. The native *pat* gene was modified and codon optimized to provide for better expression *in planta.* The expressed Cry1Ac (synpro) protein effectively controls certain lepidopteran pests, e.g., *Heliothis virescens* (tobacco budworm) and cotton bollworm (*Helicoverpa zea*). The presence of the transgenes (*cry1Ac* and *PAT*) was verified by Southern blot analysis. Segregation analysis was performed using glufosinate to identify the presence of absence of the PAT selectable marker protein and qualitative ELISA strips for the Cry1Ac and Cry1F proteins. Single gene Events consisted of: Events 281-24-236 (Cry1F), Events 3006-210-23 (Cry1Ac), and Events 3006-48-81 (Cry1Ac). The stacked Events consisted of: Events 281-24-236/3006-210-23 and Events 281-24-236/3006-48-81. Based on the segregation analyses, transgenic cotton lines carrying *cry1F* (synpro) alone or stacked with *cry1Ac* (synpro) exhibited stable Mendelian inheritance of the insect resistance traits.

CLASSIFICATION: ACCEPTABLE

455423-01 **Molecular Characterization of Cry1F (synpro) Transgenic Cotton Event 281-24-236**

The purpose of this study was to characterize the insertion of the transgenes (*cry*1F and *pat*) in transgenic cotton (Event 281-24-236) and to demonstrate the absence of the gene encoding the erythromycin resistance (ery^R). DNA extracted from cotton leaf tissue was used in the Southern blot analysis to determine integration pattern, gene copy number, and the absence of ery^R.

Southern blot data of restriction enzyme digests suggest that there was a single, unique, insertion of the insecticidal resistance gene, *cry*1F, and the plant selectable marker gene, *pat*, in the transgenic cotton event 281-24-236. In addition, data suggest that a second *pat* hybridizing fragment may be present. However, it was not possible to determine the precise manner in which the additional copy of *pat* was integrated into the plant genome. The data confirm that the bacterial erythromycin resistance gene, ery^R, on plasmid pAGM281 was not integrated in transgenic cotton event 281-24-236.

CLASSIFICATION: SUPPLEMENTAL, PENDING FINAL REPORT

456079-03 **Molecular Characterization of Cry1Ac (synpro) Transgenic Cotton Events 3006-48-81 and 3006-210-23**

The purpose of this study was to characterize the insertion of the transgenes (*cry1Ac* and *pat*) in transgenic cotton (Events 3006-48-81 and 3006-210-23) and to demonstrate the absence of the gene encoding the erythromycin resistance (ery^R). DNA extracted from cotton leaf tissue was used in the Southern analysis to determine integration pattern, gene copy number, and the absence of ery^R.

Southern blot data of restriction enzyme digests suggest that there is a single, unique, insertion of the insecticidal resistance gene, *cry1Ac*, and the plant selectable marker gene, *pat*, in both transgenic cotton events 3006-48-81 and 3006-210-23. These data also indicate that a single, intact copy of both the *cry1Ac* gene and *pat* gene have been integrated into the cotton genome of events 3006-48-81 and 3006-210-23. The data confirm that the bacterial erythromycin resistance gene, ery^R, on plasmid pMYC 3006 has not been integrated in either transgenic cotton event 3006-48-81 or 3006-210-23.

CLASSIFICATION: SUPPLEMENTAL, PENDING FINAL REPORT

455423-02 Molecular Characterization of Cry1F (synpro)/Cry1Ac (synpro) Stacked Transgenic Cotton Events 281-24-236/3006-48-81 and 281-24-236/3006-210-23

The purpose of this study was to characterize the insertion of the transgenes (*cry1Ac*, *cry1F*, and *pat*) in transgenic cotton (Events 281-24-236/3006-48-81 and 281-24-236/3006-210-23) and to demonstrate the absence of the gene encoding the erythromycin resistance (ery^R). DNA extracted from cotton leaf tissue was used in the Southern analysis to determine integration pattern, gene copy number, and the absence of ery^R.

