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

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OFFICE OF
PREVENTION, PESTICIDES AN
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Review of Product Characterization, Acute Oral Toxicity and *In vitro* Digestibility for Dekalb's Corn Expressing the Cry IA(c) δ-endotoxin

TO: Michael Mendelsohn
Regulatory Action Leader
Biopesticides & Pollution Prevention Division
(7501W)

FROM: John L. Kough, Ph.D., Biologist
Biopesticides & Pollution Prevention Division
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THROUGH: Roy D. Sjoblad, Ph.D., Senior Scientist
Biopesticides & Pollution Prevention Division
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THIS REVIEW CONTAINS FIFRA CONFIDENTIAL BUSINESS INFORMATION

DATA REVIEW RECORD

Active Ingredients:	Cry IA(c) δ-endotoxin
Product Name:	DBT 418 transformation event
Company Name:	Dekalb Genetics Corporation, Mystic, CT
ID No:	069575-E, 6F04711, 6E04710
Submission No:	S506675, S506690, S506694
Chemical No:	006430
DP Barcode:	D226923, D226926, D226927
MRID:	439995-01: Acute Oral Toxicity of Cry IA(c) Protein in Mice. 439995-02: Acute Oral Toxicity of PAT Protein in Mice. 439995-03: *In vitro* Digestibility of Cry IA(c) and PAT Proteins. 439995-04: Molecular Characterization of Introduced Traits in Corn.

439995-05: Stable Mendelian Inheritance of *cryIA(c)* and *bar* Genes in Corn
439995-07: Characterization of Cry IA(c) in Plants and Equivalence to Microbial Cry IA(c).
439995-08: Characterization of PAT in Plants and Equivalence to Microbial PAT.

ACTION REQUESTED
Review the submitted data to support an experimental use permit and a food tolerance determination for CryIA(c) and PAT.

BACKGROUND
Dekalb Genetics Corporation has been testing and developing their corn lines under an APHIS permit.

RECOMMENDATIONS
These studies demonstrate the activity and structural similarity of the microbially produced CryIA(c) and PAT proteins to those produced in DBT418 plants. This similarity allows the use of bacterially produced CryIA(c) and PAT proteins as test substances for the acute toxicology studies. The lack of significant test substance related toxicity with acute oral exposure in rodents and rapid degradation in the presence of artificial gastric fluids suggests these proteins are processed as typical nutrients in mammals. Rapid degradation in gastric fluid would suggest that these proteins are not likely to be food allergens. The studies' results support the conclusion that there is no expected increased risk for infants and children, nor an aggregate risk factor to consider for these proteins. These findings would support an exemption from the requirement for a food tolerance for both the CryIA(c) and PAT proteins.

SUMMARY OF REVIEWS

439995-01: One male died on day 1. Gross necropsy revealed a perforated trachea, mottled lungs and fluid in the thoracic cavity and it was assumed that the animal died as a result of trauma during dosing. The remaining animals all gained weight and showed no abnormalities at gross necropsy on day 14.
CLASSIFICATION: Acceptable.

439995-02: No animals died during this study. One male had decreased feces production on day 12 and showed a slight body weight loss between day 7 and day 14. All the other animals displayed weight gain, no abnormal clinical signs and nothing of note with gross necropsy at scheduled sacrifice on day 14. A non-dosed control group was not included in this study.
CLASSIFICATION: Acceptable.

439995-03: Both the CryIA(c) and PAT proteins (as judged by absence of bands to comigrating standards) were degrade to the point they were no longer recognized by specific antibodies in a western blot assay. This degradation occurred at essentially the 0 timepoint in the full strength pepsin assays and by 5 minutes in the 0.01X concentration pepsin.
CLASSIFICATION: Acceptable.

439995-04: Corn line DBT418 was transformed with three plasmids (pDPG165, pDPG320 and pDPG699) which potentially allowed for the incorporation and expression of the *bar*, *pinII* and *cryIA(c)* genes. The southern hybridization analyses of DBT418 indicate the presence of approximately 2 copies of the *cryIA(c)* and *bar* genes and a partial copy of the *pinII* gene. The results of probing the various genomic digests of DBT418 also indicated the single *pinII* gene present was an incomplete copy and one of the *bar* genes was rearranged. Data showed that over 96% of the 190 progeny tested from the generation of elite lines yielded DNA patterns as expected from a stable insertion event for the *cryIA(c)* gene.
CLASSIFICATION: Acceptable.

439995-05: The results of PCR testing for one early generation, three late generation and non-transformed genotypes indicate that the transgenes *bar* and *cryIA(c)* segregate as closely linked traits and in a 1:1 fashion when crossed with non-transformed lines. Such tight linkage stably maintained during several backcross generations indicate that the *cryIA(c)* and *bar* genes were probably inserted at the same location during the transformation process.
CLASSIFICATION: Acceptable.

439995-07: Data from western blots indicates the plant expressed CryIA(c) comigrates and is recognized by specific antibodies to tryptic core of bacterial CryIA(c). N-terminal amino acid

sequencing showed similar sequences between the bacterial tryptic core and DBT419 plant expressed CryIA(c) except that the plant sequence starts at glycine 26 rather than the expected methionine 1. No detectable glycosyl residues could be found in either the DBT418 or bacterial preparations even though other proteins did test positive for carbohydrates in the extracts. Bioassays establishing the similar activity of plant and microbial CryIA(c) were cited but no results could be confirmed.
CLASSIFICATION: Acceptable.

