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

MAR 8 1994

OFFICE OF
PREVENTION, PESTICIDES
AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Review of the Product Analysis Data Submitted by Monsanto Corporation to Support the Registration for Transgenic Potato Plants Expressing the δ-endotoxin from Bacillus thuringiensis var. tenebrionis

TO: Willie Nelson/ Phil Hutton (PM-18)
Insecticide-Rodenticide Branch
Registration Division (7505C)

FROM: John L. Kough, Ph.D., Biologist
Biological Pesticides Section
Science Analysis Branch
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THROUGH: William Burnam, Chief
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DATA REVIEW RECORD

Active Ingredient:	CryIIIA δ-endotoxin from Bacillus thuringiensis var. tenebrionis expressed in potato (Solanum tuberosum)
ID No:	000524-UTU
Submission No:	S450750
DP Barcode:	D195921
MRID No:	429322-01 (Vol. 2- Molecular Characterization of CPB Resistant Russet Burbank Potatoes) 429322-03 (Vol. 4- Equivalence of Microbially-Produced and Plant Produced B.t.t. Protein) 429322-04 (Vol. 5- Characterization of Colorado Potato Beetle Active Bacillus thuringiensis subsp. tenebrionis Protein Produced in Escherichia coli) 429322-05 (Vol. 6- Characterization of the Major Tryptic Fragment from Colorado Potato Beetle Active Protein from Bacillus thuringiensis subsp. tenebrionis (B.t.t.) 429322-06 (Vol. 7- Compositional Comparison of

(1)

Colorado Potato Beetle (CPB) Active Bacillus thuringiensis subsp. tenebrionis Proteins Produced in CPB Resistant Potato Plants and Commercial Microbial Products)
429322-07 (Vol. 8- Sensitivity of Selected Insect Species to the Colorado Potato Beetle Active Protein From Bacillus thuringiensis subsp. tenebrionis)

ACTION REQUESTED
To review the submitted data to support the registration and request for an exemption from the requirement of a tolerance for the CryIIIA δ-endotoxin from B.t.t. expressed in potato.

BACKGROUND
Monsanto has previously presented data to support an EUP for their transgenic potatoes expressing B.t.t. δ-endotoxin. Much of the toxicology data was not developed for the EUP because the potatoes were contained to the test sites and the crop was to be destroyed or saved and used for further product development. In the EUP, Monsanto described 14 different DNA constructs used to transform potatoes for pest resistance along with many other constructs for improved food quality. These pesticidal traits ranged from variants of the B.t.t. toxin to viral coat protein or viral replicase, all with introduced herbicide resistance or NPTII as a marker gene (detailed description of the DNA constructs is found in memoranda from J. Kough to L. Dye, June 10 & 16 and November 23, 1992).

For full registration of transgenic potato with a pesticidal trait, Monsanto has chosen a construct (PV-STBT02) that codes for the native CryIIIa δ-endotoxin from B.t.t. and carries NPTII as a marker. This review covers the majority of the information submitted for product analysis. Other pertinent reviews for mammalian toxicology include data on expression levels reviewed by Dr. L. LaSota and toxicology studies reviewed by C. Schaffer.

CONCLUSIONS
The submitted information along with an acceptable review of the plant pesticide expression data (MRID 429322-02) is an adequate description of the plant pesticidal substance as expressed in potato. The microbially expressed and purified compound is sufficiently similar to that expressed in the plant to be used for mammalian toxicological purposes.

SUMMARY OF REVIEWS

MRID 429322-01 Molecular Characterization of CPB Resistant Russet Burbank Potatoes Equivalence of Microbially-Produced and Plant Produced B.t.t. Protein. -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 entire introduced plasmid PV-STBT02 as the probe. These southern blots provide information about the number of copies of introduced DNA, the lack of significant amounts of DNA introduced outside the border regions and the integrity of the introduced DNA near the endonuclease cut sites. Acceptable.

MRID 429322-03 Equivalence of Microbially-Produced and Plant Produced B.t.t. Protein also called Colorado Potato Beetle Active Protein from Bacillus thuringiensis subsp. tenebrionis. -Microbially produced δ-endotoxin from the CryIIIA gene as expressed in Escherichia coli and in potato tubers was compared. The data consists of SDS-PAGE comigration, Western blot analysis, staining for carbohydrate residues, N-terminal amino acid sequence analysis and biological equivalence against Leptinotarsa decemlineata. Acceptable.

MRID 429322-04 Characterization of Colorado Potato Beetle Active Bacillus thuringiensis subsp. tenebrionis Protein Produced in Escherichia coli.-The method of preparing by fermentation the δ-endotoxin from B.t.t. in E. coli was presented. This protein was characterized for purity and stability after purification. Acceptable.

MRID 429322-05 Characterization of the Major Tryptic Fragment from Colorado Potato Beetle Active Protein from Bacillus thuringiensis subsp. tenebrionis.-The purity and activity of a 55kD protein released with tryptic digestion of the B.t.t. δ-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 δ-endotoxin from B.t.t. Acceptable.

MRID 429322-06 Compositional Comparison of Colorado Potato Beetle (CPB) Active Bacillus thuringiensis subsp. tenebrionis Proteins Produced in CPB Resistant Potato Plants and Commercial Microbial Products.-The Cry IIIA δ-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 δ-endotoxin. Acceptable.

MRID 429322-07 Sensitivity of Selected Insect Species to the Colorado Potato Beetle Active Protein From Bacillus thuringiensis subsp. tenebrionis-The range of non-target insect sensitivity to the Cry IIIA δ-endotoxin as expressed in E. coli was estimated. The tested species were all pest species that included Colorado Potato

Beetle (CPB) and 2 other coleopterans [boll weevil (BW) and southern corn rootworm (SCRW)], 4 lepidopterans [European corn borer (ECB), tobacco hornworm (THW), corn earworm (CEW), tobacco budworm (TBW)], a dipteran [yellowfever mosquito (YM)], an orthropteran [German cockroach (GC)] and a hemipteran [green peach aphid (GPA)]. The results show that no species other than CPB displayed significant mortality. There was a slight reduction in the amount of honeydew produced by the GPA indicating reduced feeding. Acceptable.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, SAB/HED JLK
Secondary Reviewer: Roy Sjoblad, Ph.D., Microbiologist, SAB/HED RD