Southern blot data of restriction enzyme digests suggest that the stacked transgenic cotton Events 281-24-236/3006-48-81 and 281-24-236/3006-210-23 contain a single, unique, insertion of the transgenic DNA from pAGM281 transgene and pMYC3006 transgene. The insert from pAGM281 contains one intact copy of *cry1F* and one intact copy of *pat* (plant selectable marker gene, phosphinothricin acteyltransferace). The insert from pMYC3006 contains one intact copy of *cry1Ac* and one intact copy of *pat*. An additional hybridizing fragment of *pat* has been integrated into the cotton genome, but it is not possible from the data to determine how it was integrated. The data confirm that the bacterial erythromycin resistance gene, ery^R, on plasmid pMYC 3006 and on plasmid PAGM281 has not been integrated in the transgenic cotton Events 281-24-236/3006-48-81 and 281-24-236/3006-210-23.

CLASSIFICATION: SUPPLEMENTAL, PENDING FINAL REPORT

455423-03 Biological Equivalency of Transgenic Cotton- and *Pseudomonas*-expressed Cry1F Proteins

The activity of Cry1F (synpro) derived from *Pseudomonas* and transgenic cotton was similar based on the insect bioassays using TBW, CBW, and BAW. TBW was the most sensitive species followed by BAW and lastly, CBW. A GI_{80} value was used to calculate valid point estimates of growth inhibition

because the data best bracketed this value. A precise GI_{80} could not be calculated for CBW because of the low concentration of Cry1F (0.053% w/w) in the transgenic cotton plant powder and the plant extract effects at high concentrations. That is, as noted in Greenplate (1999), there are other insecticidal compounds in cotton leaves that affect the control of lepidopteran species such as CBW, TBW, or BAW, and contribute to the effects of the bioassay.

CLASSIFICATION: ACCEPTABLE

455423-04 **Biological Equivalency of Transgenic Cotton- and *Pseudomonas*-expressed Cry1Ac Proteins**

The activity of Cry1Ac (synpro) derived from *Pseudomonas* and transgenic cotton was similar based on the insect bioassays using TBW, CBW, and BAW. TBW was the most sensitive species followed by CBW and lastly, BAW. A GI_{50} value was used to calculate valid point estimates of growth inhibition because the data from the two test substance sources best bracketed this value. Results of the insect bioassay using transgenic cotton-expressed Cry1Ac were highly variable.

CLASSIFICATION: ACCEPTABLE

455423-05 **Biochemical Characterization of Cry1F derived from Transgenic Cotton and *Pseudomonas fluorescens***

Several biochemical methods were used to assess the equivalency of the plant-expressed and *P. fluorescens*-expressed Cry1F proteins. Based on the results of these biochemical analyses, it can be determined that the plant-expressed (Event 281) and *P. fluorescens*-expressed Cry1F proteins are biochemically equivalent.

SDS-PAGE and Western blot analysis demonstrated that the molecular weight of the truncated Cry1F from the bacterial and plant sources was equivalent. The full length Cry1F was sensitive to protease cleavage, resulting in truncated form(s). The full length Cry1F has a theoretical molecular weight of 130 kDa. A single core protein following trypsin digestion at an apparent MW of 65 kDa was found in the transgenic cotton (Event 281) leaf extract. This 65 kDa protein was immunoreactive to Cry1F and was equivalent to the trypsinized, truncated *P. fluorescens*-expressed Cry1F protein. This 65 kDa protein is resistant to protease digestion. Transgenic cotton (Event 281)-expressed and *P. fluorescens*-expressed Cry1F proteins had no detectable post-translational glycosylation.

Results from the Edman degradation showed that the 15 N-terminal amino acid residues (XTGRLPLDISLSLTR, corresponding to residues #28 to #42 of the full length Cry1F (synpro)) determined from the truncated Cry1F from *P. fluorescens* and from transgenic cotton (Event 281) were the same. These results indicate that by exposing the full length Cry1F (produced by *P. fluorescens*) to trypsin caused truncation not just at the C-terminus resulting in removal of the chimeric nontoxic C-terminal domain between residue 27 and 28, but also at the N-terminus removing the first

27 amino acid residues.

