II. Science Assessment

A. PRODUCT CHARACTERIZATION

Product characterization is critical to understanding the way in which the registered products were made and the unique characteristics that need to be assessed for each *Bt* plant-incorporated protectant. The product characterization data provide information on the specific transformation systems used for each product, on the actual DNA inserted into the plant, on the inheritance and stability of these traits in the plant, on biochemical characteristics of the *Bt* protein and on *Bt* protein expression levels for various plant tissues. Specific information and data for each of the registrations seeking renewal are included in tabular and descriptive formats.

The classifications that are found for each data submission are assigned by the EPA science reviewer and are an indication of the usefulness of the information contained in the documents and if the data meet the intent of the test guidelines. A rating of "ACCEPTABLE" indicates the study is scientifically valid and has been satisfactorily performed according to accepted EPA guidelines or other justified criteria. A "SUPPLEMENTAL" rating indicates the data provide some information that can be useful for risk assessment. However, the studies may either have certain aspects not determined to be scientifically acceptable (SUPPLEMENTAL. UPGRADABLE) or that the studies have not been done to fulfill a specific EPA guideline requirement. If a study is rated as "SUPPLEMENTAL. UPGRADABLE," EPA always provides an indication of what is lacking or what can be provided to change the rating to "ACCEPTABLE." If there is simply a "SUPPLEMENTAL" rating, the reviewer will often state that the study is not required by current EPA guidelines or does not need to be reclassified as "ACCEPTABLE." Both ACCEPTABLE and SUPPLEMENTAL studies may be used in the risk assessment process as appropriate.The following table summarizes the registered *Bt* protein-containing plant-incorporated protectant products being evaluated.

Table A1 - *Bt* Plant-incorporated protectant Products

Common Name and Cry Protein	OPP Chemical Code	Company	Plasmid ID	Plant/ Trade Name
*Bt*11 Cry1Ab *Bt* Corn	006444	Novartis	pZO1502	YieldGard, Attribute
MON810 Cry1Ab *Bt* Corn	006430	Monsanto	pvZMCT01* pZMBK07 pZMGT10**	YieldGard

Cry1F *Bt* Corn	006481	Mycogen-Dow	PHI 8999	Herculex
Cry1Ac *Bt* Cotton	006445	Monsanto	pvGHBK04	BollGard
Cry3A *Bt* Potato	006432	Monsanto	pvSTBT02	NewLeaf

* pvZMCT01 was a mixture of two plasmids
** Plasmid contains marker gene.

Transformation systems: Registered corn products were transformed using protoplast electroporation to introduce the desired DNA or methods employing bombardment of particles coated with DNA encoding the intended insert. *Agrobacterium tumefaciens*-mediated transformation was used for both the cotton and potato products.

Each plasmid description includes a reference to the strains of *Bacillus thuringiensis* used as the source of the DNA sequence for the toxin protein. In addition, the sources for marker proteins, promoters, terminators and enhancers, as well as the fragment size, orientation and any modifications to the original DNA sequence to enhance expression in the plant are given. All the other DNA sequences introduced to improve or restrict expression of the introduced traits are also described. Finally, the plasmid discussion includes a description of any modifications made to the DNA (e.g., codon modifications to improve eukaryotic expression).

Characterization of the DNA Inserted in the Plant: Inserted DNA is characterized with Southern blot data of the DNA in the plant genome. The analysis usually consists of DNA isolation from the transformed plant, digestion of this DNA with several different endonucleases and hybridization of these restriction endonuclease fragments with labeled-DNA which is complementary to the introduced traits. This analysis includes not only probes specific for the entire insert, but also probes recognizing just the coding regions of the traits or DNA elements outside the coding region. Polymerase chain reaction (PCR) assays utilizing various specific and non-specific primers, genome walking, cosmid libraries and DNA sequencing have also been employed with sensitive Southern blotting techniques to more completely describe the inserted DNA and surrounding regions. The information available from these blots can indicate the presence of all the elements of the expected insert as well as information about the possibility of deletions and other errors associated with DNA introduction by transformation. Comparison of Southern blots of genomic DNA, digested using a range of restriction endonucleases, can also reveal the copy number of the genes introduced and suspected linkage of the traits. Alternatively, the intensity of the radioactive label from binding the probe DNA can also estimate the number of insert copies incorporated in the plant genome. When the inserted DNA construct includes traits expressed only in bacteria and not expected to be expressed in the plant, data have been presented to indicate that there is no transcription or translation of the bacterial trait (e.g., *ori* and *amp*^{r} - discussed further in the horizontal gene transfer section).

Inheritance and Stability after Transformation: The data generated for this endpoint examine progeny from crosses between selected elite lines with the transformed *Bt* expressing line, looking for the independent segregation of the introduced traits in the progeny. Traditional breeding work done during the development of the plant line by backcrossing can reveal the linkage of the introduced traits as well as changes in trait expression. The inheritance data is the ratio of progeny expressing the hemizygous trait based on expected Mendelian inheritance. Stability data implies an examination of either the expression of the trait or tracking of the DNA itself over several plant generations. One of the main concerns with stability is spontaneous loss of the inserted DNA or loss of efficacy due to gene silencing. None of the *Bt* plant-incorporated protectant products showed independent assortment of the introduced traits (usually the marker protein and the *Bt* protein were examined). This indicates that the traits were on the same chromosome and closely linked (crossover events between the two traits were not detected).

The submissions that covered characterization of the actual DNA insert and stability/inheritance data are listed in the MRIDs for each product. These submissions are acceptable and fulfill this data requirement. It should be noted that stability and inheritance were not addressed with the registrations for MON810 (006430) and Cry3A (006432). However, considering the use of these crops for several growing seasons and the lack of reports relating to loss of efficacy due to *Bt* protein expression, this specific endpoint can be considered to have been addressed through commercial use.

Protein Characterization and Expression: For the *Bt* plant-incorporated protectants, data has been presented to demonstrate that the protein expressed from the inserted DNA is similar to what was produced in the source bacterium and is active as expected against the intended target insect. Some protein characterization data demonstrate that microbially produced *Bt* protein is the equivalent to that expressed in the plant. This apparent scientific tautology (where plant produced protein is the same as microbial protein is the same as the plant produced protein) has been used to justify the use of the microbially-produced protein as a test substance in toxicity tests. Because the expression level of these proteins is so low in plants, and the maximum hazard dose acute oral toxicity test is required as part of the human health risk assessment for these proteins, the ability to produce the protein in an industrial microbe is essential. The acute oral test requires between 2000 and 5000 mg of protein per kg bodyweight of test animal. Isolating the amount of purified protein required to dose several animals from *Bt*-expressing plants would be a tremendous burden involving harvesting and processing large volumes of plant material (ecological effects testing differs and is addressed in the ecological effects section of this document). Proper characterization of the equivalency between these microbial proteins and plant expressed proteins provides an alternative to purifying the test material as the plant-produced protein from large volumes of tissue. These equivalency data were generated for all products registered to date.

Much of the characterization data describes the procedures used to isolate the protein or a highly *Bt* protein enriched fraction of plant extract. The tests done to support the equivalence of microbial and plant-produced *Bt* protein include: molecular sizing by SDS-PAGE and western blot analysis; immunorecognition using ELISA and western blot analysis; N-terminal amino acid sequencing; confirmation of the lack of glycosylation in the plant-produced protein; and bioactivity against a range of insects (often pest species including the target pest). Since the issues surrounding non-target effects are considered essential for the ecological effects assessment, these non-target pest tests are also covered in the ecological effects assessment.

The *Bt* protein expression level in various tissues throughout the growing season has been determined for each product. As this is a major aspect of the high-dose strategy determination for insect resistance management, the impact of protein expression levels are also covered in that section of the *Bt* crops reevaluation. The nominal protein expression levels as determined by field and/or greenhouse conditions are described below. Note that there may be variation between the *Bt* protein values reported by each company due to differences in the antibody-based reagents used for quantifying the *Bt* protein. There are also differences due to reporting *Bt* protein values based on tissue fresh weight. While these differences may make direct comparisons between the tissue expression levels reported by different companies difficult, the reported levels provide enough information to be used for risk assessment purposes especially when considered along with the reported tissue bioactivity values. However, to provide consistent reporting of protein expression data, these data should be determined and presented, in terms of dry weight, as the amount of protein present in the given tissue. Tissues for which expression data should be provided include: leaf, root, pollen, seed, root and whole plant.

Table A2 Cry Protein Tissue Expression

Active Ingredient	Leaf	Root	Pollen	Seed	Whole Plant
Cry1Ab-*Bt*11 (006444)	3.3 ng/mg	2.2-37.0 ng/mg protein	< 90 ng Cry1Ab/ g dry wt. pollen	1.4 ng/mg (kernel)	_
Cry1Ab-MON810 (006430)*	10.34 ng / mg	_	< 90 ng Cry1Ab/ g dry wt. pollen	0.19-0.39 ng / mg (grain)	4.65 ng / mg
Cry1F (006481)	56.6 - 148.9 ng / mg total protein	_	113.4 - 168.2 ng / mg total protein or 31 to 33 ng / mg pollen	71.2 - 114.8 ng / mg total protein	803.2 - 1572.7 ng / mg total protein

Cry1Ac (006445)	2.04 ng / mg	_	11.5 ng/g	1.62 ng / mg	_
Cry3A (006432)	28.27 ng / mg	0.39 ng / mg (tuber)	_	_	3.3 ng / mg

* 1994 Field Data ** All values reflect fresh tissue weight unless otherwise noted.

Residue Analytical Methods

Analytical methods and method validation (under OPPTS Guidelines OPPTS 860.1340) for the Cry proteins in Cry1Ab corn, Cry1Ac cotton, and Cry3A potatoes listed in this BRAD in corn, cotton, and potatoes are necessary to complete the database. Analytical methods have been submitted for Cry1F corn and is in the process of being formally reviewed by the Agency. Additional confirmatory methods and standard EPA laboratory method validation are also necessary. This includes thorough characterization of the antisera used in these methods.

1. Product Characterization of Bt 11 Cry1Ab Corn (006444)

The corn line Bt 11 was produced by transforming another proprietary corn line with plasmid PZO1502 which contained *cry1Ab, pat* and *amp*^{r} genes. The registrant submission stated that prior to the transformation which resulted in Bt11, the plasmid pZO1502 was digested with the restriction endonuclease *Not* I with the intention to remove the *amp*^{r} gene from pZO1502. While no data was submitted to confirm removal of the *amp*^{r} gene from the transforming DNA, subsequent analysis showed that the *amp*^{r} gene was not present in Bt11 corn. The *cry1Ab* gene was also altered to improve its GC ratio for expression in corn and coded for a truncated form of the original protein. Both field corn and sweet corn containing the plant-incorporated protectant descend from the original Bt 11 transformant.

Data showed that the truncated Cry1Ab toxin could be extracted from corn leaf tissue and this purified material displays characteristics and activities similar to that produced in *E. coli* transformed to produce Cry1Ab. The purified tryptic core proteins from both plant and microbe were shown to be similar in molecular weight by SDS-PAGE, immunorecognition in western blots and ELISA, partial amino acid sequence analysis, lack of glycosylation and bioactivity against either European corn borer or corn earworm. This analysis justified the use of the microbially produced toxin as an analogue for the plant produced protein in mammalian toxicity testing.

The product characterization data supporting the registration of *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material necessary for its production (plasmid vector pZO1502) in corn is listed below.