STUDY TYPE:	Product Identity
MRID NO:	429322-01
TEST MATERIAL:	Cry IIIA δ-endotoxin from B.t.t. expressed in Russet Burbank potato
PROJECT NO:	92-01-37-14
TESTING FACILITY:	Monsanto Company; Agricultural Group/ New Products Division; St. Louis, MO
TITLE OF REPORT:	Molecular Characterization of CPB Resistant Russet Burbank Potatoes
AUTHOR:	Pamela J. Keck
STUDY COMPLETED:	August 31, 1993
CONCLUSION:	Southern blots using PV-STBT02 as the probe were used to show that Bt 6, 12, 17, 18 and 23 contained a single copy of the projected DNA insert. Line Bt 10 contains an estimated 2 copies of the insert apparently as a tandem repeat. Line Bt 16 contains apparently 2 copies of the insert at some distance from each other. The data also shows the lack of introduced DNA outside the border regions of the vector plasmid.
CLASSIFICATION:	Acceptable.

I. STUDY DESIGN

Test material: DNA's from leaf tissue of 7 different strains of field grown transgenic Russett Burbank potatoes (Bt 6, 10, 12, 16, 17, 18, and 23) were obtained from a 1992 EUP site in Hancock, WI. The DNA from approximately 1 gram of frozen leaf tissue was extracted by grinding with liquid nitrogen, adding 7.0 ml of extraction buffer (100 mM Tris, 50 mM EDTA, 10 mM β-mercaptoethanol, pH 8.0) then adding 10% SDS, heating to 65°C for from 10 to 75 minutes to disrupt the plant cells and precipitating the plant cell walls with 1.3 M potassium acetate. The nucleic acids were further purified from the crude extract by techniques employing isopropanol, Tris EDTA (TE) buffer and sodium acetate. The RNA is removed from the mixture by treatment with RNAse and DNA is further purified with phenol, chloroform/isoamyl alcohol, sodium acetate and ethanol washing. The final pellet was resuspended in TE buffer and quantified using a fluorometer.

Test method: Approximately 10 μg of genomic DNA from each of the seven transgenic lines was digested with three different restriction endonucleases systems to liberate DNA fragments that could characterize the introduced DNA. The restriction endonuclease systems used were the following: *Eco*RI alone, *Hin*dIII with *Not*I and *Hin*dIII with *Xho*I. *Eco*RI digestion should release a portion of the CryIIIA insert as it recognizes restriction sites within the T-DNA

region of PV-STBT02 (bp 1622 and 2556 from sequence map in Appendix 2). *Hin*DIII with *Not*I should release most of the inserted DNA since its restriction sites are near the left and right border regions of the T-DNA (bp 76 and 4715, Appendix 2). *Hin*DIII and *Xho*I will also release the majority of the inserted DNA as its restriction sites are near the border regions (bp 76 and 3237 & 4706, Appendix 2).

Each digestion mixture plus additional control DNA preparations (Molecular weight markers, plasmid vector and non-transgenic parent plant) were run in agarose gels (SOP No. PST-90-PRO-003-00), converted to single stranded DNA by denaturation, transferred to a membrane and hybridized with radioactively labelled probes (ie., Southern blotting SOP No. PST-92-PRO-025-00). The specific protocols referenced by SOP numbers were not provided although general literature references for the procedures were included. The plasmid control differed from the other mixtures by being digested in 2 separate preparations: one using *Eco*RI to release the 935 bp fragment from CryIIIA and the other with *Hin*DIII and *Nhe*I to release a 4619 bp sequence that represents the majority of the T-DNA. These two digestions were subsequently mixed and run as the DNA plasmid preparation control in order to show both the fragments in one lane.

The probes were either the entire plasmid PV-STBT02 which vectored the inserted sequences or specific regions of this plasmid generated by polymerase chain reaction (PCR). The plasmid PV-STBT02 was purified from an E. coli culture preparation. Neither the specific PCR primers employed nor the method used to generate the labelled PCR probes was described in the submission. The radioactive labelling with ^{32}P was by random primer. No specific activity of the resulting probes was given.

The autoradiograms resulting from the Southern blots were analyzed for unique banding patterns that would indicate the presence of single or multiple copies of the inserted regions and determine if the inserted genes were stably integrated as a single unit.

II. RESULTS AND DISCUSSION

The seven transgenic lines of Russet Burbank potatoes resulting from transformation of potato stem tissue with the binary vector plasmid PV-STBT02 (approximately 10.1Kb) all showed the presence of genes between the T-DNA border regions of the plasmid and a lack of genes known to occur outside the borders. Five of the seven lines (Bt 6, 12, 17, 18, and 23) appear to have a single copy of the inserted T-DNA whereas lines Bt 10 and 16 have 2 copies. Line Bt 10 apparently has the 2 copies present as a tandem repeat and line Bt 16 has two inserts of the T-DNA located at some distance from each other.

EcoRI digests

The *Eco*RI digestions should liberate a single 935 bp fragment from within the T-DNA borders consisting of the 3' end of the CryIIIA and 2 additional unique fragments for each transgenic line consisting of the remaining T-DNA fragments plus plant genomic DNA up to the next *Eco*RI restriction site. The PV-STBT02 plasmid probe also hybridized to three background *Eco*RI fragments from the non-transformed plant genome (molecular weights ~4500, 3300 and 700 bp).

Lines 6, 12, 17, 18 and 23 all had two hybridizing bands besides the expected 935 bp fragment. One of these 2 bands has the NptII gene as shown by a separate gel hybridized with a PCR generated probe for NptII. Line 6= (6310 bp [NptII fragment] and 3390 bp); line 12= (2190 bp [probably contains NptII] and 1995 bp); line 17= (2300 bp [NptII fragment] and 3185 bp); line 18= (4100 with shadow at 4800 bp [both NptII hybridizing fragments] and 1740 bp); line 23= (10,000 bp [NptII fragment] and 2515 bp). Line 10= tandem insert (apparently 2 fragments around 3800 bp, one of which hybridizes with NptII and a 5620 fragment which also hybridizes with NptII). Line 16= 2 independent inserts (5888 bp and 9000 bp hybridizing with NptII and 2 other fragments 3185 and 1905 bp). Only the radiographs from line 18 and line 10 give ambiguous results.