Cryptic peptide mass fingerprints of the truncated Cry1F from both transgenic cotton (Event 281) and *P. flourescent* determined by MALDI-TOF MS revealed 23 to 25 peptides matched the theoretical deduced peptide masses of Cry1F (synpro). Two internal peptides from the trypsin digests were also sequenced using MS/MS, one with a sequence of TLSDPVFVW matching the residues #358 to #366 of Cry1F (synpro), and the other with a sequence of GPGFTGGDILR matching the residues #484 to #494.

CLASSIFICATION: ACCEPTABLE

455423-06 **Biochemical Characterization of Cry1Ac derived from Transgenic Cotton and *Pseudomonas fluorescens***

Several biochemical methods were used to assess the equivalency of the plant-expressed and *P. fluorescens*-expressed Cry1Ac proteins. Based on the results of these biochemical analyses, it cannot be determined unequivocally, without further analysis, that the plant-expressed (Event 3006-48-81) and *P. fluorescens*-expressed Cry1Ac proteins are biochemically equivalent. Expression of Cry1Ac in transgenic cotton appears to be too low for effective SDS-PAGE and Western blot analyses, Edman degradation, and peptide mass fingerprinting using MALDI-TOF MS. Only 9 peptides from the trypsin digests of transgenic Cry1Ac cotton tissue were identified as matching the theoretical deduced peptide masses using MALDI-TOF MS pesticide mass fingerprinting; while, 21 or 27 peptides were identified from the trypsin digests of *P. fluorescens*-expressed material. No N-terminal sequence was obtained for the Cry1Ac transgenic cotton samples. SDS-PAGE and Western blot analyses showed different size truncated forms of the transgenic Cry1Ac cotton material versus the *P. fluorescens*-expressed material. Additional biochemical and/or molecular data are needed to clarify the biochemical equivalency of plant-expressed and *P. fluorescens*-expressed Cry1Ac proteins.

SDS-PAGE and Western blot analysis demonstrated that the molecular weight of the truncated Cry1Ac from the bacterial and plant sources were similar, but not identical (Figures 1, 5, 7, and Appendix A). The Cry1Ac expression is so low in transgenic cotton (Event 3006-48-81) that typical SDS-PAGE analysis and Western blot analysis do not yield detectable Cry1Ac protein. The expression of Cry1Ac from fresh leaf tissue extracts from transgenic cotton (Event 3006-48-81) (TSN 102625) was low based on ELISA results. Cry1Ac was barely detectable by Western blot analysis even with the more sensitive chemiluminescent detection reagents (Method two, Figure 2).

The full length Cry1Ac was sensitive to trypsin cleavage, resulting in truncated form(s) in both the microbially-derived Cry1Ac protein and the plant-derived Cry1Ac protein. There were several intermediate forms of truncation observed during isolation and purification (see Figure 1, p. 29). The full length Cry1Ac has a theoretical molecular weight of approximately 131 kDa. A single core protein following trypsin digestion of the *P. fluorescens*-expressed full-length Cry1Ac (TSN 102337) had an

apparent MW of 65 kDa and was immunoreactive to Cry1Ac based on Western blot analysis, while the immunoaffinity-purified truncated Cry1Ac (synpro) for transgenic cotton tissue had an apparent MW of 60 kDa (Figures 5 and 7). The *P. fluorescens*-expressed full-length Cry1Ac 65 kDa protein was resistant to further trypsin cleavage (i.e., fully-truncated, trypsin-resistant core protein). In transgenic 3006-48-81 cotton plants, both the full-length (130 kDa) and partially truncated version (approximately 68-70 kDa) of Cry1Ac were present in fresh leaf tissues (see Appendix A, p. 47 and Figure 1, p. 29). Dow Agrosciences (e-mail from Y. Gao to S. Matten, 6/29/02) offers the following explanation as to why the molecular weights of the truncated forms of Cry1Ac isolated from transgenic cotton were different from the fully-truncated, trypsin-resistant microbially-derived core protein:

> "In Appendix A, it should be pointed out that band A in lane 2 was the fully-truncated trypsin-resistant core toxin of Cry1Ac with an approximate MW of 65 kDa. Band C in cotton (lanes 4 to 6) was not the fully-truncated core toxin. Our sequencing data of the microbially-derived Cry1Ac revealed that during proteolytic truncation, in addition to the removal of a large piece of the C-terminal domain, the first 28 amino acid residues (~3 kDa) from the N-terminus of Cry1Ac were also cleaved (Table 1 of study 010022). It is very likely that band C in cotton was an incomplete truncated form with an intact N-terminus. This phenomenon was not unique to Cry1Ac cotton. A similar observation was obtained with Herculex I Cry1F corn. The fully-truncated Cry1F had an MW of 65 kDa of which the first 27 amino acid residues at N-terminus was removed, and an N-terminus intact Cry1F was about 68 kDa. Depending on the growth stages of plant tissues and sample extraction procedure, either the doublet bands (68 and 65 kDa) or a major 65 kDa band can be detected by Western blot (Dow AgroSciences reports MYCO98-001 and GH-C 5294)."

The Cry1Ac expression level in transgenic cotton ranges from 0.05 to 3 µg/g dry tissue weight, or less than 1 µg/g fresh tissue weight (Dow Agrosicences study #010015, MRID# 455423-22). Cry1Ac levels are about 100 times lower than Cry1F levels in transgenic cotton (see discussion of biochemical characterization of Cry1F derived from transgenic cotton (Event 281) and *P. fluorescens*, MRID# 455423-05). Dow AgroSciences attempted to further purify the Cry1Ac protein using ammonium sulfate precipitation, and immunoaffinity chromatography using Cry1Ac-specific rabbit polyclonal antibodies. An immunoreactive band of approximately 60 kDa, was detected using SDS-PAGE and Western blot analysis of lyophilized cotton (event 3006) leaf extracts that were further purified using immunoaffinity chromatography (Figures 3-7, p. 31-35). The 60 kDa fragment did not produce a distinct band on SDS-PAGE (Figure 6, p. 34). Based on the Western blot data, the 60 kDa fragment is probably a degraded fragment of Cry1Ac. Transgenic cotton (Event 3006-48-81)-expressed and *P. fluorescens*-expressed Cry1Ac proteins had no detectable post-translational glycosylation.

Because the Cry1Ac expression is so low in transgenic cotton (Event 3006-48-81) to clearly show biochemical equivalency, authors cite the biological equivalency study (MRID 455423-04, Dow AgroSciences study #010082) conducted with tobacco budworm, cotton bollworm, and beet armyworm. Results indicate that there is apparent biological equivalency of Cry1Ac derived from transgenic cotton (Event 3006-48-81) and *P. fluorescens*. These results strengthen the argument that

there is biochemical equivalency between the Cry1Ac derived from transgenic cotton (Event 3006-48-81) and *P. fluorescens*.

Results from the Edman degradation showed that the first 15 N-terminal amino acid residues of the full length Cry1Ac were XDNNPNINEXIPYNX and matched the residues #1-#15 of the expected N-terminal sequence (Table 1). The N-terminal amino acid residues determined from the truncated Cry1Ac from *P. fluorescens* (65 kDa protein) were the same as the expected theoretical sequence: XETGYTPIDISLSLT (Table 1) of residues #29 to #43 of the full length Cry1Ac (synpro) protein. These results indicate that by exposing the full length Cry1Ac (produced by *P. fluorescens*) to trypsin caused truncation not just at the C-terminus resulting in removal of the chimeric nontoxic C-terminal domain, but also at the N-terminus removing the first 28 amino acid residues. Results from the Edman degradation indicated that the amino acid residues determined from the full length Cry1Ac from *P. fluorescens* matched the expected sequences as noted above. No N-terminal sequence was obtained for the Cry1Ac protein samples from the affinity chromatography preparations of transgenic cotton (event 2006) leaf tissue. The study authors surmise that the amino acid signals were too weak to interpret because there was too little Cry1Ac in the preparation. The N-terminus was blocked in all tests, as the methionine or isoleucine are never well identified using Edman degradation.