Study Type	Result	MRID #
Transformation System	Corn line HE89 was transformed with plasmid *pZO1502* which contains genes for a truncated Cry1Ab, PAT and AMP^r. The *cry1Ab* gene was also altered to improve its GC ratio for expression in corn. (See MRID No. 437548-01 below which indicates the absence of the *amp^r* gene in the Bt11 and control plants.) CLASSIFICATION: ACCEPTABLE	431308-01
Inheritance and Stability after Transformation	The linkages of the *pat* and *cry1Ab* genes were shown by examining the progeny of two selfed generations derived from a population of corn plants segregating for the desired traits. None of the 2320 plants examined showed the two traits independently assorting which indicates that the loci are tightly linked. CLASSIFICATION: ACCEPTABLE	433526-02
Transformation System	The lack of any positive probe recognition for the plant genomic DNA samples indicate the absence of the *amp^r* gene in the Bt11 and control plants. The positive *amp^r* gene probe results for the plasmid DNA digest samples confirm that a fragment of a size consistent with the 7.2 Kb pZO1502 plasmid contained the *amp^r* gene. This would also be appropriate for any digest which had a single restriction cut site as these enzymes did according to the pZO1502 map. The probe results also indicate that a Not I digest would release the *amp^r* gene from the pZO1502 plasmid CLASSIFICATION: ACCEPTABLE	437548-01
Protein Characterization and Expression	Data is presented showing that the truncated Cry1Ab toxin can be extracted from corn leaf tissue and this purified material displays characters and activities similar to that produced in *E. coli*. The similarities are shown in molecular weight after SDS-PAGE, immunorecogniton in western blots and ELISA of trypsin resistant core proteins, partial amino acid sequence analysis, lack of glycosylation and bioactivity against either European corn borer of corn earworm. CLASSIFICATION: ACCEPTABLE	433972-02

2. Product Characterization of MON810 Cry1Ab Corn (006430)

Monsanto's corn line MON 810 was produced by ballistically transforming another proprietary corn line with plasmid construct PV-ZMCT01. Plasmid construct PV-ZMCT01 consists of plasmids PV-ZMBK07 & PV-ZMGT10 ballistically introduced together. The MON 810 line of corn is similar to MON 801 corn in that they both were derived from transformation events utilizing PV-ZMCT01. The MON 810 only expresses a truncated version of Cry1Ab delta-endotoxin. MON801 expresses the full length version of Cry1Ab and the marker gene products. MON 810 and MON 801 were each transformed with the same plasmid construct (PV-ZMCT01). The MON 810 progeny express a slightly truncated version of Cry1Ab compared to MON 801, but the active site is still retained. The MON 810 progeny do not express detectable levels of the marker gene products found in MON 801 progeny. Some of the data used to

evaluate MON810 corn was generated from MON801 corn. To justify this bridging of data from one corn transformation event to another, the company provided product characterization data to demonstrate the similarities and differences between the two transformation events.

Study Type	Result	MRID #
Transformation System Characterization of the DNA Inserted in the Plant Protein Characterization and Expression	The digests of genomic DNA from corn line MON 80100 revealed that the two plasmids PV-ZMBK07 and PV-ZMGT10 had been inserted apparently at two locations. Full length copies of the cry1Ab, gox, nptII and cp4 epsps genes were found. Less than full length copies of all these genes were also found. Western blot analysis revealed that only Cry1Ab and CP4 EPSPS proteins were expressed at detectable levels in the corn plant. CLASSIFICATION: ACCEPTABLE	435332-01
Protein Characterization and Expression	The antiserum reactions revealed many western blot bands in both the Dipel® and the ECB resistant corn extracts not treated with trypsin. No bands clearly related to the Cry1Ab toxin were seen in the non-transformed plant extracts whereas a band comigrating with the full length Cry1Ac standard (similar in size to Cry1Ab) was seen in both Dipel® and ECB resistant corn. The tryptic digests of Dipel® and ECB resistant corn extracts revealed intensified bands that comigrated with the Cry1Ab tryptic core standard. Together these data infer that the same Cry1Ab protein is being produced in ECB resistant corn plants as is found in the microbial product. CLASSIFICATION: ACCEPTABLE	435332-03
Characterization of the DNA Inserted in the Plant	The Southern blots with the two transforming plasmids PV-ZMBK07 and PV-ZMGT10 indicate that only a portion of the PV-ZMBK07 plasmid was successfully integrated. Western blots indicate that all the constructs tested (MON801, 802, 805, 809, 810, 813 and 814) produce delta endotoxin detectable as tryptic core with anti-Cry1Ac antiserum. The genes of the second plasmid used to transform the corn lines, PV-ZMGT10, which include *CP4 EPSPS* and *gox*, were not detected by Southern blot analysis using the PV-ZMGT10 plasmid as probe. These genes which confer glyphosate tolerance were apparently lost during development of the MON810 line since they had to be present for the original callus culture selection process but were not found in the final line described here. CLASSIFICATION: ACCEPTABLE	436655-01
Protein Characterization and Expression	The results of the western blot showed the trypsinized extracts of corn lines MON 802, 805, 809, 810, 813, and 814 expressed proteins that comigrated with the Cry1Ab protein as found in MON 801 and the same Cry1Ab protein purified from *E. coli.* These bands also reacted with antiserum #B6 specific for the tryptic core protein of Cry1Ab. These results indicate the trypsinized proteins found in all these plants were of same molecular size (63 kD) and immunoreactivity with the reference standards of Cry1Ab expressed in *E. coli* and corn line MON801. CLASSIFICATION: ACCEPTABLE	436655-03

Study Type	Result	MRID #
Protein Characterization and Expression	The Cry1Ab protein produced in *E. coli* was shown by SDS-PAGE, western blot, N-terminal amino acid sequencing, glycosylation and bioactivity to be substantially equivalent to the plant produced Cry1Ab. The test results showed the tryptic core of the plant and microbial protein were of essentially identical SDS-PAGE mobility, immunoreactivity in western blot analysis and N-terminal amino acid sequence for the first 15 positions. A comparison of the dose response relationship of plant and microbial extracts against *Heliothis virescens* and *Helicoverpa zea* indicates that the tested proteins are of similar bioactivity. CLASSIFICATION: ACCEPTABLE. These results allow the substitution of the microbially produced Cry1Ab protein for the plant source in toxicology testing.	435332-04

3. Product Characterization of Cry1F Corn (006481)

A proprietary corn line of Pioneer Hi-Bred International and Dow Agrosciences / Mycogen was ballistically transformed with a linear *Pme*I fragment from plasmid pP8999 to produce line TC1507. This plasmid contains genes *cry1F*, *pat* and *kan*^{r} encoding the δ-endotoxin from *Bacillus thuringiensis* var. *aizawai* PS811, phosphinothricin acetyl transferase, and resistance to the antibiotic kanamycin, respectively. The *Pme*I fragment (6235 bp) derived from this plasmid was purified after plasmid digestion and used in the transformation process to eliminate the *kan*^{r} antibiotic resistance gene. The Cry1F protein expressed in transformed maize lines is a modified (synthetic, less than full length) form as compared to that from the bacterial isolate from which it is derived. This insecticidal protein confers resistance to the European corn borer (*Ostrinia nubilalis*) and feeding damage is significantly reduced or eliminated following expression of this gene in corn line TC1507. Expression of *cry1F* is under the control of the maize polyubiquitin promoter in line TC1507. The CaMV 35S promoter controls expression of the *pat* gene in this construct. The *pat* gene from *Streptomyces viridochromogenes* confers resistance to the herbicide glufosinate in corn lines accumulating this protein. Hybridization patterns indicate that one full length copy each of the *cry1F* and *pat* genes was integrated into the genome of line TC1507 and that no *kan*^{r} DNA was integrated. This suggests that one *Pme*I fragment from pP8999 integrated into the maize genome. In addition, there are one or two partial copies of the *cry1F* gene integrated into the genome which are most likely non-functional based upon the size of the fragments detected.

Study	***Result***	***MRID #***

Quantitative ELISA analysis of Cry1F expression levels in maize MPS inbreds and hybrid lines 1360, 1365, 1366, and 1369. (Interim report)	Maize plants (hybrids) from two locations grown under standard agronomic practices of the Midwestern Corn Belt were analyzed by ELISA for Cry1F protein content. The youngest leaf of expanding whorls at the V9 stage were collected from five plants per entry. Values of Cry1F protein for all four hybrids were similar, ranging from 1.52 to 2.63 pg/mg dry weight. Control hybrid A_M was negative for Cry1F as determined by ELISA. CLASSIFICATION: ACCEPTABLE	447148-04
Product characterization data for Bacillus thuringiensis var. aizawai Cry1F as expressed in maize.	A modified (synthetic, less than full-length) form of the cry1Fa2 gene and the phosphinothricin acetyl transferase (pat) gene were inserted into maize plants by microprojectile bombardment. Three transformation events resulting from microprojectile bombardment will be evaluated under the proposed EUP: TC 1360, TC 1362 and TC 1507. Plants were analyzed for Cry1F by ELISA and PAT by application of glufosinate herbicide. Using a chi square analysis with a 95 % confidence interval, the expected Mendelian ratio of 1:1 was observed for both first and second generations for five inbreds with one exception; first generation TC 1632. Event TC 1507 has been analyzed for only the first generation and ratios (1:1) were as expected. CLASSIFICATION: SUPPLEMENTARY. The registrant should clarify the source of the ubiquitin exon and intron as being from the ubiquitin gene and not the promoter region. A determination of expression of the ubiquitin exon sequence is also needed and whether it alters the sequence of Cry1F.	447148-01
Supplement to MRID 447148-01: Supplemental Data – Product Characterization Data for Bacillus thuringiensis var. aizawai Cry1F Insect Control Protein as Expressed in Maize	This submission represents a clarification of nomenclature as presented in a previous submission and review. Labeling (in a previous submission) of the Ubi DNA fragment on the plasmid map should have indicated that it includes the Ubi ZM promoter and the first exon and intron of the Ubi ZM gene. The Ubi exon and intron are included in this construct (PHI8999), however, they have no effect on the structure of the Cry1F product, only on the expression of the gene. Exon 1 contains no ATG start site for translation. A translation initiation sequence (Kozak consensus sequence) situated just upstream from the start site (first translated ATG) drives translation of the mature, spliced mRNA. CLASSIFICATION: ACCEPTABLE	450201-17
Characterization of gene inserts – Bacillus thuringiensis var. aizawai Cry1F insect control protein as expressed in maize.	The integration pattern of cry1F and pat genes introduced into event TC 1360 was analyzed by Southern blotting. Within the Southern analysis, two types of digests are employed to determine the complexity of DNA integration into the maize genome and to determine the copy number of integrated transgenes. Analysis of four of the progeny from event TC 1360 revealed the presence of two bands hybridizing to the cry1F probe; both bands appeared to hybridize with similar intensity. Hybridization to internal controls on the blot gave an indication of single copy integration and certainly no more than two copies of the insert integrated into the maize genome. When control plant DNA was probed, no hybridization was noted. TC 1360 and control DNA probed with the kan^r gene indicated no hybridization within these samples. CLASSIFICATION: ACCEPTABLE	447148-02

Characterization of inserted genes in Cry1F maize line 1507	A modified (synthetic, less than full-length) form of the cry1F gene and the phosphinothricin acetyl transferase (pat) gene were inserted into maize plants by microprojectile bombardment. Digestion of the genomic DNA of maize line 1507 with NheI or HindIII and Southern hybridization with probes specific for cry1F, kan^r and pat genes yielded indications of the complexity of the gene integration pattern and copy number. Hybridization patterns suggested that the copy number of introduced / integrated cry1F and pat genes is one. It is most likely that the TC 1507 line contains one functional cry1F gene and partial copies (1 or 2) of the gene which are non-functional. It is not possible with this technique, however, to discern the functionality of probed sequences. No kan^r DNA was introduced into line 1507 during transformation, as indicated by the lack of signal when 1507 genomic DNA was probed with the kan^r gene. There was no hybridization signal when the non-transformed maize line 13-1 was probed with pat or cry1F or kan^r. CLASSIFICATION: ACCEPTABLE	450201-02
Characterization of expressed Cry1F protein in maize tissues (pollen, grain, grain-containing feed, and purified maize-expressed Cry1F protein) and microbial expressed Cry1F delta endotoxin by biological and biochemical procedures.	Cry1F protein from maize 1507 pollen, grain, grain-derived feeds and a microbial source was evaluated biochemically using ELISA, SDS-PAGE and Western Blotting, and for bioactivity using insect bioassays. Control maize tissues were used to prepare comparable samples. Pollen from line 1507 contained Cry1F at 31 to 33 ng / mg pollen, while no Cry1F protein was detected in pollen from non-Cry1F plants. The purified maize-expressed Cry1F test substance was approximately 32 ng / mL extract. The comparable extract from non-Cry1F maize did not show any detectable Cry1F protein; the limit of detection (LOD) was 0.04 ng / mg sample. Coomassie stained gels indicated similar profiles for both control maize and Cry1F maize samples following SDS-PAGE. Antibodies directed against Cry1F detected this protein (64 kDa) in the Cry1F maize grain samples while there was no indication of any Cry1F protein in the control samples of grain. Pollen, maize-expressed Cry1F and microbially derived Cry1F were all active against the European Corn Borer larvae at the times tested. For the Tobacco Budworm larval bioassay, substances tested included maize grain, maize grain derived fish feed, and maize grain derived quail feed. Samples containing Cry1F maize grain and quail feed made from this grain had identical amounts of Cry1F protein based upon the GI_{50}s calculated. Comparison of control and Cry1F fish feed over four separate bioassays indicated that there was no statistical difference ($p = 0.05$) based upon ANOVA. Preparation of the fish feed sample reduced the biological activity of the Cry1F protein below sensitivity for the assay. CLASSIFICATION: ACCEPTABLE	450201-03