HinDIII/XhoI digests

The digestions with *Hin*DIII and *Xho*I should yield 2 fragments (3162 and 1468 bp) from within the T-DNA borders that hybridize with PV-STBT02. Lines 6, 17 and 23 did yield these fragments along with three background untransformed, plant genomic fragments (~7500, 1000 and 700 bp) that hybridized with the plasmid. Line 12 lacks a *Hin*DIII restriction site and therefore yielded a 8500 bp fragment instead of the 3162 one and line 18 lacks a *Xho*I site so yielded a 1800 bp fragment rather than the 1468 one. Lines 10 and 16 with more than one insert gave a different pattern of hybridizing bands. Line 10 had 3 bands the expected 1468 bp and 3162 bp bands plus a band at 4260 bp. Line 16 also gave 1468 and 3162 bp bands plus a band at 4500 bp. In lines 10 and 16 the unexpected fragments were both attributed to the loss of the *Hin*DIII restriction site with resulting larger fragments.

HinDIII/NotI digests

The final digestions presented were with *Hin*DIII and *Not*I which should excise practically the entire T-DNA insert or 4639 bp. Lines 6, 17 and 23 yielded the same results as seen in the *Hin*DIII/*Xho*I digests: the expected 4639 bp fragment and at least 2 background bands from the plant genome (~7500 and 4400 bp) strongly hybridizing with the PV-STBT02. Line 12 which was shown in the

*Hin*DIII/*Xho*I digest to be lacking a *Hin*DIII site yielded a large fragment of about 10,000 bp. Line 18 lacking a *Xho*I site gave a fragment of about 5000 bp. Lines 10 and 16 with 2 copies of the T-DNA inserts had more than the expected 4639 bp fragment hybridizing with PV-STBT02. Line 10 also had a 5800 bp fragment that hybridized and line 16 had a 9,500 bp hybridizing with PV-STBT02.

Aad is a gene for spectinomycin and streptomycin resistance used for maintenance of the plasmid in bacterial cells OriV provides a bacterial origin of replication for the plasmid. Both these genes while present in the PV-STBT02 plasmid are outside the T-DNA region and should not have been transferred to the transgenic potatoes. Autoradiographs of *Hin*DIII/*Xho*I digests hybridized with PCR generated probes for either Aad or OriV indicate their absence, therefore, a lack of incorporation of significant portions of the PV-STBT02 plasmid outside the T-DNA region.

In order to verify that the NptII and CryIIIA were inserted on the same fragment of DNA, two Southern blots of *Hin*DIII/*Not*I digestions were hybridized with PCR generated probes of these specific genes. The autoradiographs gave identical banding results indicating that the 2 genes were migrating on the same fragment. This identical migration pattern is evidence that the characters are closely linked and probably present as a single insert.

III. SAB COMMENTS

In combination, the data presented indicates that, in five of the seven lines of Russet Burbank potato (Bt 6, 12, 17, 18 & 23), the T-DNA insert containing B.t.t. δ-endotoxin (CryIIIA) and NptII occurs as a single intact copy in the potato genome. In two lines (Bt 10 &16), the data suggests that the inserts occur as two copies in the plant genome. All these lines gave a lack of hybridization for bacterial genes Aad and OriV outside the expected T-DNA insert region indicating the expected lack of incorporation of plasmid DNA outside the T-DNA borders. The majority of the DNA losses in T-DNA inserts reported in the literature occur near the left border of the T-DNA rather than the right border. Yet three of the lines are apparently missing base pairs from the right border (seen as loss of *Hin*DIII site in 10, 12, & 16) versus only one with missing base pairs from the left border (*Xho*I and *Not*I in line 18).

There are some features of the data that are problematical. In the *Eco*RI digests, there appears to be a discrepancy in line 18 showing two bands hybridizing with the NptII probe when there should ideally be only one hybridizing band. Line 10 is odd in that the internal fragment of the tandem insert released and the fragment released with plant genomic DNA were both 3800 bp. This overlap of fragment sizes is unfortunate as the appearance of two distinct bands would add to the tandem insert argument. In addition, the released internal fragment from the tandem repeat should be larger according to the given gene maps (from 4100 to 4600 bp instead of

the reported 3800 bp).

In the *Hin*DII/*Xho*I digests lines 12 and 18 showed a loss of one of the two restriction sites and resulting larger fragments being released. Line 12 also had a weaker hybridizing small band (1468 bp). Lines 10 and 16, both with two copies of the T-DNA insert, each appear to have lost the *Hin*DIII site in one of the two copies. The *Hin*DIII/*Not*I digests showed similar patterns to the *Hin*DIII/*Xho*I digests. The intensity of the autoradiograph however made the interpretation of the closer bands more difficult.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, SAB/HED JLK
Secondary Reviewer: Roy Sjoblad, Ph.D., Microbiologist, SAB/HED RS

STUDY TYPE:	Product Identity
MRID NO:	429322-03
TEST MATERIAL:	Cry IIIA δ-endotoxin from B.t.t. expressed in Russet Burbank potato
PROJECT NO:	92-01-37-07
TESTING FACILITY:	Monsanto Company; Agricultural Group/ New Products Division; St. Louis, MO
TITLE OF REPORT:	Equivalence of Microbially-Produced and Plant-produced B.t.t. Protein also called Colorado Potato Beetle Active Protein from Bacillus thuringiensis subsp. tenebrionis
AUTHORS:	David E. Bartnicki, Paul B. Lavrik, Richard M. Leimgruber, Christine E. Smith, Steven R. Sims
STUDY COMPLETED:	August 31, 1993
CONCLUSION:	δ-endotoxin from the CryIIIA gene as expressed in Escherichia coli or in potato tubers (line Bt 23) was evaluated by apparent molecular weight determination, immunological recognition, protein glycosylation, partial amino acid sequencing and insecticidal activity. The results are consistent with the microbial and plant protein being equivalent.
CLASSIFICATION:	Acceptable.

