A

OPP-2002-0350

3

REPORT TITLE

Petition For A Temporary Exemption
From The Requirement Of A Tolerance
For
BACILLUS THURINGIENSIS (B.t.) Cry34/35Ab1 Insecticidal Crystal Protein
As Expressed In All Raw Agricultural Commodities

DATA REQUIREMENT

None/Not Applicable

DATE

March 23, 2001

REVISED
January 9, 2002

REVISED
October 22, 2002

SUBMITTED BY

Mycogen c/o Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis IN 46268

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

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

Company: Mycogen c/o Dow AgroSciences

Company Agent: Penny L. Hunst, Ph.D.

Title: Regulatory Manager

Signature: Penny L. Hunst

Date: 10/22/2002

The data summarized in this document are the property of Mycogen, and as such, are considered confidential for all purposes other than compliance with FIFRA Section 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other statute or in any other country.

CERTIFICATION OF GOOD LABORATORY PRACTICE

This document contains only summaries of data submitted by Mycogen not required to comply with EPA FIFRA Good Laboratory Practice standards as outlined in 40 CFR Part 160.

Penny L. Hunst

Penny L. Hunst, Ph.D.
Mycogen Seeds c/o Dow AgroSciences
Sponsor/Submitter

10/22/2002
Date

TABLE OF CONTENTS

SECTION A

THE AMOUNT, FREQUENCY, AND TIME OF APPLICATION OF THE PLANT PESTICIDE

Bacillus thuringiensis (B.t.) Cry34/35Ab1 insecticidal crystal protein (ICP) is contained in corn seed that has been genetically engineered to produce resistance to a key corn pest, Western corn rootworm. Cry34/35Ab1 transgenic plants are derived from transformation events that contain the insecticidal genes via plasmid insert. Cry34/35Ab1ICP as expressed in field corn provides highly efficacious control of key pests such as the Western corn rootworm.

Corn seeds contain 1.08 – 32.5 ug/g (Construct 17658) and 0.036 – 46.9 ug/g (Construct 17662) Cry34Ab1 protein/gram and 10.4-16.9 ug/g (Construct 17658) and 0.211-1.22 ug/g (Construct 17662) of Cry35Ab1 protein/gram of corn kernel tissue. Corn seeds containing the binary ICP are planted and grown according to conventional agricultural practices in regions where corn is typically grown.

The proposed label is included in this section of the petition document.

SECTION B

PRODUCT IDENTITY

The *B.t.* Cry34/35Ab1 binary ICP has been adequately characterized. In studies conducted to analyze the differences between the microbially-derived and plant-derived *B.t.* Cry34/35Ab1 ICP, no significant differences were detected. The specific data summarizing these studies were provided to EPA as part of Mycogen's EUP submissions (68467-EUP-T; 68467-EUP-I) on October 17, 2002:

- Product Characterization Data for *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 Proteins Expressed in Transgenic Maize Plants (PHP17662) (Study ID #PHI-2002-046)

- Product Characterization Data for *Bacillus thuringiensis* Cry34Ab1 and Cry35Ab1 Proteins Expressed in Transgenic Maize Plants (PHP17658) (Study ID # PHI-2002-047)

- Characterization of DNA Inserted into Transgenic Corn Events E4497.42.1.18, E4497.42.1.34, E4497.45.2.16, E4497.59.1.10, E4497.67.1.47, E4497.66.1.27, E4497.71.1.29, and E4497.71.1.33 (Study ID #GH-C 5550)

- Characterization of Cry34Ab1 and Cry35Ab1 from Recombinant *Pseudomonas fluorescens* and Transgenic Maize (Study ID #GH-C 5545)

SECTION C

FULL REPORTS OF TESTS AND INVESTIGATIONS MADE WITH RESPECT TO THE SAFETY OF THE PLANT PESTICIDE

Toxicology studies conducted to determine the toxicity of *Bacillus thuringiensis* PS149B1 ICPs demonstrated that the proteins have very low toxicity. The test material demonstrated low acute toxicity and a high degree of digestibility in an *in vitro* study. Amino acid sequences of the PS149B1 ICPs exhibited no significant homology to the amino acid sequences of proteins that are known food allergens or toxins. Specifically, the following studies were conducted:

- PS149B1 14 KDA Protein: Acute Oral Toxicity Study in CD-1 Mice (MRID #45242207)
- PS149B1 44 KDA Protein: Acute Oral Toxicity Study in CD-1 Mice (MRID #45242208)
- PS149B1 14 KDA and 44 KDA Proteins: Acute Oral Toxicity Study in CD-1 Mice (MRID #45242209)
- *In Vitro* Digestibility of PS149B1 Proteins (MRID #45242212)
- *In Vitro* Simulated Gastric Fluid Digestibility Study of Microbially Derived Cry34Ab1 Protein (MRID #45584502)
- SDS-PAGE Senstivity Analysis for Cry35Ab1 in Support of the Simulated Gastric Fluid Digestion Study MRID #45242212 (Study ID GH-C 5513; submitted on October 17, 2002)
- Comparison of Amino Acid Sequence of the *Bacillus thuringiensis* Strain PS149B1 13.6 kDa and 43.8 kDa Insecticidal Crystal Proteins to Known Protein Allergens (MRID #45242205)
- Thermolability of PS149B1 Binary Delta-Endotoxin (MRID #453584-01)
- Heat Lability of Individual Proteins of PS149B1 Binary ICP (MRID #45584501)

Results from these studies are summarized below.

Acute Oral Toxicity Study in Mice (MRID #45242207, #45242208 and #45242209)

B.t. Cry34/35Ab1 binary insecticidal crystal proteins were evaluated for acute toxicity potential in mice, alone and in combination. In the experiments evaluating the toxicity of the Cry34Ab1 and Cry35Ab1 ICP individually, five male mice received 5000 mg of test material/kg body weight The test material was administered as a 20% mixture in a 0.5% aqueous methylcellulose vehicle by single dose gavage (25ml/kg). Parameters evaluated during the two-week observation period included body weights and detailed clinical observations. All animals were examined for gross pathologic changes.

No mortality was noted during the course of the study. In the Cry34Ab1 test, three mice lost weight between days 1 and 2 but gained weight over the course of the study. In the Cry35Ab1 test, two mice lost weight between days 1 and 2 but gained weight over the course of the study. No clinical signs were noted during the study, and no findings were noted at necropsy. The acute oral LD_{50} of Cry34Ab1 is greater than 5000 mg/kg, and at 54% purity, the acute LD_{50} for pure protein is greater than 2700 mg/kg. The acute oral LD_{50} of Cry35Ab1 is greater than 5000 mg/kg, and at 37% purity, the acute LD_{50} for pure protein is greater than 1850 mg/kg.

7 of 18

In the experiment evaluating the toxicity of Cry34Ab1/Cry35Ab1 ICP together, five male mice and five female mice received 5000 mg of test material/kg body weight. The test material contained 482 mg/kg of Cry34Ab1 (54% pure) and 1520 mg/kg Cry35Ab1 (37% pure) (a 1:3 ratio to provide an equimolar mixture of the pure proteins). The test material was administered as a 20% mixture in a 0.5% aqueous methylcellulose vehicle by single dose gavage (25ml/kg). Parameters evaluated during the two-week observation period included body weights and detailed clinical observations. All animals were examined for gross pathologic changes.

No mortality was noted during the course of the study. One female mouse had protruding or enlarged eyes on test days 6 and 7, however, this was not considered to be treatment related. No other clinical signs were observed. Two mice lost weight between days 1 and 2, but gained weight over the remainder of the study, and the remaining mice gained body weight throughout the study. There were no treatment-related gross pathologic observations. The acute oral LD_{50} of Cry34Ab1 and Cry35Ab1 proteins in male and female mice is greater than 5000 mg/kg, and greater than 2000 mg/kg of an equimolar (1:3) mixture of pure proteins.

In Vitro Digestibility (MRID No. 45242212, 45584502 and Study ID GH-C 5513)

Previous gastric digestion studies for *Bt* products focused on the time required for the protein of interest to become undetectable via a specific analytical method. This criterion is primarily a function of the sensitivity of the analytical technique and not the digestion rate of the protein and is not useful for comparing the digestion rates among ICPs derived from *Bacillus thuringiensis* that are currently registered and/or in the registration process, because the sensitivities of the analytical techniques used to track the proteins were not equivalent. For example, some previously registered plant-incorporated protectants used analytical techniques for heat lability evaluation that lost sensitivity when approximately 10% of the protein remained (www.monsantoinfo.dk/nyhedsbrev/YieldGardCornProductSafetySummary.pdf). Thus, the assay lost sensitivity at the DT_{90} (time until 90% digestion). The analytical method used for Cry34Ab1 is more sensitive than this (MRID #45242212), allowing less than 0.38% protein residue to be detected. Using the disappearance of the protein as the criterion for digestibility will make the same protein appear to digest more than 2.4-times slower using this more sensitive technique, when in fact the digestibility is identical.