Quantitative ELISA analysis of Cry1F and PAT expression levels in compositional analysis of maize inbred and hybrid lines 1362 and 1507	Protein expression values indicated substantial variability in protein levels for Cry1F in the tissues sampled. No definitive conclusions could be reached from the data presented when comparing levels of Cry1F in hybrid 1507 and inbred 1507 when examining pollen, silk, stalk, leaf, grain, whole plant and senescent whole plant samples. Since these hybrids and inbreds were grown in areas of Chile with similar climatic extremes to the maize growing areas of the U.S., it is anticipated that these values will represent those to be expected in the U.S. cornbelt. PAT expression was also not readily distinguishable when comparing inbred and hybrid expression values. The inability to detect PAT protein in the majority of samples, except leaf, is somewhat puzzling in that the plants demonstrated clear glufosinate tolerance at all field sites. Given the generally strong, non-tissue specific expression levels typically associated with the CaMV 35S promoter (driving pat expression), it is not readily apparent why more PAT protein was not detected in more samples. Its presence in leaf tissue was expected, however, the reason for the absence in many of these samples is less than clear. CLASSIFICATION: ACCEPTABLE	450201-04
Quantitative ELISA Analysis of Cry1A(b) Expression Levels in and Compositional Analysis of Hybrid Lines Derived from Event 176	General agronomic performance, nutrient analysis and Cry1A(b) expression data suggest the equivalence of maize plants grown in South and North America and the feasibility of using winter grow out plots to extend the breeding season or evaluate further traits on either continent. Results from the compositional analysis for fatty acids, amino acids, and minerals for whole plant and grain samples demonstrated that the maize grown on either continent were comparable, however, values for amino acids in Chile were beyond the previously observed ranges for 7 of the 18 examined; with these amino acids, the percent differences ranged from 2 to 13 % higher. Given the typical biological variability observed in any field or natural situation, these differences, while above what was expected, are not substantially out of line with what was previously known. When total protein levels were measured between countries, there were some statistically different values. Except for the leaf expression data from Chile, in all cases the ranges overlapped between growing regions and the differences observed (1 to 2 fold in some cases) were not totally unexpected for a biological system grown in an environment with several variables (e.g., water relations, GDU, soil type). CLASSIFICATION: ACCEPTABLE	451311-04

Cry1F Lateral Flow Test Kit Procedure for Analyzing Cry1F Corn Grain	A double antibody sandwich test was developed to detect the Cry1F protein in homogenized maize grain samples using a rapid test method. A double antibody sandwich technique is used in the Lateral Flow Test Kit for Cry1F. Antibodies raised against the Cry1F protein are incorporated into the Lateral Flow test strip and coupled to a color reagent. When in contact with Cry1F protein, the antibodies bind Cry1F and a sandwich is formed, however, not all of the antibodies are coupled to the color reagent. The test strips contain two zones wherein capture of color reagent or antibodies can occur. One zone captures bound Cry1F and the other captures color reagent. Both zones display a reddish color when protein-antibody sandwich and / or unreacted color reagent are captured. When only one line (control) line is present, a negative sample is indicated, while the presence of two lines indicates the presence of Cry1F. The Cry1F Lateral Flow Test Kit accurately detected Cry1F protein in 30 of 30 corn kernels from Cry1F maize and indicated negative reactions for the 30 control maize kernels. This finding demonstrates the utility of using the Cry1F Lateral Flow Test Kit for detection of Cry1F protein in maize grain samples. This kit allows for a rapid qualitative determination of the presence of Cry1F protein. CLASSIFICATION: ACCEPTABLE	452793-01
Method Validation Report for the Determination of Cry1F Delta-endotoxin Protein in Corn Grain by Enzyme Linked Immunosorbent Assay	The results of this assay validation indicate that the ELISA based assay was suitable for the analysis of Cry1F as found in maize grain. Average recoveries from samples spiked with Cry1F protein (truncated microbial form) were between 67 and 107 %. Extractions from known Cry1F maize grain samples demonstrated that a sample as small as 50 mg could be properly extracted and quantified. CLASSIFICATION: ACCEPTABLE	452793-02
Thermolability of Cry1F (truncated) Delta-Endotoxin	The Cry1F test substance was prepared in 10 mM potassium phosphate buffer (pH 7.5) and placed into a water bath at either 60, 75 or 90 °C for 30 minutes, or into the refrigerator at 4 °C. Application of treated Cry1F to the surface of an insect diet and measurement of growth inhibition of neonate tobacco budworm larvae, indicated that the Cry1F protein was labile to heat at and above 75 °C. CLASSIFICATION: ACCEPTABLE	452748-01
Compositional Analysis of Maize MPS Hybrid Line 1507	Protein and nutritional parameters were measured in grain and whole plant samples of hybrid 1507 (expressing Cry1F) and a genetically similar control hybrid, both grown at 4 locations in Chile. Fatty acids, ash, vitamins, fiber, moisture, amino acids, minerals and antinutrients were examined using standard tests. No difference was observed between levels of these constituents in the hybrid 1507 when compared to commercial hybrids not encoding this gene, however, the non-essential amino acid, glutamic acid, was slightly above the known ranges for both the control and test lines. CLASSIFICATION: ACCEPTABLE	452748-02

Equivalency of microbial and maize expressed Cry1F protein; Characterization of test substances for biochemical and toxicological studies.	Standard techniques of protein chemistry were used to assess similarities between the bacterial and plant sources of the Cry1F protein. Additionally, insect mortality assays were performed to determine *in vitro* toxicity. An *in vitro* digestibility assay was done to determine that Cry1F was unstable under conditions simulating the gastric environment. This simulation of gastric conditions indicated that the toxin (from microbial source) was readily digested by pepsin. SDS-PAGE and Western blotting of plant and bacterial sources determined the presence of a 65 kDa protein corresponding to the trypsinized core of the δ-endotoxin. Plant extracts contained 0.158 % Cry1F as determined by ELISA; control plants were negative. N-terminal sequencing of 5 aa determined that the microbial and plant expressed protein maintained this sequence intact. Glycosylation was not evident in Cry1F from either source. CLASSIFICATION: ACCEPTABLE	447149-03

4. Product Characterization of Cry1Ac Cotton (006445)

Monsanto submitted information which adequately described the Cry1Ac delta-endotoxin from *Bacillus thuringiensis* subsp. *kurstaki*, as expressed in cotton, along with the genetic material necessary for its production. Because it would be difficult, or impossible, to extract sufficient biologically-active toxin from the plants to perform toxicology tests, Monsanto used delta-endotoxin produced in bacteria. Product analysis data was submitted to show that the microbially expressed and purified Cry1Ac delta-endotoxin is sufficiently similar to that expressed in the plant to be used for mammalian toxicological purposes. Plant and microbially produced Cry1Ac delta-endotoxin were shown by these studies to have similar molecular weights and immunoreactivity (SDS-PAGE and western blots), to lack detectable post-translational modification (glycosylation tests), to have identical amino acid sequences in the N-terminal region and to have similar results in bioassays against *Heliothis virescens* and *Helicoverpa zea*. While it is difficult to prove that two proteins are identical, the combined results of the below studies indicate a high probability that these two sources produce proteins that are essentially identical by available protein analytical assays.

Study Type	Result	MRID #
Protein Characterization and Expression Characterization of the DNA Inserted in the Plant	Southern blot analysis on restriction digests of DNA extract from cotton line 531 and the parental Coker 312 showed that there is probably only one insert of the *cry1Ac* gene cassette present in the transformed line. The introduced gene appears to be genetically stable in cotton according to the results of progeny selfing and backcrosses with elite lines. The amino acid sequence is homologous to the *cryIAb* gene from HD-1 for positions 1-466 and homologous to *cry1Ac* for positions 467-1178 with a single exception of a leucine-serine 766 in the crystal portion of the protein cleaved prior to toxin activation. Western blot analysis of purified toxin, leaf tissue from cotton line 531 and the parental Coker 312 shows that trypsinized extracts have comigrating bands similar to that found in *B.t.k.* HD-73 protein reference material and commercial preparations. CLASSIFICATION: ACCEPTABLE.	431452-01
Protein Characterization and Expression	*B.t.k.* HD-73 toxin isolated from either cotton line 531 or 931 were compared to the toxin expressed in *Escherichia coli* (*E. coli*) by SDS-PAGE, western blot, glycosylation and bioactivity. The data presented suggests the bacterially produced protein and that found in cotton are equivalent and suggests the bacterially produced *B.t.k.* HD-73 toxin can serve as a surrogate test substance in the toxicological tests to support the registration of transgenic cotton. The original data package for this study did not have a section describing the purification method to obtain the plant standard, and was classified as supplementary on that basis. Additional information on the purification method as described in "Assessment of Equivalence Between *E. coli*-produced and cotton-produced *B.t.k* HD-73 Protein..." MRID 43152-02 were provided. The additional information was sufficient to clarify the extraction procedure and the study is now ACCEPTABLE.	431452-02
Protein Characterization and Expression	The delta-endotoxin from *B.t.k.* HD-73 (lot # 5025385) produced in *E. coli* containing plasmid (pMON10569) was purified, lyophilized and found to have the following characteristics: 4.5% moisture, 75.6% protein (amino acid analysis), 70% protein (BCA), 88% HD-73 specific protein (ELISA), 80% HD-73 specific protein (coomassie blue PAGE), 1.6 micrograms gram-negative endotoxin/mg and no significant trace metals except for sodium, potassium and phosphate. The molecular weight of the *B.t.k.* HD-73 toxin was estimated to be 134.8 kD for the full length species and 77.1 kD for the tryptic fragment. The functional activity was found to be an LC_{50} of 0.28 ppm against *Heliothis virescens*. CLASSIFICATION: ACCEPTABLE.	431452-03

Study Type	Result	MRID #
Protein Characterization and Expression	Ten insect pest species from 5 families were tested for their sensitivity to *B.t.k.* HD-73 protein. Only in the lepidopteran species was there significant mortality. In one study, the green peach aphid showed marginal effects from treatment with a tryptic digest of the Cry1Ac toxin from *B.t.k.* HD-73 which was not reproducible in a repeat test. The tryptic digest preparation positive control from a *B.t.k* species also showed higher mortality in the tobacco budworm test than that produced in *E. coli.* CLASSIFICATION: ACCEPTABLE.	431452-04

5. Product Characterization of Cry3A Potato (006432)

Monsanto submitted information which adequately described the plant-pesticidal substance, *Bacillus thuringiensis* subsp. *tenebrionis* Cry3A delta endotoxin as produced in potato. Because it would be difficult, or impossible, to extract sufficient biologically-active toxin from the plants to perform toxicology tests, Monsanto used an endotoxin produced in bacteria. Product analysis data were submitted to show that the microbially expressed and purified *Bt* Cry3A delta endotoxin is sufficiently similar to that expressed in the plant to be used for mammalian toxicological purposes.