I. STUDY DESIGN

Protein Purification

The company has described a method for extracting CryIIIA δ-endotoxin from potato tubers and another for protein inclusions from E. coli engineered to produce the toxin. The extraction procedure for plant protein in the tuber was as follows:

Ten kg of peeled and diced tuber tissue (line Bt 23 grown in Hermiston, OR in 1991) were placed in ice cold 50mM Tris, pH 7.8 with 0.1% sodium metabisulfite. Two kg of fresh cut tuber pieces were then placed in a one gallon Waring blender with 2 liters of crushed ice, 200 ml of 10X grinding buffer (0.5M Tris, 20mM EDTA, 10mg/l leupeptin, 5mM benzamidine/PMSF, to pH 7.8 with boric acid), 10 ml of 10% sodium metabisulfite, 40 ml of 50X reducing cocktail (5% ascorbate acid, 20mM DTT, 20mM thioglycolate to pH 7.5 with sodium hydroxide) and 100 g of insoluble polyvinylpolypyrrolidone (Sigma P6755). This mixture was homogenized for 3 X 60 second bursts then 100 g of Celite (Sigma D3877) was added and mixed for 20 seconds.

The mixture was filtered through a Buchner funnel precoated with 50 g of Celite. This procedure was repeated until all 10 kg of tuber pieces were processed and the homogenate pooled (~18 L). This pool

was concentrated by ultrafiltration with a 30 kD cutoff to 7.5 L. Ammonium sulfate was added to the retenate (70% saturation solution at 550g/L), was precipitated overnight and spun at 11,000 RPM for 30 minutes. The 800 ml of precipitate was stored at 4°C until needed.

The next step was an immunoaffinity column prepared with polyclonal antibodies pooled from rabbits and goats immunized with δ-endotoxin from B.t.t. The ammonium sulfate slurry was dialyzed (12,000-14,000 MW cutoff) against 25mM Tris buffer with 1mM EDTA, 0.1M NaCl pH 7.8 with 500 ml resulting. Effluent from the first pass of the solution through the column was stored for another pass. The bound B.t.t. protein was eluted with 50mM diethylamine, pH 11.5 and the UV absorbing fractions collected and frozen until needed.

From initial analysis of the collected fractions, it was decided that another pass through the immunoaffinity column was needed to remove the patatin protein that was appearing as a significant contaminant. After several washings with column equilibration buffer, the bound B.t.t. protein was eluted as described above.

Preparation of 55 kD tryptic fragment

Fifty mg of B.t.t. protein from E. coli was dissolved in 10mL of sodium carbonate buffer (pH 10.5) and 50mg of trypsin was added and incubated at 37°C for 16 hours. A second dose of trypsin was added, incubated as above and the pH adjusted to pH 6.0. The solution was cooled overnight and the crystals were collected by centrifugation. The pellet was washed with cold 0.1M NaCl followed by water.

The crystals were centrifuged prior to column injection for further purification by cation ion exchange FPLC using 2M urea (pH 4.6). The column was run with an NaCl gradient of 0.1M to 0.5M in 2M urea. The collected fractions were dialyzed against 50mM sodium carbonate (pH 10.5) and checked by SDS-PAGE. The preparation was given the lot # 5214557a.

SDS-PAGE Analysis

The gels were prepared in the range of from 10 to 11.5% acrylamide and run with B.t.t. proteins purified from either E. coli or transgenic potato tubers and molecular weight standards (SOP #MSL-11054). Gels were stained with either Coomassie blue or silver stain. The apparent molecular weights of the bands were calculated by digital scanning compared to the co-electrophoresed standards (SOP #MSL 120698).

Western Blot Analysis

B.t.t. proteins from E. coli or transgenic potato tubers was run side-by-side on 11.5% SDS-PAGE gels and eletrophoretically

transferred to a polyvinylidene difluoride (PVDF) membrane. The samples were loaded from 5 to 500ng per well and the membranes were developed after reacting with primary detection antibody for B.t.t. protein (lot # DD-78 at 1:30,000 dilution; SOP's MSL-11054 & MSL-12068).

Protein Glycosylation Anlaysis

SDS-PAGE gels were run with samples from 0.5 to 2 μg per well of B.t.t. protein purified from both E. coli and potato tubers. Positive controls included horseradish peroxidase and transferrin enzyme preparations both known to be glycosylated (0.3 μg per well), a clarified potato tuber homogenate (9.8μg total protein per well from line Bt10, lot# 5214435) and molecular weight standards. These gels were electroblotted onto PVDF membranes. The membranes were stained for the presence of carbohydrate using a Glyco Track kit (Oxford Systems, Rosedale, NY). This system uses a periodate oxidation of bound carbohydrate, followed by biotinylation, reaction with streptavidin-linked alkaline phosphatase and visualization with a NBT/BCIP substrate.

Determination of N-terminal Amino Acid Sequence

The N-terminal amino acid sequence was determined by an automated Edmand degradation chemistry of PVDF membrane transferred SDS-PAGE protein bands visualized with Poinceau S. An Applied Biosystems Model 470A gas phase sequencer generated the PTH-amino acid derivatives (cycle 03RPTH according to SOP #GEN-EQPQ-015-00). The identity of these individual amino acids was determined by reverse phase HPLC with a Brownlee 2.1mm PTH-C_{18} column.

Biological Activity

The lethality of the purified B.t.t. proteins was determined by an assay employing incorporation of the toxins into the diet of first instar larvae of Leptinotarsa decemlineata. Five to 7 days after feeding the survivors of the 22 larvae used at each dose level were counted. Dosing levels ranged from 0.05 to 2.0μg/ml and were determined by BCA protein assay and ELISA (SOP# PST-91-PRO-007-01 AND BtP-PRO-032-02). The dose-response of the CPB larvae was analyzed by a LOGIT plot and a LC_{50} was calculated.

II. RESULTS

The intent of this study was to compare purified protein preparations produced by transgenic potatoes and E. coli to verify their equivalence using five chemical and biological criteria: apparent molecular weight, immunological recognition, protein glycosylation, amino acid sequencing and biological activity.

The banding patterns on five different gels stained with either

silver stain or Coomassie blue revealed similar migration patterns for the E. coli and tuber purified B.t.t. protein. Only a reproduction of one of the Coomassie gels was submitted but this gel showed that the tuber purified fraction gave 2 bands analogous to the "68kD" and "55kD" bands generated from the E. coli whole protein and tryptic digestion fragment. The apparent molecular weight of the larger protein was 66.2kD from image analysis and comparison to the molecular weight standards. The company claims that the 66.2kD band was split with a smaller band apparently 63kD. This band was not apparent in the image provided. The second band that appears in the lanes loaded with protein purified from transgenic potato has an apparent molecular weight of 56.9kD. Using densitometry image analysis, this lighter band constituted 20-30% of the tuber purified B.t.t. protein. This 55kD species was barely visible in the SDS-PAGE bands from E. coli produced B.t.t. protein. To add weight to the argument that this is simply a byproduct of tryptic digestion, the company also ran a sample of the digested E. coli protein in the gels which gave a band comigrating with the 56.9kD component from the tuber.