Tryptic peptide mass fingerprints of the truncated Cry1Ac from transgenic cotton (Event 3006) and *P. fluorescens* determined by MALDI-TOF MS revealed that 27 peptides from *P. fluorescens* and 9 peptides from the transgenic cotton affinity purified preparations matched the theoretical deduced peptide masses under residues #682 of Cry1Ac (synpro) (Table 2, p. 28). There were a considerable number of unmatched peptides from *P. fluorescens* and from transgenic cotton preparations. These results are far less conclusive than those determined by MALDI-TOF MS of the Tryptic peptide mass fingerprints of the truncated Cry1F from transgenic cotton (Event 281) and *P. fluorescens* (MRID 455423-05). Factors such as over digestion, self-digestion products of trypsin, or random breakage of peptides during ionizations, were noted by the study authors. In addition, the authors comment that the Cry1Ac in the affinity chromatography fractions was still quite low even after concentration and there may have been contaminant proteins that contributed to excess unmatched peptides. However, these data do provide another piece of evidence that the Cry1Ac protein was produced in cotton.

Two internal peptides from the *P. fluorescens* derived truncated Cry1Ac digest were also sequenced using MS/MS, one with a sequence of WGFDAATINSR matching the residues #182 to #192 of Cry1Ac (synpro), and the other with a sequence of IVAQLGQGVYR matching the residues #350 to #360. No internal peptides from transgenic cotton (Event 3006-48-81) were sequenced using MS/MS.

Dow Agrosciences (e-mail Y. Gao to S. Matten, 6/29/02) indicates that it is in the process of collecting additional biochemical characterization data to more definitively show that cotton-produced Cry1Ac is equivalent or comparable to the microbially-derived Cry1Ac. The work includes: further quantitation of Cry1Ac in cotton by ELISA using a specific polyclonal antibody and a monoclonal antibody in a sandwich format and measuring the Cry1Ac expression levels in leaves, squares, flowers, roots, seeds,

bolls, pollen, and whole plants at various growth stages in different geographic locations in the U.S. (Dow Agrosciences study 010015, MRID# 455423-22, this study presents some preliminary data). Work is also underway to qualitatively detect Cry1Ac on Western blots with the transgenic cotton event (3006-210-23) in different tissues (leaves, flowers, roots, etc.). A study using Northern blot analysis of Cry1Ac cotton is underway to determine whether the correct Cry1Ac mRNA is transcribed in cotton. This will provide information on gene transcription and prediction of protein translation. Another study is in progress to clone and sequence the *cry1Ac* gene insert and its border sequences. Southern blot analysis has previously demonstrated that Cry1Ac cotton events 3006-48-81 and 3006-210-23 each contain a single insert of transgenic DNA which contains one intact copy of the *cry1Ac* gene (Dow AgroSciences study 010053, MRID# 455423-03). Both the Northern blot analysis and cloning work would indicate the correct amino acid sequence of the translated Cry1Ac protein in cotton. In conclusion, additional data are needed to clarify the biochemical equivalency of the plant-expressed and bacterially-expressed Cry1Ac proteins.