Study Type	Result	MRID #
Characterization of the DNA Inserted in the Plant	The relative size and number of copies of the DNA inserted into potatoes was demonstrated with endonuclease digested chromosomal DNA from field grown potato plants Southern blotted with the introduced plasmid as the probe. These Southern blots provided information about the number of copies of introduced DNA, the lack of significant amount of DNA introduced outside the border regions and integrity of the introduced DNA near the endonuclease cut site. These results indicate that only the DNA necessary to produce the Cry3A delta endotoxin were introduced into the plant. CLASSIFICATION: ACCEPTABLE	429322-01
Protein Characterization and Expression	Microbially-produced delta endotoxin from the *cry3A* gene as expressed in Escherichia coli and in potato tubers was compared. The data consist of SDS-PAGE co-migration, western blot analysis, staining for carbohydrate residues, N-terminal amino acid sequence analysis and biological equivalence against *Leptinotarsa decemlineata.* These data are adequate to support the equivalence of the microbially- and plant-produced protein for use in the toxicology studies. CLASSIFICATION: ACCEPTABLE	429322-02

Study Type	Result	MRID #
Protein Characterization and Expression	The purity and activity of a 55kD protein released with tryptic digestion of the *Bt* Cry3A delta endotoxin purified from *E. coli* was shown to have a similar size, immunoreactivity and amino acid sequence to the 55kD fragment found in potato tubers. The 55kD protein had somewhat higher bioactivity than the 68kD full-length delta endotoxin from *B.t.t*. These data support the contention that both the 55kD and 68kD forms of the Cry3A delta endotoxin found in the plant were similar to those occurring in *B.t.t*. CLASSIFICATION: ACCEPTABLE	429322-05
Characterization of *E. coli*-Produced Cry3A Protein	The method of preparing by fermentation the delta endotoxin from *B.t.t.* in *E. coli* was presented. The protein was characterized for purity and stability after purification. This data indicates that normal fermentation techniques were used to produce the plant equivalent, microbial Cry3A delta endotoxin. CLASSIFICATION: ACCEPTABLE	429322-04
Protein Characterization and Expression	The Cry3A delta endotoxin as expressed in potato tissue or an *E. coli* alternative gives a similar immunoreactivity and electrophoretic mobility to registered microbial products producing the same delta endotoxin. CLASSIFICATION: ACCEPTABLE	429322-06

B. HUMAN HEALTH ASSESSMENT

1. Background

The basic premise relied on for the toxicology assessment is the fact that all the *Bt* plant-incorporated protectants are proteins. Proteins are commonly found in the diet and, except for a few well described phenomena, present little risk as a mammalian hazard. In addition, for the majority of *Bt* proteins currently registered, the source bacterium has been a registered microbial pesticide which has been approved for use on food crops without specific restrictions. Because of their use as microbial pesticides, a long history of safe use is associated with many *Bt* products.

Several types of data are required for the *Bt* plant-incorporated protectants to provide a reasonable certainty that no harm will result from the aggregate exposure to these proteins. The information is intended to show that the *Bt* protein behaves as would be expected of a dietary protein, is not structurally related to any known food allergen or protein toxin, and does not display any oral toxicity when administered at high doses. These data consist of an *in vitro* digestion assay, amino acid sequence homology comparisons and an acute oral toxicity test. The acute oral toxicity test is done at a maximum hazard dose using purified protein of the plant-incorporated protectant as a test substance. Due to limitations of obtaining sufficient quantities of pure protein test substance from the plant itself, an alternative production source of the protein

is often used such as the *Bacillus thuringiensis* source organism or an industrial fermentation microbe. The justification for employing this alternative source of pure protein is the equivalence data discussed above under product characterization.

EPA believes that protein instability in digestive fluids and the lack of adverse effects using the maximum hazard dose approach in general eliminate the need for longer-term testing of *Bt* protein plant-incorporated protectants. Dosing of these animals with the maximum hazard dose, along with the product characterization data should identify potential toxins and allergens, and provide an effective means to determine the safety of these protein. The adequacy of the current testing requirements was discussed at the June 7, 2000 Scientific Advisory Panel (SAP) meeting. In their final report, the SAP agreed in principle with the methods used by EPA to assess the toxicity of proteins expressed in plants especially the maximum hazard dose approach.

2. *In vitro* Digestibility Assay

The intent of this assay is to demonstrate that the *Bt* protein is degraded into small peptides or amino acids in solutions that mimic digestive fluids. Usually only gastric fluid is tested since Cry protein is known to be stable in intestinal fluid, but in the initial *Bt* products registered, gastric and intestinal fluids were examined separately. In order to track the breakdown, the proteins were added to a solution of the digestive fluids and a sample was either removed or quenched at given time points (usually at time 0, one to several minutes later and one hour later). The time point samples were then electrophoresed on either an SDS-PAGE gel and further analyzed by western blot or tested in a bioassay against the target pest. All were degraded in gastric fluid in 0-7 minutes. All the *Bt* proteins tested in intestinal fluid were not affected by trypsin digestion as would be expected since this is similar to their behavior in the insect gut. In intestinal fluid, those *Bt* plant-incorporated protectants that are expressed as protoxin molecules broke down into the active toxin moiety and degraded no further.

As has been stated in several public fora, the *in vitro* digestibility test is basically a test to confirm the biochemical characteristic of instability of the protein in the presence of digestive fluids. The digestibility test is not intended to provide information on the toxicity of the protein or imply that similar breakdown will happen in all human digestive systems. The *in vitro* digestibility assay may also provide information about the potential of a protein to be a food allergen. The *in vitro* digestion assays confirm that the protein is being broken down in the presence of typical digestive fluids and is not unusually persistent in the digestive system. One of the limitations of the test is that it usually only tracks protein breakdown to fragments still recognized by the immunological reagents employed.

3. Heat Stability and Amino Acid Homology

Two additional characteristics that are considered as an indication of possible relation to a food allergen are a protein's ability to withstand heat or the conditions of food processing and its amino acid sequence when compared to known food allergens. For a few of the protein plant-incorporated protectants registered to date there is information about the heat/processing stability of the delta-endotoxins as expressed in bioactivity or immunological recognition after typical food processing. The Cry1Ab protein in one corn product and the Cry1Ac protein were demonstrated to be inactive in processed corn. These results were used to justify waivers for the fish feed toxicity studies. No heat stability studies were available for Cry3A. A full-length amino acid sequence homology comparison for one Cry1Ab product against the database of known proteins (allergens and gliadins) has been formally reviewed by the Agency. No amino acid sequence homology comparison data has been submitted for Cry3A.

4. Acute Oral Toxicity

One of the bases for addressing the toxicity of proteins primarily through the use of acute oral toxicity is that, when demonstrated to be toxic, proteins are toxic at low doses (Sjoblad, *et al*., 1992). Therefore, when no effects are shown to be caused by the protein plant-incorporated protectants, even at relatively high dose levels in the acute oral exposure, the proteins are not considered toxic. The acute oral toxicity test is performed in mice with a pure preparation of the plant-incorporated protectant protein at doses from 3280 to over 5000 mg/kg bodyweight. None of the tests performed to date have shown any significant effects on the treated animals.

5. EPA Recommendation

The mammalian toxicity data continue to support the registrations of the *Bt* products described. EPA believes the data it currently has is sufficient to support the *Bt* plant-incorporated protectant registrations.

The data listed below is confirmatory and is the type of data that will bring each product up to current standards needed to make a regulatory decision. The data/information listed below include heat/processing stability of the subject protein as well as amino acid sequence homology comparisons. There are several types of amino acid sequence comparisons. A full length sequence comparison of the subject protein is made against a data base of the known sequences of protein toxins and allergens. More specialized comparisons including a stepwise series of overlapping 8 amino acid peptides along the entire sequence of the subject protein and screening for potential sites for post-translational modification (e.g., potential glycosylation sites) are also needed. As valid methods become available, more complete analyses of the expressed protein (e.g. MALDI TOF) should be utilized to confirm the expressed amino acid sequence.

None of the products registered at this time, all of which have tolerance exemptions for food use, show any characteristics of toxins or food allergens.

Table B1 Confirmatory Studies Needed to Complete Product Database

Common Name and Cry Protein	OPP Chemical Code	Study Type
*Bt*11, Cry1Ab *Bt* Corn	006444	Amino Acid Sequence Homology*
MON810, Cry1Ab *Bt* Corn	006430	Additional Amino Acid Sequence Homology* Processing and/or Heat Stability
Cry1Ac cotton	006445	Additional Amino Acid Sequence Homology*
Cry3A Potato	006432	Additional Amino Acid Sequence Homology*

* as described in section IIB.5.

6. Human Health Assessment of Cry1Ab Crops, Including But Not Limited To: Bt11 Cry1Ab Bt Corn (006444) and MON810 Cry1Ab Bt Corn (006430)

a. Toxicology Assessment

Mammalian toxicology data are available to examine the potential effects of Cry1Ab on human health and assess if the data support the registration of *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material necessary for its production (plasmid vector pZO1502) in corn and *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material necessary for its production in corn (OPP PC code 006430). *Bt* microbial pesticides, containing Cry proteins other than Cry1Ab, have been applied for more than 30 years to food and feed crops consumed by the U.S. population. These data would also support other Cry1Ab plant-incorporated protectants' human health assessments provided adequate information was submitted to show that the Cry1Ab proteins in question were biochemically and functionally similar to the proteins of the plant-incorporated protectants already examined.

1) Acute Toxicity

Study Type	Result	MRID #
Acute Oral Toxicity in Mice	Five male and five female mice received a single dose of 3,280 mg/kg of Cry1Ab protein by oral gavage. No animals died nor were there significant clinical signs as a result of the exposure. One female failed to gain weight between day 7 and day 14. All animals gained weight by the end of the study. Males gained more weight over the study than females. CLASSIFICATION: ACCEPTABLE Test substance is given a TOXICITY CATEGORY IV rating although highest dose administered is 3280 mg/kg due to lack of any evidence of a dose/effect relation.	433236-08
Acute Oral Toxicity Study of *B.t.k.* HD-1 Tryptic Core Protein In Albino Mice	No test substance related deaths occurred. One female died within a day of BSA dose administration due to a perforated trachea. The majority of the animals failed to gain weight or showed a slight weight reduction. No treatment related trends in these losses was apparent. CLASSIFICATION: ACCEPTABLE. Test substance is given a TOXICITY CATEGORY IV rating although highest dose administered is 4000 mg/kg due to lack of any evidence of a dose/effect relation.	434680-01

2) Mutagenicity and Developmental Toxicity, Subchronic Toxicity, and Chronic Exposure and Oncogenicity Assessment

Data demonstrating no mammalian toxicity at high levels of exposure confirm the safety of the product at levels well above any possible maximum exposure levels anticipated for a plant-incorporated protectant. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.740(b).] For microbial products, further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study.

The acute oral toxicity data submitted support the determination that the Cry1Ab protein is non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, *et al*.1992). Since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry1Ab delta-endotoxin protein is not considered toxic. Because these proteins break down into their constituent amino acids, there would be no chronic exposure to the protein and therefore no need for chronic toxicity testing. Therefore, the mutagenicity, developmental toxicity, subchronic toxicity, chronic exposure and oncogenicity assessment studies were not required.

3) Effects on the Immune System

Since Cry1Ab is a protein, allergenic potential was considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, to be glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry1Ab delta-endotoxin is degraded in two minutes by gastric fluid *in vitro* and is non-glycosylated. Studies submitted to EPA done in laboratory animals have not indicated any potential for allergic reactions to *B. thuringiensis* or its components, including the delta-endotoxin in the crystal protein. Despite decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)2 have been made for various *Bacillus thuringiensis* microbial products claiming dermal allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins. The reported reactions were determined to be due to non-Cry proteins produced during fermentation or to added formulation ingredients. Thus, the Cry1Ab protein is not expected to be a food allergen.

Allergenicity Endpoints of Cry1Ab Crops [*Bt*11 and MON810 *Bt* Corn (006444 & 006430)]

Study Type	Result	MRID #
In vitro Digestibility	The Cry1Ab protein from either maize or B.t.k. HD1-9 is rapidly degraded in the presence of pepsin. Using 1/1000 strength pepsin, a time course study shows that the introduced protein from either source degrades within 10 minutes to lack of any recognition in a western blot assay. CLASSIFICATION: ACCEPTABLE.	433236-06
In vitro Digestibility	The tryptic core Cry1Ab protein is significantly degraded by 2 minutes incubation in gastric fluid but not significantly affected by 19.5 hours in intestinal fluid as monitored by western blot. The decrease in bioactivity of these digestions against tobacco budworm is similar to its loss of immunorecogniton in western blots CLASSIFICATION: ACCEPTABLE	434392-01
Amino Acid Sequence Homology	An amino acid database was constructed containing amino acid sequences of known protein allergens and gliadins. The B.t.k. HD-1 protein was compared to this database and no significant sequence similarity was identified. Based upon this data, there does not appear to be significant sequence similarity between HD-1 and known protein allergens and gliadins.	453849-01

4) Effects on the Endocrine System

The pesticidal active ingredients are proteins, derived from sources that are not known to exert an influence on the endocrine system. Therefore, the Agency is not requiring information on the endocrine effects of these plant-pesticides at this time.

5) Dose Response Assessment

No toxicological endpoints were identified, therefore a dose response assessment was not required.

6) Dietary Risk Characterization

a) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry1Ab protein include information on the characterization of the expressed Cry1Ab delta-endotoxin in corn, the acute oral toxicity, and *in vitro* digestibility of the delta-endotoxin.