The classical technique for measuring enzymatic digestibility is to quantify the residue (Brussock and Currier 1990) at various time points and to model the decay (degradation). Degradation rates for enzymatic reaction are known to follow the Michaelis-Menten Rate Law (Rawn 1989). At the concentrations of proteins historically used in these studies, the reaction is first order. Using this simple model, it is possible to determine point estimates of decay such as half lives and DT_{90} values. The use of DT_{90} values is especially useful since at this point, proteins have been substantially digested. This is also the point where the analytical techniques used for the digestibility evaluation of some registered plant-incorporated protectants lose their ability to detect the protein of interest (see above), and thus the DT_{90} provides a reference point for comparison with these proteins.

The digestibility of the Cry34/35Ab1 ICP was determined via exposure of equimolar solutions of the Cry34Ab1 and Cry35Ab1 proteins to simulated gastric fluid (SGF) containing approximately 0.3% (w/v) pepsin at pH 1.2 for both studies. The digestions were performed at time intervals of 1,5,7,10,15,20,30 and 60 minutes (MRID #45242212) at 37°C and 1, 2, 3, 5, 7.5, 10, 15, and 20 at 37°C with shaking of the reaction vials (MRID #45584502). In MRID #45584502, the wetted gels, after destaining, were placed on the glass platter of the densitometer and each gel was scanned using 100 micron and 12 bit settings. This provided the required detail when the protein bands were viewed under 4x magnification. Quantitation was achieved by using rectangular volume integration (optical density x area) of the individual Cry34Ab1 bands. The densitometric quantification is expressed as peak volume per band. After correcting the volumes for background, the peak volume is directly proportional to the quantity of protein that is present (Brussock and Currier 1990).

The digestion rate of Cry34Ab1 in simulated gastric fluid was determined. The DT_{50} and DT_{90} for this protein were found to be 1.9 and 6.2 minutes, respectively, indicating that the Cry34Ab1 protein is substantially digested by simulated gastric fluid in less than 7 minutes. This is consistent with the digestion profile reported for other registered Bt products (≤7 minutes). The digestion rate of the Cry35Ab1 (MRID #45242212) was also rapid, with the protein band being undetectable on the western blot between the 1 and 5 minute SGF time points translating into >97% of the protein being degraded in less than 5 minutes (Study ID #GH-C 5513).

Comparison of Amino Acid Sequence of the *Bacillus thuringiensis* Strain PS149B1 13.6 kDa and 43.8 kDa Insecticidal Crystal Proteins to Known Protein Allergens (MRID #45242205)

To determine the potential risk of allergic response to *B.t.* Cry34/35Ab1 ICP from dietary exposure to humans, relevant databases were searched determine the similarity of the test material to known allergens was conducted. A sequence evaluation scheme based on that formulated by Gendel (1998) was used. An immunologically signficant sequence identity requires a match of at least eight contiguous identical amino acids. No immunologically significant sequence identity was detected for either the Cry34Ab1 or Cry35Ab1 proteins.

Thermolability of PS149B1 Binary Delta-Endotoxin (MRID #453584-01 and 45584501)