CLASSIFICATION: SUPPLEMENTAL, UPGRADEABLE WITH SUBMISSIONS OF MORE DATA TO DEMONSTRATE BIOCHEMICAL EQUIVALENCY

455423-07 **Cotton-Insect-Pest Susceptibility Study - Microbial B.t. Cry1F (synpro) Protein**

The laboratory insect bioassay provides a crude measurement of the relative sensitivities (susceptibility) of six cotton-feeding insects to the purified Cry1F (synpro, full-length) bacterial endotoxin. Based on the results from this study, there is no significant activity of pure Cry1F (synpro, full-length) delta endotoxin against pink bollworm (PBW), boll weevil (BW), and western tarnished plant bug (WTPB) The relative sensitivity of the six cotton-feeding insects to the purified Cry1F (synpro, full-length) delta endotoxin tested in this study and the three cotton-feeding insects (cotton bollworm (CBW), fall armyworm (FAW), tobacco budworm (TBW)) used in a biological equivalency study are as follows: (sensitive) soybean looper (SBL)>TBW, cabbage looper (CL), FAW> beet armyworm (BAW)>CBW>>PBW, BW, WTPB (insensitive).

CLASSIFICATION: ACCEPTABLE

455423-08 **Cotton-Insect-Pest Susceptibility Study - Microbial B.t. Cry1Ac (synpro) Protein**

The laboratory insect bioassay provides a crude measurement of the relative sensitivities (susceptibility) of eight cotton-feeding insects to the purified Cry1Ac (synpro) bacterial endotoxin. Based on the results from this study, there is no significant activity of pure Cry1Ac (synpro) delta endotoxin against pink bollworm (PBW), boll weevil (BW), and cotton aphid (CA). The relative sensitivity of the eight cotton-feeding insects to the purified Cry 1Ac (synpro) delta endotoxin tested in this study are as follows: (sensitive species) tobacco budworm (TBW)> cabbage looper (CL)>cotton bollworm

(CBW), beet armyworm (BAW)>fall armyworm (FAW)>>BW, PBW, CA (insensitive species). PBW is expected to be sensitive to Cry1 proteins as demonstrated in published data for Cry1Ac (the delta endotoxin expressed in Bollgard® cotton) (Perlak et al. 2001, Patin et al. 1999).

CLASSIFICATION: SUPPLEMENTAL, NOT UPGRADEABLE (DO NOT REDO)

455423-24 **Field Efficacy of Cry1F (synpro), Cry1Ac (synpro) and Cry1F + Cry1Ac Stack Insecticidal Crystalline proteins from *Bacillus thuringiensis* var. *aizawai* strain PS811 and *Bacillus thuringiensis* subspecies *kurstaki* strain HD73 Cotton Events Against Tobacco Budworm and Pink Bollworm**

Four field efficacy studies were conducted at four locations to examine the efficacy of Cry1F (synpro), Cry1Ac (synpro), and Cry1F + Cry1Ac stacked insecticidal crystalline proteins from *Bacillus thuringiensis* var. *aizawai* strain PS811 and *Bacillus thuringiensis* subspecies *kurstaki* strain HD73 cotton events against tobacco budworm and pink bollworm.

Overall, the three cotton efficacy trials conducted for tobacco budworm showed that Cry1F cotton (event 281-24-236), Cry1Ac cotton (events 3006-210-23 and 3006-48-81) and the Cry1F+Cry1Ac cotton (events 281-24-236/3006-210-23 and 281-24-236/3006-48-81) areas gave statistically significantly better control of tobacco budworm than untreated controls by exhibiting little cotton square damage or boll damage and limited survival of neonate budworms. Preliminary data indicate the efficacy of transgenic cotton was not significantly different from the chemically treated non-transgenic control. Preliminary data on efficacy of Cry1Ac cotton (event 3006-210-23) and Cry1F+Cry1Ac cotton (event 281-24-236/3006-210-23) indicate virtually complete control of the pink bollworm in cotton bolls. However, the efficacy of the Cry1F event 281-24-236 for control of pink bollworm did not differ from that of the control. Further efficacy studies should be performed during the course of the Experimental Use Permit.