Adequate information was submitted to show that the Cry1Ab test material derived from microbial cultures were biochemically and functionally similar to the proteins produced by the plant-incorporated protectant ingredients in corn. Production of microbially produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the conclusion that the Cry1Ab protein is non-toxic to humans. Therefore, because no effects were shown to be caused by these plant-incorporated protectants, even at relatively high dose levels (4000 mg/kg), the Cry1Ab delta-endotoxin protein is not considered toxic. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.740(b).] Further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are only triggered by significant acute effects in studies such as the mouse oral toxicity study. Because the acute testing showed no toxicity, higher tier testing is not required.

Because Cry1Ab is a protein and the major exposure is dietary, food allergenic potential was considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, are glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry1Ab delta-endotoxin is degraded in two minutes by gastric fluid *in vitro* and is non-glycosylated. After decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the

delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)(2) have been made for various *Bacillus thuringiensis* microbial products claiming dermal allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins. Thus, the Cry1Ab protein is not expected to be a food allergen.

Both (1) available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children) and (2) safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food additives, are generally recognized as appropriate for the use of animal experimentation data were not evaluated because the lack of mammalian toxicity at high levels of exposure demonstrates the safety of the product at levels above possible maximum exposure levels.

The genetic material necessary for the production of the plant-incorporated protectants active ingredients are the nucleic acids (DNA) which comprise (1) genetic material encoding these proteins and (2) their regulatory regions. "Regulatory regions" are the genetic material (termed promoters, terminators and enhancers) that control the expression of the DNA encoding proteins. DNA is common to all forms of plant and animal life and the Agency knows of no instance where these nucleic acids have been associated with toxic effects related to their consumption as a component of food. These ubiquitous nucleic acids as they appear in the subject active ingredient have been adequately characterized by the applicant. Therefore, no mammalian toxicity is anticipated from dietary exposure to the genetic material necessary for the production of the subject active plant pesticidal ingredients.

Residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity in the acute exposures.

b) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408 provides that EPA shall apply an additional tenfold margin of exposure (safety) for infants and children in the case of threshold effects to account for pre- and post-natal toxicity and the completeness of the database unless EPA determines that a different margin of exposure (safety) will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that infants and children will consume minimal residues of this plant-pesticide and that there is a finding of no toxicity.

Thus, there are no threshold effects of concern and, as a result the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

c) Aggregate Exposure (Not Including Occupational Exposure) Risk Conclusions

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectants chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Oral exposure, at very low levels, may occur from ingestion of processed corn products and drinking water. However, a lack of mammalian toxicity and the digestibility of the plant-incorporated protectants has been demonstrated. The use sites for Cry1Ab delta endotoxin are all agricultural for control of lepidopteran insects. Therefore, exposure via residential or lawn use to infants and children is not expected. Even if negligible exposure should occur, the Agency concludes that such exposure would present no risk due to the lack of toxicity.

d) Cumulative Effects Risk Conclusions

The Agency has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity to these plant-incorporated protectants, there are no cumulative effects.

e) Dietary Risk Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the United States population, including infants and children, to the Cry1Ab protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. We have arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed for the currently registered plant-incorporated protectants.

f) Occupational Exposure and Risk Characterization

Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Worker exposure to the Cry protein via seed dust is also expected to be negligible because of the low amount of protein expressed in transformed plants. If such exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity. If any unreasonable adverse effects caused by exposure to Cry1Ab are identified, these effects must be reported to the Agency as required by Sec. 6(a)(2) of FIFRA.

BPPD RECOMMENDATION:
There is a reasonable certainty that no harm will result from exposure to *Bacillus thuringiensis* Cry1Ab delta-endotoxin and the genetic material necessary for its production in corn. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. Therefore, EPA considers that the Cry1Ab tolerance exemption has been reassessed and meets the 408(c)(2) standard.

7. Human Health Assessment of Cry1F Corn (006481)

a. Mammalian Toxicity and Allergenicity Assessment

Data have been submitted demonstrating the lack of mammalian toxicity at high levels of exposure to the pure Cry1F protein. These data demonstrate the safety of the products at levels well above maximum possible exposure levels that are reasonably anticipated in the crops. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.740(b)(2)(i).] For microbial products, further toxicity testing and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study, to verify the observed effects and clarify the source of these effects (Tiers II & III).

The acute oral toxicity data submitted support the prediction that the Cry1F protein would be non-toxic to humans. Male and female mice (5 of each) were dosed with 15 % (w/v) of the test substance, which consisted of *Bacillus thuringiensis* var. *aizawai* Cry1F protein at a net concentration of 11.4 %. Two doses were administered approximately an hour apart to achieve the dose totaling 33.7 mL / kg body weight. Outward clinical signs and body weights were observed and recorded throughout the 14 day study. Gross necropsies performed at the end of the study indicated no findings of toxicity. No mortality or clinical signs were noted during the study. An LD_{50} was estimated at >5050 mg / kg body weight of this microbially produced test

material. The actual dose administered contained 576 mg Cry1F protein / kg body weight. At this dose, no LD_{50} was demonstrated as no toxicity was observed. Cry1F maize seeds contain 0.0017 to 0.0034 mg of Cry1F / gram of corn kernel tissue.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels [Sjoblad, Roy D., *et al*. "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)]. Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry1F protein is not considered toxic. Further, amino acid sequence comparisons showed no similarity between Cry1F protein to known toxic proteins available in public protein databases.

Since Cry1F is a protein, allergenic sensitivities were considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, may be glycosylated and present at high concentrations in the food.

Data has been submitted which demonstrates that the Cry1F protein is rapidly degraded by gastric fluid *in vitro* and is non-glycosylated. In a solution of Cry1F:pepsin at a molar ratio of 1:100, complete degradation of Cry1F to amino acids and small peptides occurred in 5 minutes. A heat lability study demonstrated the loss of bioactivity of Cry1F protein to neonate tobacco budworm larvae after 30 minutes at 75 °C. Studies submitted to EPA done in laboratory animals have not indicated any potential for allergic reactions to *B. thuringiensis* or its components, including the δ-endotoxin of the crystal protein. Additionally, a comparison of amino acid sequences of known allergens uncovered no evidence of any homology with Cry1F, even at the level of 8 contiguous amino acids residues.

The potential for the Cry1F protein to be a food allergen is minimal. Regarding toxicity to the immune system, the acute oral toxicity data submitted support the prediction that the Cry1F protein would be non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels [Sjoblad, Roy D., et al. "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)]. Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry1F protein is not considered toxic.]

b. Aggregate Exposures

Pursuant to FFDCA section 408(b)(2)(D)(vi), EPA considers available information concerning aggregate exposures from the pesticide residue in food and all other non-occupational exposures,

including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectant chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectant is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Oral exposure, at very low levels, may occur from ingestion of processed corn products and, potentially, drinking water. However a lack of mammalian toxicity and the digestibility of the plant-incorporated protectants have been demonstrated. The use sites for the Cry1F protein are all agricultural for control of insects. Therefore, exposure via residential or lawn use to infants and children is not expected. Even if negligible exposure should occur, the Agency concludes that such exposure would present no risk due to the lack of toxicity demonstrated for the Cry1F protein.

c. Cumulative Effects

Pursuant to FFDCA Section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity to these plant-incorporated protectants, we conclude that there are no cumulative effects for the Cry1F protein.

d. Determination of Safety for U.S. Population, Infants and Children

1) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry1F protein include the characterization of the expressed Cry1F protein in corn, as well as the acute oral toxicity, heat stability, and *in vitro* digestibility of the proteins. The results of these studies were determined applicable to evaluate human risk and the validity, completeness, and reliability of the available data from the studies were considered.

Adequate information was submitted to show that the Cry1F test material derived from microbial cultures was biochemically and, functionally similar to the protein produced by the plant-incorporated protectant ingredients in corn. Production of microbially produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the prediction that the Cry1F protein would be non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels [Sjoblad, Roy D., et al. "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)]. Since no effects were shown to be caused by Cry1F protein, even at relatively high dose levels (>5,050 mg test substance / kg body weight; 576 mg Cry1F / kg body weight), the Cry1F protein is not considered toxic. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.740(b)(2)(i).] For microbial products, further toxicity testing and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study to verify the observed effects and clarify the source of these effects (Tiers II & III).

Although Cry1F expression level data was required for an environmental fate and effects assessment, residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity.

Both (1) available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children); and (2) safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food additives, are generally recognized as appropriate for the use of animal experimentation data were not evaluated. The lack of mammalian toxicity at high levels of exposure to the Cry1F protein demonstrates the safety of the product at levels well above possible maximum exposure levels anticipated in the crop.

The genetic material necessary for the production of the plant-incorporated protectants active ingredients are the nucleic acids (DNA, RNA) which comprise (1) genetic material encoding these proteins and (2) their regulatory regions. "Regulatory regions" are the genetic material, such as promoters, terminators, and enhancers, that control the expression of the genetic material encoding the proteins. DNA and RNA are common to all forms of plant and animal life and the Agency knows of no instance where these nucleic acids have been associated with toxic effects related to their consumption as a component of food. These ubiquitous nucleic acids, as they appear in the subject active ingredient, have been adequately characterized by the applicant. Therefore, no mammalian toxicity is anticipated from dietary exposure to the genetic material

necessary for the production of the subject active plant pesticidal ingredients.

2) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408(B)(2)(C) also provides that EPA shall apply an additional tenfold margin of safety for infants and children in the case of threshold effects to account for pre- and post-natal toxicity and the completeness of the database unless EPA determines that a different margin of safety will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that there is a finding of no toxicity for the Cry1F protein and the genetic material necessary for its production. Thus, there are no threshold effects of concern and, as a result, the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

3) Overall Safety Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry1F protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information.

The Agency has arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed for the plant-incorporated protectants.

e. Other Considerations

1) Endocrine Disruptors

The pesticidal active ingredients are proteins, derived from sources that are not known to exert an influence on the endocrine system. Therefore, the Agency is not requiring information on the endocrine effects of these plant-pesticides at this time.

2) Analytical Method(s)

A validated method for extraction and direct ELISA analysis of Cry1F in corn grain has been submitted and found acceptable by the Agency.

3) Codex Maximum Residue Level

No Codex maximum residue levels exists for the plant-incorporated protectants *Bacillus thuringiensis* Cry1F protein and the genetic material necessary for its production in corn.

f. Tolerance Exemption

Therefore, 40 CFR chapter I was amended to add section 180.1217.

Section 180.1217 *Bacillus thuringiensis* Cry1F Protein and the Genetic Material Necessary for its Production in Corn; exemption from the requirement of a tolerance.