The western blots indicate the presence of comigrating bands in both the E. coli and tuber isolated proteins for the larger of the two bands in the tuber. A second smaller band was apparent in the tuber tissue extracts which presumably represents the 55kD fragment seen in the SDS-PAGE gels. There are also several less intensely staining bands of lower molecular weight appearing below the 55kD band. These bands may also be found in the SDS-PAGE of fractions collected from the immunoaffinity columns after either the first or second pass. One of these lower weight bands is attributed to patatin and is a major contaminant of the immunoaffinity purification process. However, other lighter bands of about the same molecular weight are also seen in western blots run during development of the ELISA assay (see MRID 429322-02). These lighter weight immunoreactive bands were considered further degradation products of the B.t.t. protein (Dr. Lavrik, pers.comm.). No molecular weight determination is possible from this gel as no standards were run.

The SDS-PAGE gels electroblotted onto PVDF and stained for the presence of bound carbohydrate showed the absence of significant carbohydrate staining bands in the vicinity of the B.t.t. proteins produced in E. coli or in the plant extracts. Carbohydrate staining bands are present both above (tuber purified protein) and below (tuber homogenate) the general location of the B.t.t. band.

Five different species of B.t.t. protein (67.6kD, 55.5kD, 66.5kD, 55.7kD and 55.1kD) were isolated from SDS-PAGE bands on PVDF membranes and sequenced. These sequences represent the first 15 residues found at the amino terminal end of the six protein species found in either tuber or E. coli purified extracts (2 from E. coli and 4 from tuber tissue). The sequences match the published theoretical sequence for the B.t.t. δ-endotoxin except for

equivocal results for the amino acids asparagine, serine and histidine.

The biological activity of the 2 different proteins was comparable on CPB larvae. The mortality for the potato B.t.t. protein was slightly higher than the E. coli protein at lower protein doses. This difference was apparently not significant as both data sets yielded essentially identical logit curves and LC_{50} values.

III. SAB COMMENTS

The value for molecular weight for the B.t.t. protein derived from comparisons to molecular weight standards (66.3kD) agrees with the value calculated from the known amino acid sequence (67.6kD). The presence of 2 bands in the B.t.t. protein isolated from tubers is apparently not a by-product of the isolation procedure itself as both bands are also found in western blots of field grown potato tissue extracted with a minimum of disruption (see MRID 429322-02, Expression Levels).

The immunorecognition of the purified B.t.t. toxins found in potato tuber by western blot is not remarkable given the fact that this protein was isolated by an immunoaffinity column using the same rabbit polyclonal antibody. Positive recognition by western blot results do indicate that the column elution and western blot methods do not destroy the protein epitopes recognized by these antibodies. Also important is the fact that the antibodies chosen recognize the 55kD tryptic digestion product for the E. coli protein and its equivalent species occurring in the tuber.

The stained PVDF membranes showed the absence of significant carbohydrate staining bands in the vicinity of the B.t.t. proteins. The gels are difficult to interpret due to the lack of staining and a clear indication where the B.t.t. band would appear in the plant extracts. Carbohydrate staining bands are present both above (tuber purified protein) and below (tuber homogenate) the general location of the B.t.t. band.

No raw data on CPB larval mortality was given and no separate logit derived LC_{50} was generated for the assay using E. coli purified B.t.t. protein alone in this comparative analysis. (Studies with the E. coli protein alone are discussed under a different review: MRID 429322-04) However, the logit plot analysis yields no significant difference by considering the microbial data separately and indicates the E. coli and tuber protein data sets give very similar dose-response curves. A second LC_{50} test with two species of the E. coli derived B.t.t. protein gave similar results but a wider 95% confidence interval. The LC_{50} value for the tryptic fragment (0.22μg/ml on 3/11/93) was nearly twofold lower and significantly different than those determined for the intact 68kD B.t.t. protein (0.44μg/ml on 12/1/92 and 0.54μg/ml on 3/11/93). While these are

large significant differences indicate differential toxicity, it is difficult to ascribe true toxicity differences to the forms given the variation of the LC_{50} determinations by this method ($\pm 0.5 \mu g/ml$) and the small total number of determinations done.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, SAB/HED JLK
Secondary Reviewer: Roy Sjoblad, Ph.D., Microbiologist, SAB/HED RDS

STUDY TYPE:	Product Identity
MRID NO:	429322-04
TEST MATERIAL:	Cry IIIA δ-endotoxin from B.t.t. expressed in E. coli
PROJECT NO:	92-01-37-10
TESTING FACILITY:	Monsanto Company; Agricultural Group/ New Products Division; St. Louis, MO
TITLE OF REPORT:	Characterization of Colorado Potato Beetle Active Bacillus thuringiensis subsp. tenebrionis Protein Produced in Escherichia coli
AUTHORS:	Paul B. Lavrik
STUDY COMPLETED:	August 31, 1993
CONCLUSION:	A description of the fermentation the δ-endotoxin from B.t.t. in E. coli was given. The δ-endotoxin protein was also characterized for purity and stability once removed from the production microbe. This protein was generated to provide alternative test material to the potato plant produced δ-endotoxin for addressing mammalian toxicology.
CLASSIFICATION:	Acceptable.

I. STUDY DESIGN

This study describes the use of an alternative production method to yield adequate quantities of the B.t.t. δ-endotoxin for toxicity testing yet functionally similar to that found in transgenic potato plants.

The microbe used was Escherichia coli strain JM101 with the plasmid pMON 5450. This plasmid allowed the microbe to produce B.t.t. δ-endotoxin as encoded by the CryIIIA gene. The B.t.t. protein was found as inclusion bodies within the bacterium. A single 1000L batch was produced by fermentation and yielded approximately 50g of B.t.t. protein that was purified, lyophilized and stored as a powder (batch #5192101).