CLASSIFICATION: ACCEPTABLE

Human Toxicity and Allergenicity

455423-12 **Acute Oral Toxicity: Cry1F (synpro) Bacterial Protein (885.3050)**

The acute oral LD_{50} of Cry1F bacterial protein in male and female CD-1 mice was estimated to be greater than 600 mg/kg body weight (test material was 2000 mg/kg body weight containing 30% pure Cry1F bacterial protein). No significant adverse effects at this level were observed. There were no deaths during the two-week observation period. There were no remarkable clinical observations, except for male 2689 who was partially lame in one leg. This was probably due to transponder implantation and not due to the effects of Cry1F. There were no pathological lesions for any animal in the study. There were no statistically significant weight changes during the course of the study and all animals gained weight during the course of the study.

CLASSIFICATION: ACCEPTABLE, TOXICITY CATEGORY III

455423-13 Acute Oral Toxicity: Cry1Ac (synpro) Bacterial Protein (885.3050)

The acute oral LD_{50} of Cry1Ac (synpro) bacterial protein in male and female CD-1 mice was estimated to be greater than 700 mg/kg body weight (test material was 5000 mg/kg body weight containing 14% pure Cry1Ac (synpro) bacterial protein). No significant adverse effects at this level were observed. There were no deaths during the two-week observation period. There were no remarkable clinical observations nor any pathological lesions for any animal in the study. There were no statistically significant weight changes during the course of the study and all animals gained weight during the course of the study.

CLASSIFICATION: ACCEPTABLE, TOXICITY CATEGORY III

455423-14 Acute Oral Toxicity: Cry1F (synpro) + Cry1Ac (synpro) Bacterial Proteins (885.3050)

The acute oral LD_{50}s of Cry1F bacterial protein and Cry1Ac bacterial protein in male and female CD-1 mice was estimated to be greater than 375 mg Cry1F/kg body weight and 350 mg Cry1Ac/kg body weight (test material was 5000 mg/kg body weight containing 15% pure Cry1F bacterial protein and 14% pure Cry1Ac bacterial protein). No significant adverse effects at these levels were observed. There were no deaths during the two-week observation period. There were no remarkable clinical observations. There were no pathological lesions for any animal in the study. Except for transient weight losses, all animals gained weight during the course of the study.

CLASSIFICATION: ACCEPTABLE, TOXICITY CATEGORY III

455423-09 Comparison of Amino Acid Sequence with Known Allergens: Cry1F (synpro) Protein as Expressed in Cotton

An amino acid sequence evaluation scheme based on that formulated by Gendel (1998) was used to assess the similarity of the Cry1F (synpro) insect control protein to known allergens. The GCG FINDPATTERNS analysis revealed no matches of eight or more contiguous amino acid residues between the Cry1F (synpro) protein sequence and any sequences in the two constructed allergen databases. These results show that the Cry1F (synpro) protein sequence does not share a linear IgE epitope of eight contiguous identical amino acids with any known protein allergen. However, because linear IgE-epitopes may be less than eight contiguous identical amino acids, shorter stretches of contiguous amino acids (e.g., four to seven amino acids) may need to be evaluated using bioinformatic methods for allergenicity assessment (see Kleter et al. 2002, Hileman et al. 2002).

CLASSIFICATION: ACCEPTABLE

455423-10 **Comparison of Amino Acid Sequence with Known Allergens: Cry1Ac (synpro) Protein as Expressed in Cotton**

An amino acid sequence evaluation scheme based on that formulated by Gendel (1998) was used to assess the similarity of the Cry1Ac (synpro) insect control protein to known allergens. The GCG FINDPATTERNS analysis revealed no matches of eight or more contiguous amino acid residues between the Cry1Ac (synpro) protein sequence and any sequences in the two allergen databases. These results show that the Cry1Ac (synpro) protein sequence does not share a linear IgE epitope of eight contiguous identical amino acids with any known protein allergen. However, because linear IgE-epitopes may be less than eight contiguous identical amino acids, shorter stretches of contiguous amino acids (e.g., four to seven amino acids) may need to be evaluated using bioinformatic methods for allergenicity assessment (see Kleter et al. 2002, Hileman et al. 2002).