Bacillus thuringiensis Cry1F protein and the genetic material necessary for its production in corn are exempt from the requirement of a tolerance when used as plant-pesticides (now known as plant-incorporated protectants) in the food and feed commodities of field corn, sweet corn and popcorn. ``Genetic material necessary for its production'' means the genetic material which comprise: genetic material encoding the Cry1F protein and its regulatory regions. ``Regulatory regions'' are the genetic material, such as promoters, terminators, and enhancers, that control the expression of the genetic material encoding the Cry1F protein.

g. Supporting Data

Study	Result	MRID #
Acute oral toxicity study in mice: Cry1F Bacillus thuringiensis var. aizawai delta-endotoxin.	Dosing of ten albino mice with bacterial cell protein containing the d-endotoxin of Bacillus thuringiensis var. aizawai at > 5050 mg/kg (0.576 g/kg of Cry1F) body weight resulted in no mortality and no observed gross abnormalities. All animals appeared normal during the study and all except one gained weight throughout the study. Classification: Acceptable. Toxicity category III based on dose given with no observable effect.	446911-01

Supplement to MRID 446911-01: Supplemental Data for Acute Oral Toxicity Study in Mice: Cry1F Bacillus thuringiensis var. aizawai delta-endotoxin	This submission represents a clarification of test substance as presented in a previous submission and review. The acute oral toxicity study dosed mice at > 5050 mg microbial protein / kg body weight. The actual dose administered contained 576 mg Cry1F protein / kg body weight. At this dose, no LD_{50} was demonstrated as no toxicity was observed. The truncated form of the protein represents amino acids 28-612 of the Cry1F toxin sequence, whereas the plant-expressed form of Cry1F contains amino acids 1-605. The truncated form used in the oral toxicity study adequately represents that toxin to be found in the plant expression system. Classification: Acceptable.	450201-18
Comparison of amino acid sequence similarity of Cry1F and PAT proteins to known allergen proteins	A modified (synthetic) form of the cry1Fa2 gene and the phosphinothricin acetyl transferase (pat) gene were inserted into maize plants by microprojectile bombardment. A database of available sequenced allergens and toxins was searched for similarity to both the less than full-length Cry1F and PAT proteins such that a level of eight, contiguous amino acid homology would be detected. This number of contiguous amino acids is considered to be the smallest antigenic portion of a protein (peptide) to induce an allergic reaction based upon T-cell recognition in a sensitized individual. The database search and comparison to known allergens from plant, bacterial, fungal and animal origins indicates that no significant amino acid homology exists for Cry1F or PAT with any of these proteins. For both proteins of interest, the lack of any significant amino acid homology indicates that the potential for an immunological response developing into a food allergy from consumption of these proteins is low. Classification: Acceptable.	449717-01
Equivalency of microbial and maize expressed Cry1F protein; Characterization of test substances for biochemical and toxicological studies.	Standard techniques of protein chemistry were used to assess similarities between the bacterial and plant sources of the Cry1F protein. Additionally, insect mortality assays were performed to determine in vitro toxicity. An in vitro digestibility assay was done to determine that Cry1F was unstable under conditions simulating the gastric environment. This simulation of gastric conditions indicated that the toxin (from microbial source) was readily digested by pepsin. SDS-PAGE and Western blotting of plant and bacterial sources determined the presence of a 65 kDa protein corresponding to the trypsinized core of the d-endotoxin. Plant extracts contained 0.158 % Cry1F as determined by ELISA; control plants were negative. N-terminal sequencing of 5 aa determined that the microbial and plant expressed protein maintained this sequence intact. Glycosylation was not evident in Cry1F from either source. Classification: Acceptable.	447149-03

Thermolability of Cry1F (truncated) Delta-Endotoxin	The Cry1F test substance was prepared in 10 mM potassium phosphate buffer (pH 7.5) and placed into a water bath at either 60, 75 or 90 °C for 30 minutes, or into the refrigerator at 4 °C. Application of treated Cry1F to the surface of an insect diet and measurement of growth inhibition of neonate tobacco budworm larvae, indicated that the Cry1F protein was labile to heat at and above 75 °C. Classification: Acceptable.	452748-01

8. Human Health Assessment of Cry1Ac Bt Crops, Including But Not Limited To Cry1Ac Bt Cotton (6455)

a. Toxicology Assessment

Mammalian toxicology data are available to examine the potential effects of Cry1Ac protein on human health and assess if the data support registration of *Bacillus thuringiensis* Cry1Ac delta-endotoxin and the genetic material necessary for its production in corn and *Bacillus thuringiensis* Cry1Ac delta-endotoxin and the genetic material necessary for its production in cotton. *Bt* microbial pesticides, containing Cry proteins other than Cry1Ac, have been applied for more than 30 years to food and feed crops consumed by the U.S. population. These data would also support other Cry1Ac plant-incorporated protectants' human health assessments provided adequate information was submitted to show that the Cry1Ac test material derived from microbial cultures were biochemically and functionally similar to the proteins produced by the plant-incorporated protectants ingredients.

1) Acute Toxicity

The acute oral toxicity data demonstrates that the Cry1Ac endotoxin is non-toxic to humans.

Toxicological Endpoints of Cry1Ac Crops

Study	**Result**	**MRID #**
Acute Oral Toxicity	Ten male and female CD-1 mice per dose level were exposed by oral gavage to 500, 1000 and 4200 mg/kg bodyweight of E. coli produced B.t.k. HD-73 toxin. The controls were given the protein equivalent of 6340 mg/kg of bovine serum albumin. No mortalities or treatment related adverse effects were seen in either the treated or control mice. There were no observable dose related effects seen upon necropsy. CLASSIFICATION: ACCEPTABLE. TOXICITY CATEGORY IV.	431452-13

2) Mutagenicity and Developmental Toxicity, Subchronic Toxicity, and Chronic Exposure and Oncogenicity Assessment

The lack of mammalian toxicity at high levels of exposure demonstrates the safety of the product at levels above possible maximum exposure levels. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.740(b).] For microbial products, further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are only triggered by significant acute effects in studies such as the mouse oral toxicity study.

The acute oral toxicity data submitted support the determination that the Cry1Ac protein is non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, *et al*., 1992). Since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry1Ac delta-endotoxin protein is not considered toxic. Because these proteins break down into their constituent amino acids, there would be no chronic exposure to the protein and therefore no need for chronic toxicity testing. Therefore, the mutagencity, developmental toxicity, subchronic toxicity, chronic exposure and oncogenicity assessment studies are not required.

3) Effects on the Immune System

Since Cry1Ac is a protein, allergenic potential was considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, are glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry1Ac delta-endotoxin is degraded between two minutes and seven minutes by gastric fluid *in vitro* (MRID# 439995-03) and is non-glycosylated. Studies submitted to EPA done in laboratory animals have not indicated any potential for allergic reactions to *B. thuringiensis* or its components, including the delta-endotoxin in the crystal protein. After decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)2 have been made for various *Bacillus thuringiensis* microbial products claiming dermal allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins found in these strains. The reported reactions were determined to be due to non-Cry proteins produced during fermentation or to added formulation ingredients. Thus, the Cry1Ac protein is not expected to have any adverse effects on the immune system.

The Agency is aware of the recent scientific publications, including those by Bernstein (1999) and Vazquez, et al (1999 & 2000), which suggest that Cry1Ac protein could potentially act as allergenic or antigenic proteins. In the case of the Bernstein study, the report does indeed indicate that farm workers have circulating antibodies (both IgG and IgE) to components of the microbial sprays. However, there were no reports of clinical allergic disease in any worker or serum reactivity to the delta-endotoxin protein component of the microbial spray. These findings are cogent since any protein has the potential to induce an immunological reaction with some subset of these reactions possibly leading to an allergic response. The fact that this subset of workers showed signs of exposure to Bt sprays without developing an allergic response is significant and adds weight to the finding that the Bt delta-endotoxin proteins considered here are not expected to induce an allergic reaction.

The intent of the Vazquez *et al.* papers was to examine the Cry1Ac protein for its ability to induce both systemic and mucosal immunity with the goal of developing improved vaccines. In the Vazquez *et al.* papers Cry1Ac was administered by both the oral route and by intraperitoneal injection. While there are indications that the Cry1Ac protein has the ability to induce immune responses as measured by IgG, IgM and IgA levels, there is no data linking this to a hypersensitive response (i.e., levels of IgE were not determined in any study). It is important to realize that any protein has the ability to induce these types of antibodies (i.e., IgG, IgM and IgA) but that IgE is the major antibody type responsible for allergic reactions. The major effect seen in the Vazquez *et al.* studies was an increased intestinal IgG level when Cry1Ac was used as an adjuvant[1]. However, the increased IgG levels were only seen when Cry1Ac was introduced by intraperitoneal injection, not by the intragastric route and therefore has no relevance to any anticipated exposure for the Cry1Ac protein in cotton. It is also important to note that Cry1Ac immunization by the intragastric route was done with large amounts of an agent to neutralize stomach acid. This antacid would be expected to significantly raise the stomach pH, lessen proteolytic activity against the protein in the stomach and enhance the likelihood that the Cry1Ac protein would survive intact to be taken up by the gut associated lymphoid tissue (GALT).

One of the issues raised in the public comment is that the Vazquez *et al.* studies show Cry1Ac "is bound by mouse intestinal cells." There is no data described in any of these studies that would indicate this specific binding response to intestinal tissue. The data in the Vazquez *et al.* papers do suggest that Cry1Ac can be absorbed by the GALT to mount an immune response. However, this GALT sampling and immune response is similarly mounted to any dietary protein and is an integral part of the process leading to oral tolerance. Oral tolerance is the normal immune process of the GALT whereby dietary proteins are sampled as they pass through the

[1]An adjuvant is an aid to boosting the protective antibody response.

digestive tract and an initial immune response to these normal dietary components is seen. This immune response is modulated by the GALT over time to non-responsiveness or tolerance to these dietary proteins. Food allergy is basically a failure of the normal process of oral tolerance. In food allergy, dietary proteins are recognized as threatening foreign proteins by the GALT which then mounts an IgE response usually reserved only for pathogens and parasites. The process of oral tolerance is a major area of research as a mechanism to treat autoimmune diseases such as multiple sclerosis, hopefully decreasing the aberrant immune recognition of self proteins. In conclusion, the Vazquez *et al.* experiments are intriguing but being cited somewhat out of the context of their intent. The researchers were examining Cry1Ac as a potential adjuvant for use to improve the efficacy of oral or injected vaccines and their results cannot be extrapolated to address the potential food allergenicity of Cry1Ac.

Allergenicity Endpoints of Cry1Ac Crops

Study	Result	MRID #
In vitro Digestibility	The B.t.k. HD-73 protein was rapidly degraded to fragments not recognized in a western blot after 7 minutes incubation in simulated gastric fluid (SGF) and was not active in a tobacco budworm (TBW) bioassay after SGF incubation. The in vitro digestibility assay provides useful information to predict the metabolic fate of the Cry1Ac protein and its potential as a food allergen. However, it is not clear how this protein assay's results relate to protein toxicity. Therefore the Agency also requested that an acute oral toxicity study be done to confirm the expected lack of toxicity indicated by the in vitro digestibility results. CLASSIFICATION: ACCEPTABLE	431452-14
Amino Acid Sequence Homology	The FASTA amino acid sequence comparison tool of GCG was used to compare the amino acid sequence of Cry1Ac protein to known protein allergens and toxins. One hundred twenty one proteins were retrieved using “allergen” as the keyword and 1,935 proteins were retrieved using “toxin” as the key word for searches of public protein databases. When compared to these proteins, B.t.k. HD-73 protein (Cry1Ac) did not share more than random sequence similarity with any of the known toxins and allergens. Based upon this data, there does not appear to be any significant sequence similarity between B.t.k. HD-73 protein (Cry1Ac) and any known protein allergen or protein toxin. Note: This analysis is not equivalent to a stepwise 8 AA analysis of the subject protein against the available databases. CLASSIFICATION: ACCEPTABLE	454155-01

Study	Result	MRID #
Amino Acid Sequence Homology	Construction of the "allergen" and "gliadin" database allowed for direct comparison of the B.t.k. HD-73 (Cry1Ac) protein sequence to the available amino acid sequences of known protein allergens present in public domain databases. As part of the assessment of potential protein allergenicity of pesticidal proteins expressed in plants, amino acid sequence comparison provides a rapid analysis of the relatedness of a protein to those known to possess allergenic properties. A lack of protein similarity to known allergens and gliadins is not, in itself, definitive of a lack of potential allergenicity, but it does provide an important initial "screen" for protein characteristics and properties. Based upon the data provided in what appears to be an adequate amino acid sequence comparison, there does not appear to significant similarity between B.t.k. HD-73 protein and known protein allergens and gliadins. Note: This analysis is not equivalent to a stepwise 8 AA analysis of the subject protein against the available databases. CLASSIFICATION: ACCEPTABLE	454155-02

4) Effects on the Endocrine System

The pesticidal active ingredients are proteins, derived from sources that are not known to exert an influence on the endocrine system. Therefore, the Agency is not requiring information on the endocrine effects of these plant-pesticides at this time.

5) Dose Response Assessment

No toxicological endpoints are identified so no dose response assessment is required.

6) Dietary Risk Characterization

a) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry1Ac protein include information on the characterization of the expressed Cry1Ac delta-endotoxin in cotton, the acute oral toxicity, and *in vitro* digestibility of the delta-endotoxin. The results of these studies were determined to be adequate to evaluate human risk and the validity, completeness, and reliability of the available data from the studies were considered.

Data was submitted to show that the Cry1Ac test material derived from microbial cultures were biochemically and functionally similar to the proteins produced by the plant-incorporated

protectant ingredients. Production of microbially-produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the determination that the Cry1Ac protein is non-toxic to humans. Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels (5,000 mg/kg), the Cry1Ac delta-endotoxin protein is not considered toxic. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.740(b).] Further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are only triggered by significant acute effects in studies such as the mouse oral toxicity study. Because the acute testing showed no toxicity, higher tier studies are not required.