Fermentation

A standard fermentation procedure was described in Appendix 1 (Monsanto Report MSL-12262). Basically, this was a glucose fed 1500L Chemap fermentor charged with 1000L of M9 medium supplemented with 2% casamino acids. After sterilization and inoculation with a 150L secondary seed lot, the fermentor was maintained at 37°C with agitation and an airflow of 1000 lpm. As required additional glucose was provided until an OD of 20 was attained and the culture

induced with 50ppm nalidixic acid to produce B.t.t. protein. Four hours post induction the culture was chilled to 5°C and centrifuged. No microbial contamination was detected in any nutrient solution, seed culture or fermentation sample

Purification

The purification of the B.t.t. δ-endotoxin from the E. coli production microbe is described in Appendix 2 (Monsanto Report MSL-12394). Basically, the 1000L batch described above was placed in a 0.2M phosphate buffered solution with 0.2M EDTA (pH 6.0) and homogenized with a Gaulin M6 homogenizer at 10,000 PSI. The crude refractile bodies were recovered by low flow rate centrifugation from the cell debris. This extract was chilled to 2°C aliquoted into 10L batches and frozen. The refractile bodies were dissolved by addition of sodium carbonate and adjustment of the pH to 10.8 in a solution held at 2-6°C. The remainder of the solid cell debris was removed by centrifugation. The B.t.t. protein was recovered by lowering the pH to 9.0, aging the solution overnight and collecting the resulting precipitate. The white waxy solid was physically separated from a softer dark, yellow solid which was considered a contaminant. The white solids were washed, resuspended in carbonate buffer at pH 10.8, filtered and frozen solid on glass plates (~1-1.5cm deep). The frozen plates were then lyophilized and the powder recovered and stored at -40°C until needed. This batch of approximately 50 g purified material was given the batch designation of No. 5192101.

Protein Characterization

Besides the normal procedures of analyzing the fermentation batch for sodium, moisture, protein, solubility and elemental composition, the E. coli produced B.t.t. protein was examined for amino acid composition and sequence, storage stability, electrophoretic banding and bioactivity. These secondary analyses can be used to determine the similarity of E. coli produced protein to that isolated from the potato tuber.

II. RESULTS

The composition of the final powder was 8.0% water, 1.7% sodium and from 91.8% to 95.9% protein by bicichoninic acid and Coomassie dye binding or amino acid composition, respectively. The carbon, hydrogen and nitrogen elemental analysis yielded 48.18% C, 6.97% H and 14.26% N for the powder which agreed closely with the expected results (after correction for water and sodium carbonate) of 48.27% C, 6.87% H and 14.35% N. The powder was found to have a solubility of 90 mg/ml in 0.1M carbonate buffer at pH10.8 and 2.4 mg/ml in 0.1M carbonate buffer at pH 8.0.

The protein characterization yielded results in good agreement with the known sequence for B.t.t. CryIIIA as published in the

literature for amino acid composition (the reported values were the means of six measurements), amino acid sequence for the first 15 N-terminal amino acids, and molecular weight by SDS-PAGE. Optical density scanning of the gels revealed that approximately 95% of the protein staining on the gel was the B.t.t. protein which was approximately 67.6kD in size. The amino acid sequence showed that approximately 75% of the B.t.t. protein lacked the N-terminal methionine, a phenomenon described in the literature for protein produced in E. coli. The bioactivity and stability were determined by diet incorporation assay with first instar larvae of CPB. These results gave an LC_{50} value range of 0.43 to 0.97μg/ml of insect diet. The protein was stable over six months storage as either a lyophilized powder at RT or in solution at pH 10.5, 4°C when examined for SDS-PAGE integrity and bioactivity.

III. SAB COMMENTS

The results of this study indicate that the E. coli produced B.t.t. protein from the CryIIIA gene yielded a preparation with properties similar to what are reported in the literature for the δ-endotoxin and this preparation was stable over a six month period when examined for gross integrity (SDS-PAGE) and bioactivity. The amount of the B.t.t. protein preparation that was in the 55kD form was not determined but none was detectable by the SDS-PAGE/Coomassie blue analysis used.

It is unclear why the LC_{50} value range reported in the text is different from that reported in table 3. It is also unclear why the lot numbers differ when the batch numbers remain the same. Both these items should be clarified by the registrant.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, SAB/HED JLK
Secondary Reviewer: Roy Sjoblad, Ph.D., Microbiologist, SAB/HED RD

STUDY TYPE:	Product Identity
MRID NO:	429322-05
TEST MATERIAL:	55kD tryptic fragment from Cry IIIA δ-endotoxin from B.t.t. expressed in E. coli
PROJECT NO:	92-01-37-10
TESTING FACILITY:	Monsanto Company; Agricultural Group/ New Products Division; St. Louis, MO
TITLE OF REPORT:	Characterization of the Major Tryptic Fragment from Colorado Potato Beetle Active Protein from Bacillus thuringiensis subsp. tenebrionis (B.t.t.)
AUTHORS:	David E. Bartnicki, Richard M. Leimgruber, Paul B. Lavrik, Christine E. Smith, Steve R. Sims
STUDY COMPLETED:	August 31, 1993
CONCLUSION:	The 55kD protein released by tryptic digestion of the B.t.t. δ-endotoxin produced in E. coli was compared to the 55kD protein found in potato tubers using apparent molecular weight determination, immunoreactivity and amino acid sequence. The data is consistent with the 55kD forms from potato tuber and E. coli being equivalent. The 55kD protein had somewhat higher bioactivity than the 68kD full-length δ-endotoxin from B.t.t.
CLASSIFICATION:	Acceptable

I. STUDY DESIGN

To address the toxicity of a 55kD protein making up about 30% of the B.t.t. protein in preparations purified from potato tubers, the company has prepared a large amount of this toxin species by tryptic digestion and purification of some microbially produced B.t.t. toxin.

Test substance

Three mg of primarily the 55kD fragment (batch # 5214557a) was obtained by tryptic digestion of 50 mg of the 68kD protein of E. coli produced B.t.t. δ-endotoxin (batch # 5192101). A sixteen hour digestion at 37°C and 50 mg trypsin/treatment was used to degrade the parent δ-endotoxin. This digestion was prolonged for another 16 hours after the addition of 50 mg trypsin. The 55kD fragment was purified from this solution by pH induced precipitation followed by cation exchange FPLC separation with a NaCl gradient (0.1 to 0.5 M) in the presence of 2M urea. This substance was made as a clear solution in 50mM carbonate buffer at pH 10.5 and stored at -30°C.