CLASSIFICATION: ACCEPTABLE

455423-11 **Comparison of Amino Acid Sequence with Known Allergens: PAT Protein as Expressed in Cotton**

An amino acid sequence evaluation scheme based on that formulated by Gendel (1998) was used to assess the similarity of the phosphinothricin acetyltransferase (PAT) protein to known allergens. The GCG FINDPATTERNS analysis revealed no matches of eight or more contiguous amino acid residues between the PAT protein sequence and any sequences in the two allergen databases. These results show that the PAT protein sequence does not share a linear IgE epitope of eight contiguous identical amino acids with any known protein allergen. However, because linear IgE-epitopes may be less than eight contiguous identical amino acids, shorter stretches of contiguous amino acids (e.g., four to seven amino acids) may need to be evaluated using bioinformatic methods for allergenicity assessment (see Kleter et al. 2002, Hileman et al. 2002).

CLASSIFICATION: ACCEPTABLE

455423-18 ***In vitro* Digestibility of Bacterially-derived Cry1F (synpro)**

In vitro digestibility is one step in a multilevel analytical process to assess allergenic potential. The simulated gastric fluid model (SGF) provides a method to assess allergenic potential of proteins introduced into food plants. The test protein, Cry1F (synpro), was digested in less than one minute in simulated gastric conditions (pH 1.2), as demonstrated by both SDS-PAGE and Western blot analysis. The positive control, BSA, was digested in less than one minute and, the negative control, $-lactoglobulin, remained undigested for 60 minutes.

CLASSIFICATION: ACCEPTABLE

455423-19 ***In vitro* Digestibility of Bacterially-derived Cry1Ac (synpro)**

In vitro digestibility is one step in a multilevel analytical process to assess allergenic potential. The simulated gastric fluid model (SGF) provides a method to assess allergenic potential of proteins introduced into food plants. The test protein, Cry1Ac (synpro), was digested in less than one minute in simulated gastric conditions (pH 1.2), as demonstrated by both SDS-PAGE and Western blot analysis. The positive control, BSA, was digested in less than one minute and the negative control, $-lactoglobulin, remained undigested for 60 minutes.

CLASSIFICATION: ACCEPTABLE

455423-21 Thermolability of Cry1F (synpro) Delta-Endotoxin

Results of the insect bioassay using *Heliothis virescens* (tobacco budworm) neonate larvae indicate that Cry1F was inactivated after exposure to 75ºC and 90ºC for 30 minutes, but not to 60ºC. SDS-PAGE and Western blot analyses confirmed that the inactivated Cry1F protein was present and undegraded after exposure to heat. Higher incubation temperatures greater than 90ºC might cause degradation of the Cry1F protein so that it was not immunoreactive. To further any assessment of allergenicity, then information directly related to the stability of the introduced protein under food processing conditions (e.g., "heat stability studies") should be provided.

CLASSIFICATION: ACCEPTABLE

455423-21 Thermolability of Cry1Ac (synpro) Delta-Endotoxin

Results of the insect bioassay using *Heliothis virescens* (tobacco budworm) neonate larvae indicate that Cry1Ac (synpro) protein was inactivated after exposure to 75ºC and 90ºC for 30 minutes, but not to 60ºC. The loss of activity of the Cry1Ac (synpro) protein after heat treatment indicates that the Cry1Ac (synpro) protein is labile at and above 75ºC when incubated for 30 minutes. SDS-PAGE and Western blot analyses confirmed that the inactivated Cry1Ac (synpro) protein was present after exposure to heat. Some degradation of the Cry1Ac (synpro) protein occurred after heat treatment at 90ºC. To further any assessment of allergenicity, then information directly related to the stability of the introduced protein under food processing conditions should be provided.

CLASSIFICATION: ACCEPTABLE