Because Cry1Ac is a protein and the major exposure is dietary, food allergenic potential was considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, are glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry1Ac delta-endotoxin is degraded between two minutes (MRID#439995-03) and seven minutes by gastric fluid *in vitro* and is non-glycosylated. Despite decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)(2) have been made for various *Bacillus thuringiensis* products claiming allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins. Thus, the Cry1Ac protein is not expected to be a food allergen.

Although Cry1Ac expression level data was required for an environmental fate and effects assessment, residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity.

Both (1) available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children) and (2) safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food additives, are generally recognized as appropriate for the use of animal experimentation data were not evaluated because the lack of mammalian toxicity at high levels of exposure demonstrate the safety of the product at levels above possible maximum exposure levels.

The genetic material necessary for the production of the plant-incorporated protectants active ingredients are the nucleic acids (DNA) which comprise (1) genetic material encoding these

proteins and (2) their regulatory regions. "Regulatory regions" are the genetic material (termed promoters, terminators and enhancers) that control the expression of the DNA encoding proteins. DNA is common to all forms of plant and animal life and the Agency knows of no instance where these nucleic acids have been associated with toxic effects related to their consumption as a component of food. These ubiquitous nucleic acids as they appear in the subject active ingredient have been adequately characterized by the applicant. Therefore, no mammalian toxicity is anticipated from dietary exposure to the genetic material necessary for the production of the subject active plant pesticidal ingredients.

As mentioned above (7a.3) the Agency is aware of the recent scientific publications, including those by Bernstein *et al.* (1999) and Vazqeuz *et al.* (1999 & 2000), which suggest that Cry1Ac protein could potentially act as allegenic or antigenic proteins. In the case of the Bernstein study, the report does indeed indicate that farm workers have circulating antibodies (both IgG and IgE) to components of the microbial sprays. However, there were no reports of clinical allergic disease in any worker or serum reactivity to the delta-endotoxin protein component of the microbial spray. These findings are cogent since any protein has the potential to induce an immunological reaction with some subset of these reactions possibly leading to an allergic response. The fact that this subset of workers showed signs of exposure to Bt sprays without developing an allergic response is significant and adds weight to the finding that the Bt delta-endotoxin proteins considered here are not expected to induce an allergic reaction.

The intent of the Vazquez *et al.* papers was to examine the Cry1Ac protein for its ability to induce both systemic and mucosal immunity with the goal of developing improved vaccines. In the Vazquez *et al.* papers Cry1Ac was administered by both the oral route and by intraperitoneal injection. While there are indications that the Cry1Ac protein has the ability to induce immune responses as measured by IgG, IgM and IgA levels, there is no data linking this to a hypersensitive response (i.e., levels of IgE were not determined in any study). It is important to realize that any protein has the ability to induce these types of antibodies (i.e., IgG, IgM and IgA) but that IgE is the major antibody type responsible for allergic reactions. The major effect seen in the Vazquez *et al.* studies was an increased intestinal IgG level when Cry1Ac was used as an adjuvant which is an aid to boosting the protective antibody response. However, the increased IgG levels were only seen when Cry1Ac was introduced by intraperitoneal injection, not by the intragastric route and therefore has no relevance to any anticipated exposure for the Cry1Ac protein in cotton. It is also important to note that Cry1Ac immunization by the intragastric route was done with large amounts of an agent to neutralize stomach acid. This antacid would be expected to significantly raise the stomach pH, lessen proteolytic activity against the protein in the stomach and enhance the likelihood that the Cry1Ac protein would survive intact to be taken up by the gut associated lymphoid tissue (GALT).

One of the issues raised in the public comment is that the Vazquez *et al.* studies show Cry1Ac "is bound by mouse intestinal cells." There is no data described in any of these studies that would indicate this specific binding response to intestinal tissue. The data in the Vazquez *et al.* papers do suggest that Cry1Ac can be absorbed by the GALT to mount an immune response. However, this GALT sampling and immune response is similarly mounted to any dietary protein and is an integral part of the process leading to oral tolerance. Oral tolerance is the normal immune process of the GALT whereby dietary proteins are sampled as they pass through the digestive tract and an initial immune response to these normal dietary components is seen. This immune response is modulated by the GALT over time to non-responsiveness or tolerance to these dietary proteins. Food allergy is basically a failure of the normal process of oral tolerance. In food allergy, dietary proteins are recognized as threatening foreign proteins by the GALT which then mounts an IgE response usually reserved only for pathogens and parasites. The process of oral tolerance is a major area of research as a mechanism to treat autoimmune diseases such as multiple sclerosis, hopefully decreasing the aberrant immune recognition of self proteins. In conclusion, the Vazquez *et al.* experiments are intriguing but being cited somewhat out of the context of their intent. The researchers were examining Cry1Ac as a potential adjuvant for use to improve the efficacy of oral or injected vaccines and their results cannot be extrapolated to address the potential food allergenicity of Cry1Ac.

b) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408 provides that EPA shall apply an additional tenfold margin of exposure (safety) for infants and children in the case of threshold effects to account for pre- and post-natal toxicity and the completeness of the database unless EPA determines that a different margin of exposure (safety) will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that infants and children will consume minimal residues of this plant-pesticide and that there is a finding of no toxicity.

Thus, there are no threshold effects of concern and, as a result the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

c) Aggregate Exposure (Not Including Occupational Exposure) Risk Conclusions

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectants chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Oral exposure, at very low levels, may occur from ingestion of processed products and drinking water. However, a lack of mammalian toxicity and the digestibility of the plant-incorporated protectants has been demonstrated. The use sites for Cry1Ac delta endotoxin are all agricultural for control of lepidopteran insects. Therefore, exposure via residential or lawn use to infants and children is not expected. Even if negligible exposure should occur, the Agency concludes that such exposure would present no risk due to the lack of toxicity.

d) Cumulative Effects Risk Conclusions

The Agency has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity to these plant-incorporated protectants, there are no cumulative effects.

e) Tolerance Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry1Ac protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. Therefore, EPA considers that the Cry1Ac tolerance exemption has been reassessed and meets the 408(c)(2) standard.

The Agency has arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed for the plant-incorporated protectants. As a result, EPA established an exemption from tolerance requirements pursuant to FFDCA section 408(j)(3) for *Bacillus thuringiensis* Cry1Ac delta-endotoxin and the genetic material necessary for its production in all plants.

Bacillus thuringiensis subspecies *kurstaki* Cry1Ac delta-endotoxin and the genetic material necessary for its production in all plants are exempt from the requirement of a tolerance when used as plant-incorporated protectants in all plant raw agricultural commodities. ``Genetic material necessary for its production'' means the genetic material which comprise (1) genetic

material encoding the Cry1Ac delta-endotoxin and (2) its regulatory regions. ``Regulatory regions'' are the genetic material that control the expression of the genetic material encoding the Cry1Ac delta-endotoxin, such as promoters, terminators, and enhancers.

f) Occupational Exposure and Risk Characterization

Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Worker exposure to the Cry protein via seed dust is also expected to be negligible because of the low amount of protein expressed in transformed plants. If such exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity. However, if any unreasonable adverse effects caused by exposure to Cry1Ac are identified, these effects must be reported to the Agency as described in Sec. 6(a)(2) of FIFRA.

BPPD RECOMMENDATION:
There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry1Ac protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because no toxicity to mammals has been observed for the plant-incorporated protectants and anticipated exposures are negligible.

8. Human Health Assessment of Cry3A Potatoes

a. Toxicology Assessment

The delta endotoxin proteins of *B. thuringiensis* have been intensively studied and no indications of mammalian toxicity have been reported. *Bt* microbial pesticides, containing Cry proteins other than Cry3A, have been applied for more than 30 years to food and feed crops consumed by the U.S. population. Furthermore, *B. thuringiensis* products containing Cry3A have been registered and in use for more than a decade, and the Agency has not received any reports of dietary toxicity attributable to their use. The Agency does not anticipate any mammalian toxicity from this protein in plants based on the use history of *B. thuringiensis* products. Therefore, EPA considers that the Cry3A tolerance exemption has been reassessed and meets the 408(c)(2) standard.

The data submitted by Monsanto indicate that this protein would be non-toxic to mammals under the proposed use. Cry3A protein was non-toxic to mice at doses up to 5220 mg/kg bodyweight. This level is >10,000 times the amount found in potato tubers. Adequate information was

submitted to show that the test material derived from microbial cultures was essentially identical to the protein as produced by the potatoes. Production of a microbial Cry 3A delta endotoxin equivalent to plant-produced delta endotoxin was chosen in order to obtain sufficient material for mammalian testing. In addition, the *in vitro* digestibility studies indicate the protein was degraded within 30 seconds in simulated gastric fluid.

Toxicological Endpoints of Cry3A Crops

Study	Result	MRID #
Acute Oral Toxicity of *B.t.t.* Protein	*Bt* Cry3A delta endotoxin was not toxic by oral gavage when mice were dosed with up to 5220 mg/kg body weight. CLASSIFICATION: ACCEPTABLE These results placed this protein in TOXICITY CATEGORY IV.	429322-17

2) Mutagenicity and Developmental Toxicity, Subchronic Toxicity, and Chronic Exposure and Oncogenicity Assessment

The lack of mammalian toxicity at high levels of exposure demonstrates the safety of the product at levels above possible maximum exposure levels. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.740(b).] For microbial products, further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are only triggered by significant acute effects in studies such as the mouse oral toxicity study.

The acute oral toxicity data submitted support the determination that the Cry3A protein is non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, *et al*., 1992). Since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels, the Cry3A delta-endotoxin protein is not considered toxic. Because these proteins break down into their constituent amino acids, there would be no chronic exposure to the protein and therefore no need for chronic toxicity testing. Therefore, the mutagencity, developmental toxicity, subchronic toxicity, chronic exposure and oncogenicity assessment studies are not required.

3) Effects on the Immune System

Since Cry3A is a protein, allergenic potential was considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, are glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry3A delta-endotoxin is degraded within 30 seconds (MRID# 429332-18) by gastric fluid *in vitro* and is non-glycosylated. Studies submitted to EPA done in laboratory animals have not indicated any potential for allergic reactions to *B. thuringiensis* or its components, including the delta-endotoxin in the crystal protein. After decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)(2) have been made for various *Bacillus thuringiensis* microbial products claiming dermal allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins. The reported reactions were determined to be due to non-Cry proteins produced during fermentation or to added formulation ingredients. Thus, the Cry3A protein is not expected to be a food allergen.

Allergenicity Endpoints of Cry3A Crops

Study Type	Result	MRID #
In-Vitro Digestibility	The 68 kD and 55kD *Bt* Cry3A proteins degraded within 30 seconds in simulated gastric fluid when analyzed by western blot and were not active against Colorado potato beetle after degradation. The 68kD *Bt* Cry3A protein degraded to 55kD within 2 hours of incubation in simulated intestinal fluid. The 55 kD form remained unchanged after 14 hours of incubation and retained its bioactivity and western blot results. These results indicate that, following ingestion by humans, the *Bt* Cry 3A proteins very likely will be degraded like other proteins to amino acids and peptides like other dietary proteins. CLASSIFICATION: ACCEPTABLE	429332-18

4) Effects on the Endocrine System

The pesticidal active ingredients are proteins, derived from sources that are not known to exert an influence on the endocrine system. Therefore, the Agency is not requiring information on the endocrine effects of these plant-pesticides at this time.

5) Dose Response Assessment

No toxicological endpoints are identified so no dose response assessment is required.

6) Dietary Risk Characterization

a) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry3A protein include information on the characterization of the expressed Cry3A delta-endotoxin in cotton, the acute oral toxicity, and *in vitro* digestibility of the delta-endotoxin. The results of these studies were determined to be adequate to evaluate human risk and the validity, completeness, and reliability of the available data from the studies were considered.

Data was submitted to show that the Cry3A test material derived from microbial cultures were biochemically and functionally similar to the proteins produced by the plant-incorporated protectant ingredients. Production of microbially-produced protein was chosen in order to obtain sufficient material for testing.

The acute oral toxicity data submitted supports the determination that the Cry3A protein is non-toxic to humans. Therefore, since no effects were shown to be caused by the plant-incorporated protectants, even at relatively high dose levels (5,000 mg/kg), the Cry3A delta-endotoxin protein is not considered toxic. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived. [See 40 CFR Sec. 158.740(b).] Further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are only triggered by significant acute effects in studies such as the mouse oral toxicity study. Because the acute testing showed no toxicity, higher tier studies are not required.