To measure the lability of the Cry34/35Ab1ICP to heat, an aqueous formulation of the ICP (1:1 active ingredient (AI) mass ratio) was divided into four aliquots. Each aliquot was placed at 60°, 75°, 90° or 4°C (positive control) for 30 minutes. The treated aliquots were applied to the surface of solid, agar-based insect diet in 128-well bioassay trays. The formulation was targeted to produce 10 ug AI/cm^2 of diet. Sixteen wells (1 insect/well) were used for each formulation. The negative control (buffer of the formulation) was replicated three times, 16 insects per replicate. Mortality results were not useful for separating treatment groups since little mortality (6%) occurred with the positive control (4°C). Growth inhibition data indicated the following: -3% at 60°C, -3% at 75°C and 1% at 90°C compared to 70% growth inhibition with the positive control (4°C). Therefore, the Cry34/35Ab1ICP is deactivated following exposure to 60°, 75° or 90°C. To further refine this to elucidate the more exact nature of the lability of the individual proteins, a second study (MRID #45584501) was conducted. After incubating aqueous formulations of the ICP at various temperatures for 30 minutes, neonate southern corn rootworm (SCR), *Diabrotica undecimpunctata howardi*, were exposed to artificial dietary substrates that had been treated with the ICP. In addition, the heated ICP was spiked with non-heated samples of the individual proteins to allow evaluation of the thermolability of the complimentary protein. As in the previous heat lability study, the activity of the ICP (mixture) was significantly reduced after 30 minutes of exposure to 60, 75 and 90° C compared to the ICP held at 4° C. There was little difference between the Cry34Ab1 spike treatments (of the heated ICP) and the treatment including the Cry34Ab1 alone. This indicates that the Cry35Ab1 (44-kDa) component of the ICP is deactivated after 30 minutes of exposure to 60, 75 and 90° C.

There was a temperature dependent effect seen for the Cry35Ab1 spike treatments (of the heated ICP indicating that the Cry34Ab1 component of the binary ICP was only partially deactivated after 30 minutes exposure to 60 and 75° C. However, activity was largely destroyed after 30 minutes exposure to 90° C. The results of this latter treatment are consistent with those observed for the deactivated ICP.

SECTION D

FULL REPORTS OF TESTS AND INVESTIGATIONS MADE WITH RESPECT TO THE AMOUNT OF RESIDUE REMAINING, INCLUDING A DESCRIPTION OF THE ANALYTICAL METHOD USED

Because a temporary exemption from the requirement of a tolerance is requested for *B.t.* Cry34/35Ab1 binary ICP as expressed in all raw agricultural commodities, this section of the petition is not applicable.

SECTION E

PRACTICABLE METHODS FOR REMOVING RESIDUE THAT EXCEEDS ANY PROPOSED TOLERANCE

Because a temporary exemption from the requirement of a tolerance is requested for *B.t.* Cry34/35Ab1 binary ICP as expressed in all raw agricultural commodities, this section of the petition is not applicable.

SECTION F

PROPOSED TOLERANCES FOR THE PLANT PESTICIDE

Because a temporary exemption from the requirement of a tolerance is requested for *B.t.* Cry34/35Ab1 binary ICP as expressed in all raw agricultural commodities, this section of the petition is not applicable.

12 of 18

SECTION G

REASONABLE GROUNDS IN SUPPORT OF THE PETITION

Reasonable grounds in support of this request for a temporary exemption from the requirement of a tolerance for *B.t.* Cry34/35Ab1 binary ICP as expressed in all raw agricultural commodities are presented in Section C of this document and the supporting studies conducted on the plant pesticide. A data matrix summarizing all studies conducted with *B.t.* Cry34/35Ab1 binary ICP is contained in this section of the document.

Aggregate Exposure

Based on the proposed use of *B.t.* Cry34/35Ab1 binary ICP as expressed in all raw agricultural commodities, and the demonstrated low toxicity and low potential for allergenicity of the ICPs, aggregate exposure is unlikely. In addition, the ICPs are not likely to be present in drinking water due to their containment within the plant and their demonstrated rapid degradation in soil. Significant dietary exposure to the protein is unlikely to occur. Dietary exposures at very low levels, via ingestion of processed commodities, although they may occur, are unlikely to be problematic because of the low toxicity and the high degree of digestibility of the ICPs.

Cumulative Exposure

Common modes of toxicity are not relevant to consideration of the cumulative exposure to *B.t.* Cry34/35Ab1 binary ICP. The product has demonstrated low toxicity and these effects do not appear to be cumulative with any other known compounds.

Safety Determination

U.S. Population

The intended use of the product, the very low toxicity of the product, the lack of potential for allergenicity, and the high degree of digestibility of the ICPs are all factors in Mycogen's assertion that no significant risk is posed by exposure of the U.S. population to *B.t.* Cry34/35Ab1 ICP.

Infants and Children

Non-dietary exposure to infants and children is not anticipated, due to the proposed use pattern of the product. Due to the very low toxicity of the product, the high degree of digestibility of the ICP, lack of homology to known protein allergens, and the low potential for dietary exposure, the Cry34/35Ab1 ICP is not anticipated to pose any harm to infants and children.