Two reference substances were employed: one a plant extracted B.t.t. protein and an E. coli derived B.t.t. protein. Both these proteins are primarily in the 68kD form as expressed.

Tests for Characterization and Equivalence

The test substance and references were subjected to analyses similar to that used before (see review for MRID 429322-04): SDS-PAGE (11.5% acrylamide), western blot, N-terminal amino acid sequence and CPB bioactivity.

II. RESULTS

The 55kD tryptic fragment of E. coli produced B.t.t. δ-endotoxin comigrates with the lower molecular weight species appearing in the tuber expressed B.t.t. protein in Coomassie blue stained SDS-PAGE gels. There is a very faint 55kD fragment found in the native E. coli produced B.t.t. protein. The 55kD fragment also appears to be the only product resulting from the digestion of the 68kD protein with trypsin with the running front of the gel detectable down to ~14kD.

The western blot analysis revealed B.t.t. protein present in the tuber purified extract, in the E. coli produced protein and the tryptic digested extract. The antibody used in this assay is a rabbit polyclonal (DD-78) also used in immunoaffinity purifying the B.t.t. protein from potato tubers. The 55kD species is not apparent in the microbially produced B.t.t. protein preparations in these blots until after trypsin digestion. The 55kD species is found along with the 68kD species in the tuber extracts. No immunorecognition of the lower weight species was found in the E. coli extracts although there were very faint bands present in SDS-PAGE.

The reported amino acid sequence for the 55kD fragment is in agreement with that reported for the CryIIIA gene from B.t.t.. Fourteen of the 15 residues were the same except for an arginine residue that was not determinable in the digested protein. The apparent tryptic cut site was at arginine 111.

The bioactivity against CPB first instar larva gave an LD_{50} value of 0.15 to 0.29 ppm (95% confidence interval) which was signifcantly lower than the undigested 68kD species (0.36 to 0.72 ppm).

III. SAB COMMENTS

The SDS-PAGE analysis indicates that there may be 55kD forms of the B.t.t. endotoxin present in both the tuber and E. coli produced preparations. However, the western blot analysis did not reveal an immunoreactive form to be present in the microbial preparation unless it was digested by trypsin. Therefore, the faint bands

appearing in the microbial preparation were either not 55kD toxin or were not concentrated enough to yield a discernable band by western blot analysis.

Assuming no loss in toxin activity with tryptic digestion, the same number of active toxin molecules would lower the LD_{50} value in the CPB assay approximately 20%. The reported LD_{50} value of the 55kD fragment is significantly lower than that of the 68kD form for this test even taking into account the concentration difference. However, it is unlikely this represents a definitively higher toxicity to CPB without having a much larger number of CPB assays with the tryptic digest as a background to judge the variability of the bioassay itself.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, SAB/HED JLK
Secondary Reviewer: Roy Sjoblad, Ph.D., Microbiologist, SAB/HED RS

STUDY TYPE:	Product Identity
MRID NO:	429322-06
TEST MATERIAL:	Cry IIIA δ-endotoxin from B.t.t. expressed in E. coli, potato tissue or commercial products
PROJECT NO:	92-01-37-17
TESTING FACILITY:	Monsanto Company; Agricultural Group/ New Products Division; St. Louis, MO
TITLE OF REPORT:	Compositional Comparison of Colorado Potato Beetle (CPB) Active Bacillus thuringiensis subsp. tenebrionis (B.t.t.) Proteins Produced in CPB Resistant Potato Plants and Commercial Microbial Products
AUTHORS:	Glennon J. Rogan and Paul B. Lavrik
STUDY COMPLETED:	August 31, 1993
CONCLUSION:	The Cry IIIA δ-endotoxin as expressed in potato tissue or an E. coli alternative was compared to registered microbial products with the same δ-endotoxin using immunoreactivity and electrophoretic mobility.
CLASSIFICATION:	Acceptable.

I. STUDY DESIGN

Test Substance

Three sources of the B.t.t. protein were used: seven lines of Cry IIIA δ-endotoxin producing potato, M-One® and Foil®. In addition, a preparation of Cry IIIA δ-endotoxin as produced by an E. coli carrying the plasmid pMON 5450 was used as a reference for the 68kD protein and a trypsin digested preparation of this 68kD protein was used as a reference for the 55kD protein.

Purification of Test Substances

The potato expressed proteins were extracted with buffer (8.1mM Na_2HPO_4, 140mM NaCl, 1.4mM KH_2PO_4, 2.7mM KCl, pH 7.4) at either 1:15 tissue fresh weight for leaf tissue or 1:2 tissue fresh weight for tuber tissue as described in SOP No. BtP-PRO-021-02. The microbial preparations were diluted in cold 0.1M carbonate buffer pH 10.5, sonicated to disrupt the cell membranes, centrifuged then filtered through 0.22μm membranes and frozen until needed. The E. coli reference substances were prepared as described in MRID 429322-05. Content of the B.t.t. protein in each extract was done by an ELISA method as described in MRID 429322-02.

Analytical Method

The extracts were loaded into the wells at approximately 1.5 ng of B.t.t. protein as determined by ELISA (except for the Foil® which was loaded at 0.6ng). The SDS-PAGE gels were gradients from 4 to 20% acrylamide. These gels were run for approximately 1.5 hours and electroblotted onto nitrocellulose membranes. The nitrocellulose membranes were blocked with non-fat dried milk and reacted with anti-B.t.t. antibody (1:2000), visualized with donkey anti-rabbit HRP and enhanced chemiluminescence then photographed.

II. RESULTS

The potato tuber and leaf tissue from the seven transformed lines all displayed detectable amounts of the 68kD species of the B.t.t. δ-endotoxin from Cry IIIA as determined by comigration with the E. coli produced standards. All tuber tissue also showed a considerable amount of the 55kD species which could only be seen in lines Bt 10 and 12 for the leaf tissue samples.

Both commercial products showed the presence of the 68kD protein. Foil® contained only the 68kD protein but M-One® also had the 55kD species plus a considerable amount of other indeterminable species between the 68kD and somewhat below the 55kD proteins.