Because Cry3A is a protein and the major exposure is dietary, food allergenic potential was considered. Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid, and proteases, are glycosylated and present at high concentrations in the food. Data has been submitted which demonstrates that the Cry1Ac delta-endotoxin is degraded within 30 seconds (MRID#429332-18) by gastric fluid *in vitro* and is non-glycosylated. Despite decades of widespread use of *Bacillus thuringiensis* as a pesticide (it has been registered since 1961), there have been no confirmed reports of immediate or delayed allergic reactions to the delta-endotoxin itself despite significant oral, dermal and inhalation exposure to the microbial product. Several reports under FIFRA § 6(a)2 have been made for various *Bacillus thuringiensis* products claiming allergic reactions. However, the Agency determined these reactions were not due to *Bacillus thuringiensis* itself or any of the Cry toxins. Thus, the Cry3A protein is not expected to be a food allergen.

Although Cry3A expression level data was required for an environmental fate and effects assessment, residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity.

Both (1) available information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children) and (2) safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food additives, are generally recognized as appropriate for the use of animal experimentation data were not evaluated because the lack of mammalian toxicity at high levels of exposure demonstrate the safety of the product at levels above possible maximum exposure levels.

The genetic material necessary for the production of the plant-incorporated protectants active ingredients are the nucleic acids (DNA) which comprise (1) genetic material encoding these proteins and (2) their regulatory regions. "Regulatory regions" are the genetic material (termed promoters, terminators and enhancers) that control the expression of the DNA encoding proteins. DNA is common to all forms of plant and animal life and the Agency knows of no instance where these nucleic acids have been associated with toxic effects related to their consumption as a component of food. These ubiquitous nucleic acids as they appear in the subject active ingredient have been adequately characterized by the applicant. Therefore, no mammalian toxicity is anticipated from dietary exposure to the genetic material necessary for the production of the subject active plant pesticidal ingredients.

b) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408 provides that EPA shall apply an additional tenfold margin of exposure (safety) for infants and children in the case of threshold effects to account for pre- and post-natal toxicity and the completeness of the database unless EPA determines that a different margin of exposure (safety) will be safe for infants and children.

In this instance, based on all the available information, the Agency concludes that infants and children will consume minimal residues of this plant-pesticide and that there is a finding of no toxicity.

Thus, there are no threshold effects of concern and, as a result the provision requiring an additional margin of safety does not apply. Further, the provisions of consumption patterns, special susceptibility, and cumulative effects do not apply.

c) Aggregate Exposure (Not Including Occupational Exposure) Risk Conclusions

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the plant-incorporated protectants chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Oral exposure, at very low levels, may occur from ingestion of processed products and drinking water. However, a lack of mammalian toxicity and the digestibility of the plant-incorporated protectants has been demonstrated. The use sites for Cry3A delta endotoxin are all agricultural for control of lepidopteran insects. Therefore, exposure via residential or lawn use to infants and children is not expected. Even if negligible exposure should occur, the Agency concludes that such exposure would present no risk due to the lack of toxicity.

d) Cumulative Effects Risk Conclusions

The Agency has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity to these plant-incorporated protectants, there are no cumulative effects.

e) Tolerance Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry3A protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. Therefore, EPA considers that the Cry3A tolerance exemption has been reassessed and meets the 408(c)(2) standard.

The Agency has arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed for the plant-incorporated protectants. As a result, EPA established an exemption from tolerance requirements pursuant to FFDCA section 408(j)(3) for *Bacillus*

thuringiensis Cry3A delta-endotoxin and the genetic material necessary for its production in all plants.

Bacillus thuringiensis subspecies *tenebrionis* Cry3A delta-endotoxin and the genetic material necessary for its production in all plants are exempt from the requirement of a tolerance when used as plant-incorporated protectants in all plant raw agricultural commodities. ``Genetic material necessary for its production" means the genetic material which comprise (1) genetic material encoding the Cry3A delta-endotoxin and (2) its regulatory regions. ``Regulatory regions" are the genetic material that control the expression of the genetic material encoding the Cry3A delta-endotoxin, such as promoters, terminators, and enhancers.

f) Occupational Exposure and Risk Characterization

Exposure via the skin or inhalation is not likely since the plant-incorporated protectants are contained within plant cells which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Worker exposure to the Cry protein via seed dust is also expected to be negligible because of the low amount of protein expressed in transformed plants. If such exposure should occur, the Agency concludes that such exposure would not be expected to present any risk due to the lack of toxicity. However, if any unreasonable adverse effects caused by exposure to Cry3A are identified, these effects must be reported to the Agency as described in Sec. 6(a)(2) of FIFRA.

BPPD RECOMMENDATION:
There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry3A protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because no toxicity to mammals has been observed for the plant-incorporated protectants and anticipated exposures are negligible.

REFERENCES

Bernstein, I.L., J.A. Bernstein, M. Miller, S. Tierzieva, D. I. Bernstein, Z. Lummus, M.K. Selgrade, D.L. Doerfler & Verner L. Seligy (1999) Immune Responses in Farm Workers after Exposure to *Bacillus thuringiensis* Pesticides, Environmental Health Perspectives Volume 107:575-582.

Sjoblad, R. D., J. T. McClintock, and R. Engler (1992) "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15:3-9.

Vázquez-Padrón, R.I., L. Moreno-Fierros, L. Neri-Bazán, A.F. Martínez-Gil, G.A. de la Riva & R. López-Revilla (2000) Characterization of the mucosal and systemic response induced by Cry1Ac protein from *Bacillus thuringiensis* HD 73 in mice, Brazilian Journal of Medical and Biological research 33:147-155.

Vázquez-Padrón, R.I., L. Moreno-Fierros, L. Neri-Bazán, G.A. de la Riva & R. López-Revilla (1999) Intragastric and intraperitoneal administration of Cry1Ac protoxin from *Bacillus thuringiensis* induces systemic and mucosal antibody responses in mice, Life Sciences 64: 1897-1912.

Vázquez-Padrón, R.I., L. Moreno-Fierros, L. Neri-Bazán, G.A. de la Riva & R. López-Revilla (1999) *Bacillus thuringiensis* Cry1Ac protoxin is a potent systemic and mucosal adjuvant, Scandinavian Journal of Immunology 49:578-584.

43130801 Williams, D. (1994) Product Characterization: *Bacillus thuringiensis* var. *kurstaki* (*Btk*) Protein in Corn: Lab Project Number: NKJNV2. Unpublished study prepared by Northrup King Co. 13 p.

43352602 Williams, D. (1994) Product Characterization: *Bacillus thuringiensis* var. *kurstaki* Protein in Corn: Supplemental Information: Lab Project Number: NK5PDCH. Unpublished study prepared by Northrup King Co. 6 p.

437548-01 Williams, D. (1995) Product Characterization: *Bacillus thuringiensis* var. *kurstaki* Protein in Corn: Registration Application. Unpublished study prepared by Northrup King Co. 23p.

43397202 Meeusen, R.; Mettler I. (1994) Equivalence of Plant and Microbially Produced *Bacillus thuringiensis kurstaki* HD-1 Protein: Lab Project Number: 1/NK5EQ. Unpublished study prepared by Nothrup King Co.; University of Wisconsin; and Kendrick Labs. 43 p.

43533201 Keck, P.; Fromm, M.; Sanders, P.; et al. (1995) Molecular Characterization of Insect Protected Corn Line MON 80100: Lab Project Number: MSL 13924. Unpublished study prepared by Monsanto Co. 100 p.

43533203 Lee, T.; Bailey, M.; Sanders, P. (1995) Compositional Comparison of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 Protein Produced in European Corn Borer Resistant Corn and the Commercial Microbial Product, DIPEL: Lab Project Number: 94-01-39-12: MSL 13876. Unpublished study prepared by Monsanto Co. 35 p.

43665501 Levine, E.; Groth, M.; Kania, J.; et al. (1995) Molecular Characterization of Insect Protected Corn Line MON 810: Lab Project Number: MSL 14204. Unpublished study prepared by Monsanto Co. 61 p.

43533204 Lee, T.; Bailey, M.; Sims, S. (1995) Assessment of the Equivalence of the *Bacillus thuringiensis* subsp. *kurstaki* HD-1 Protein Produced in *Escherichia coli* and European Corn Borer Resistant Corn: Lab Project Number: 94-01-39-09: MSL 13879. Unpublished study prepared by Monsanto Co. 94 p.

43145201 Keck, P. (1994) Determination of Copy Number and Insert Integrity for Cotton Line 531: Lab Project Number: 92/01/36/12: 92/427/713. Unpublished study prepared by Monsanto Agricultural Group. 66 p.

43145202 Sammons, D. (1994) Assessment of Equivalence Between *E. coli*-Produced and Cotton-Produced *B.t.k.* HD-73 Protein and Characterization of the Cotton-Produced *B.t.k.* HD-73 Protein: Lab Project Number: 92/01/36/14: 13170. Unpublished study prepared by Monsanto Agricultural Group. 45 p.

43145203 Sammons, D. (1994) Characterization of Purified *B.t.k.* HD-73 Protein Produced in *Escherichia coli*: Lab Project Number: 92/01/36/18: 92/427/721: 13171. Unpublished study prepared by Monsanto Agricultural Group. 84 p.

43145204 Sims, S. (1994) Sensitivity of Insect Species to the Purified CryIA(c) Insecticidal Protein from *Bacillus thuringiensis* var. *kurstaki* (*B.t.k.* HD-73): Lab Project Number: 92/01/36/17:92/427/720: 13273. Unpublished study prepared by Monsanto Agricultural Group. 39 p.

42932201 Keck, P. (1993) Molecular Characterization of CPB Resistant Russet Burbank Potatoes: *Bacillus thuringiensis* var *tenebrionis*: Lab Project Number: 92-01-37-14: 93-081E:92-448-715. Unpublished study prepared by Monsanto Co. 325 p.

42932202 Rogan, G.; Anderson, J.; McCreary, J.; et al. (1993) Determination of the Expression Levels of *B.t.t* and NPTII Proteins in Potato Tissues Derived from Field Grown plants: Lab Project Number: 92-01-37-02: 93-081E: 12735. Unpublished study prepared by Monsanto Co. 349 p.

42932205 Bartnicki, D.; Leimgruber, R.; Lavrik, P.; et al. (1993) Characterization of the Major Tryptic Fragment from Colorado Potato Beetle Active Protein from *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t*): Lab Project Number: 92-01-37-15:93-081E: 12994. Unpublished study prepared by Monsanto Co. 53p.

42932204 Lavrik, P. (1993) Characterization of Colorado Potato Beetle Active *Bacillus thuringiensis* subsp. *tenebrionis* Protein Produced in *Escherichia coli*: Lab Project Number: 92-01-37-10:93-081E: 92-448-711. Unpublished study prepared by Monsanto Co. 82 p.

42932206 Rogan, G.; Lavrik, P. (1993) Compositional Comparison of Colorado Potato Beetle (CPB) Active *Bacillus thuringiensis* subsp. *tenebrionis* (*B.t.t.*) Protein Produced in CPB Resistant Potato plants and Commercial Microbial Products: Lab Project Number: 92--01-37-17: 93-081E: 12988. Unpublished study prepared by Monsanto Co. 46 p.

43468001 Naylor, M. (1992) Acute Oral Toxicity Study of *Btk* HD-1 Tryptic Core Protein in Albino Mice: Lab Project Numbers: 92069: 11985:ML92069. Unpublished study prepared by Monsanto Co. 264 p.

43439201 Ream, J. (1994) Assessment of the In vitro Digestive Fate of *Bacillus thuringiensis* subsp. *kurstaki* HD-1 Protein: Lab Project Number: 93-01-39-04. Unpublished study prepared by Monsanto Co. 44 p.

43145213 Naylor, M. (1993) Acute Oral Toxicity of *Bacillus thuringiensis* var. *kurstaki* (Cry IA(c)) HD-73 Protein in Albino Mice: Lab Project Number: 92197: ML/92/493. Unpublished study prepared by Monsanto Agriculture Group. 187 p.

43145214 Ream, J. (1994) Assessment of the In vitro Digestive Fate of *Bacillus thuringiensis* var. *kurstak*i HD-73 Protein: Lab Project Number: 92/01/36/22: 92/427/728. Unpublished study prepared by Monsanto Agriculture Group. 43 p.

42932217 Naylor, M. (1993) Acute Oral Toxicity Study of *B.t.t.* Protein in Albino Mice: Lab Project Number: 92170: ML-92-407. Unpublished study prepared by Monsanto Co. 191 p.