Effects on the Immune and Endocrine System

Given the high degree of digestibility of *B.t.*Cry34/35Ab1 binary ICP, no chronic effects are expected. *B.t.*Cry34/35Ab1 binary ICP or metabolites of the binary ICP are not known to, or are expected to have any effect on the immune or endocrine systems. Proteins in general are not carcinogenic, therefore, no carcinogenic risk is associated with the *B.t.* Cry34/35Ab1 ICP.

Existing Tolerances or Exemptions from Tolerance

There are no existing tolerances or exemptions from tolerance for *B.t.* Cry34/35Ab1 binary ICP.

Form Approved OMB No. 2070-0060

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
401 M Street, S.W.
WASHINGTON, D.C. 20460

DATA MATRIX

EPA Reg No./File Symbol 68467-EUP-I | Page 1 of 2

Date October 22, 2002

Applicant's/Registrant's Name & Address: Mycogen Seeds c/o Dow AgroSciences LLC
9330 Zionsville Road, Indianapolis, IN 46268

Product: Mycogen Brand B.t. Cry34/35Ab1 Construct 17662 Corn

Ingredient: Bacillus thuringiensis Cry34/35Ab1 insecticidal crystal protein and the genetic material necessary for its production in maize

Guideline Reference Number	Guideline Study Name	MRID Number	Submitter	Status	Note
[redacted]			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	

Signature: Penny L. Hurst

Name and Title: Penny L. Hurst, Regulatory Manager

Date: October 22, 2002

Public File Copy

EPA Form 8570-35 (9-97) Electronic and Paper versions available. Submit only Paper version.

Form Approved OMB No. 2070-0060

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
401 M Street, S.W.
WASHINGTON, D.C. 20460

DATA MATRIX

Date October 22, 2002	EPA Reg No./File Symbol 68467-EUP-I	Page 2 of 2
Applicant's/Registrant's Name & Address: Mycogen Seeds c/o Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268	Product: Mycogen Brand B.t. Cry34/35Ab1 Construct 17662 Corn	

Ingredient: Bacillus thuringiensis Cry34/35Ab1 insecticidal crystal protein and the genetic material necessary for its production in maize

Guideline Reference Number	Guideline Study Name	MRID Number	Submitter	Status	Note
[redacted]			68467	own	
			68467	own	
			68467	own	
			68467	own	
			68467	own	
			68467	own	
			68467	own	
			68467	own	
			68467	own	
			68467	own	
			68467	own	
			68467	own	

Signature: Penny L. Hurst

Name and Title: Penny L. Hurst, Regulatory Manager

Date: October 22, 2002

EPA Form 8570-35 (8-97) Electronic and Paper versions available. Submit only Paper version.

Public File Copy

Form Approved OMB No. 2070-0060

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
401 M Street, S.W.
WASHINGTON, D.C. 20460

DATA MATRIX

Date October 22, 2002	EPA Reg No./File Symbol 68467-EUP-T — Page 1 of 2
Applicant's/Registrant's Name & Address: Mycogen Seeds c/o Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268	Product: Mycogen Brand B.t. Cry34/35Ab1 Construct 17658 Corn

Ingredient: Bacillus thuringiensis Cry34/35Ab1 insecticidal crystal protein and the genetic material necessary for its production in maize

Guideline Reference Number	Guideline Study Name	MRID Number	Submitter	Status	Note
[redacted]			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	

Signature: Penny L. Hurst

Name and Title: Penny L. Hunst, Regulatory Manager

Date: October 22, 2002

Public File Copy

Form Approved OMB No. 2070-0060

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
401 M Street, S.W.
WASHINGTON, D.C. 20460

DATA MATRIX

Date: October 22, 2002

EPA Reg No./File Symbol: 68467-EUP-T

Applicant's/Registrant's Name & Address: Mycogen Seeds c/o Dow AgroSciences LLC
9330 Zionsville Road, Indianapolis, IN 46268

Product: Mycogen Brand B.t. Cry34/35Ab1 Construct 17658 Corn

Ingredient: Bacillus thuringiensis Cry34/35Ab1 insecticidal crystal protein and the genetic material necessary for its production in maize

Guideline Reference Number	Guideline Study Name	MRID Number	Submitter	Status	Note
[redacted]			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	
			68467	OWN	

Signature: Penny K. Hurst

Name and Title: Penny L. Hunst, Regulatory Manager

Date: October 22, 2002