III. SAB COMMENTS

The Foil® product showed a remarkably homogenous preparation of only the 68kD species although an unsubstantiated statement was made that the 55kD species was detectable when the lanes were loaded at higher amounts. The E. coli produced B.t.t. protein showed a detectable amount of the 55kD species in these tests prior to trypsin digestion which was not as apparent in previous western blot analyses (MRID 429322-04 & 05).

The gross variability of the B.t.t. protein amount expressed in the potato lines either in the leaf or the tuber was very evident in these western blots. The statement that generally there was less of the 55kD species appearing in the leaf tissue compared to the tuber is confirmed by these blots.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, SAB/HED JLK
Secondary Reviewer: Roy Sjoblad, Ph.D., Microbiologist, SAB/HED RD

STUDY TYPE:	Product Identity
MRID NO:	429322-07
TEST MATERIAL:	Cry IIIA δ-endotoxin from B.t.t. expressed in E. coli
PROJECT NO:	92-01-37-05
TESTING FACILITY:	Monsanto Company; Agricultural Group/ New Products Division; St. Louis, MO
TITLE OF REPORT:	Sensitivity of Selected Insect Species to the Colorado Potato Beetle Active Protein from Bacillus thuringiensis subsp. tenebrionis
AUTHORS:	Steven R. Sims
STUDY COMPLETED:	September 3, 1993
CONCLUSION:	The range of non-target insect sensitive to the Cry IIIA δ-endotoxin as expressed in E. coli was examined by bioassay. The tested species were all pest species that included Colorado Potato Beetle (CPB) and 2 other coleopterans [boll weevil (BW) and southern corn rootworm (SCRW)], 4 lepidopterans [European corn borer (ECB), tobacco hornworm (THW), corn earworm (CEW), tobacco budworm (TBW)], a dipteran [yellowfever mosquito (YM)], an orthropteran [German cockroach (GC)] and a hemipteran [green peach aphid (GPA)]. The results show that no species other than CPB displayed significant mortality. There was a slight reduction in the amount of honeydew produced by the GPA indicating reduced feeding.
CLASSIFICATION:	Acceptable.

I. STUDY DESIGN

Test substance

The B.t.t. protein test substance purified from E. coli (Batch # 5192101, lot # 5002059) was incorporated into the diet of the test species at 50 ppm as a single high dose for 1 to 7 days exposure. The diluent, water or carbonate buffer, was used at the same level of diet incorporation as a control. Three different diets were used to orally expose the test insects to the substance: southern corn rootworm diet (SOP No. BUG-PRO-044-00), pinto bean diet (SOP No. BUG-PRO-022-02) and green peach aphid diet (SOP No. BUG-PRO-041-00).

Assays for test insect species

For the coleopterans CPB, BW and SCRW, southern corn rootworm diet was used and either neonate larvae or ova (BW) were exposed to the diets poured into 96-well insect assay trays (1 larva/well). The BW eggs were from GAST, Mississippi State University Insectary and introduced in a 0.15% agar suspension. The SCRW and CPB were from Monsanto colonies. Twenty four larvae were used per treatment replicate with 3 replicates per treatment. Larval mortality was assayed at 5 and 7 days after the start.

For the lepidopterans ECB, THW, CEW and TBW, southern corn rootworm diet was poured into 96-well insect assay plates and one neonate larvae was placed in each well. The ECB can from USDA-ARS in Ankeny, IA. THW came from the Entomology Department, North Carolina State University. CEW came from the USDA-ARS, Stoneville, MS. TBW was provided from Monsanto insect colonies. Twenty four larvae were used per treatment replicate with 3 replicates per treatment. Larval mortality was assayed at 5 and 7 days after the start.

For the dipteran YM, southern corn rootworm diet was used as a 0.2 g sample added to 20ml of water in a 50 ml centrifuge tube with ten 4th instar larvae added to each tube. YM was provided by American Biological Supplies, Finksburg, MA. There were 5 replicate tubes per treatment kept at 28°C. Larval survival was determined after 24 hours.

For the orthropteran GC, the toxin was incorporated into the pinto bean diet and poured into 50-well insect assay trays. The GC was obtained from American Biological Supplies, Finksburg, MA. One GC nymph from the 3rd or 4th instar was added to each well. Fifteen nymphs were tested per treatment replicate with 2 replicates per treatment. Survivors were tallied after 5 days exposure.

The GPA diet required special preparation and handling since this is a siphon feeder. The GPA was from Monsanto insect colonies. The liquid diet was prepared and placed in 24-well culture plates covered with parafilm then placed over identical trays with 12 aphids (maturity unspecified). The aphids fed on the overhead solution. Individual wells were considered replicates. There were 15 to 16 replicates per treatment and the test was repeated on 2 separate days. The trays were incubated at 22 to 24°C and evaluated after 4 days exposure for survivors.

II. RESULTS

There was no significant mortality associated with dietary exposure to the B.t.t. protein purified from E. coli in any insect species tested except the target CPB. There were treatment related effects and mortality in the first assays with ECB and GPA. Due to these unexpected findings, the assays with ECB and GPA were repeated.

The original test and the repetition were analyzed as a single data set with the result being that day-related variation explained most

of the variation between the treatment and control for GPA. For ECB, there were no significant treatment related effects or day related variation when the 2 data sets were combined as the survivorship was identical between treatment and control for the repeated test.

A complication of the GPA assay was that there appeared to be an effect of the B.t.t. protein on the feeding behavior of the GPA. The treatment group had a reduction in their honeydew production which indicates lower feeding. This effect was determined by a rating scale of 0 to 3 (3 indicating no honeydew production, no feeding). B.t.t. treated GPA had a mean rating of 0.40 and 0.56 compared to a mean control GPA rating of 0.19 and 0.06.

III. SAB COMMENTS

These tests indicate that the B.t.t. protein has a limited spectrum of activity when considering the few species of animals tested here. The mildly repellent effect of B.t.t. protein on siphon feeding GPA is an interesting phenomenon considering the low likelihood of this organism encountering B.t.t. through its normal feeding behavior. The effect is barely detectable and occurs at concentrations well above those expected in transgenic potatoes even if the toxin occurred in the phloem sap. It would be unlikely that there will be any negative impact on this pest species in practice.