439995-08: PAT derived from DBT418 plants, from *E. coli* as a histidine-TAG PAT form and from *E. coli* as histidine-TAG PAT but digested with thrombin migrate with similar mobilities and are all immunoreactive with anti-PAT rabbit antibodies. The histidine-TAG PAT has 88% of the activity of the plant extracted form. The amino acid analysis of the three forms yielded the expected sequence for each form save the initial methionine in the plant sequence.
CLASSIFICATION: Acceptable.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD

Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD

STUDY TYPE:	Acute Oral Toxicity
MRID NO:	439995-01
CHEMICAL NO:	006430
TEST MATERIAL:	CryIA(c) δ-endotoxin
STUDY NO:	SLS 3406.1 (Dekalb DGC-95-A17)
SPONSOR:	Dekalb Genetics Corp., Mystic, CT
TESTING FACILITY:	Springborn Laboratories, Spencerville, OH
TITLE OF REPORT:	An Acute Oral Toxicity Study in Mice with *Bacillus thuringiensis* subsp. *kurstaki* CryIA(c) Delta Endotoxin
AUTHOR:	Todd N. Merriman, B.S., LATG
STUDY COMPLETED:	March 26, 1996
CONCLUSION:	One male died on day 1. Gross necropsy revealed a perforated trachea, mottled lungs and fluid in the thoracic cavity and it was assumed that the animal died as a result of trauma during dosing. The remaining animals all gained weight and showed no abnormalities at gross necropsy on day 14. A non-dosed control group was not included in this study.
CLASSIFICATION:	Acceptable

STUDY DESIGN

A single dose of 5000 mg/kg of CryIA(c) protein was administered to each of five male and female mice which were monitored for 14 days for signs of toxicity.

STUDY METHODS

Test substance: CryIA(c) δ-endotoxin was isolated from a fermentation of *Bacillus thuringiensis* subsp. *kurstaki* ATCC 35866. The purified toxin was subjected to trypsin digestion and purified to yield the test substance, the tryptic core protein of CryIA(c) (lot #CSV-102695, received 11/28/95). The equivalence of this microbially produced protein to that found in DBT418 corn plant is demonstrated elsewhere (MRID 439995-07).

Test animal: Young adult CD-1(ICR)BR mice were received from Charles River Laboratories, Portage, MI. Five male and five female (nulliparous, non-pregnant) mice were identified by ear

tags and caged individually after a five day acclimation. The animals were fed and given water *ad libitum* throughout the study except for the fasting period prior to dosing and maintained in controlled environment room (13-53%R.H., temperature 65-70°F, 12-hour light/12-hour dark).

Dosing and observations: The animals were dosed to a level of 5000 mg/kg using a 250 mg/ml solution (diluted with carboxymethylcellulose) and utilizing a ball tipped stainless steel gavage needle with a syringe. After dosing the animals were returned to their cages and observed twice on the day of dosing and once a day thereafter. The animals were weighed on days 0, 7 and 14 after dosing. A the time of death or at scheduled sacrifice the animals were subjected to a gross necropsy.

RESULTS

One male displayed labored breathing and decreased activity on the day of dosing and was found dead on day 1. Upon necropsy this animal was found to have a perforated trachea, mottled lungs and fluid in the thoracic cavity. It was assumed from these findings that the animal died as a result of trauma suffered during the dosing procedure. All the remaining dosed animals gained weight during the study and displayed no clinical signs or abnormalities at necropsy.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD RDS

STUDY TYPE:	Acute Oral Toxicity
MRID NO:	439995-02
CHEMICAL NO:	006430
TEST MATERIAL:	Phosphinothricin Acetyltransferase protein
STUDY NO:	SLI 3406.2 (Dekalb DGC-95-A18)
SPONSOR:	Dekalb Genetics Corp., Mystic, CT
TESTING FACILITY:	Dekalb Genetics Corp., Mystic, CT
TITLE OF REPORT:	An Acute Oral Toxicity Study in Mice with Phosphinothricin Acetyltransferase (PAT) Protein
AUTHOR:	Todd N. Merriman, B.S., LATG
STUDY COMPLETED:	March 26, 1996
CONCLUSION:	No animals died during this study. One male had decreased feces production on day 12 and showed a slight body weight loss between day 7 and day 14. All the other animals displayed weight gain, no abnormal clinical signs and nothing of note with gross necropsy at scheduled sacrifice on day 14. A non-dosed control group was not included in this study.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

A single dose of 2500 mg/kg bodyweight of phosphinothricin acetyltransferase (PAT) was administered to five male and five female mice by oral gavage.

TEST METHODS

Test substance: A histidine labelled form of the phosphinothricin acetyltransferase (PAT) enzyme was purified from a fermentation with *E. coli* (batch #1, received 1/23/96). This purified histidine labelled PAT containing 20 extra amino acids at the 5' end has been shown to be equivalent to that expressed in the plant (MRID 439995-08).

Test animal: Young adult CD-1(ICR)BR mice were received from Charles River Laboratories, Portage, MI. Five male and five female (nulliparous, non-pregnant) mice were identified by ear

tags and caged individually after a five day acclimation. The animals were fed and given water *ad libitum* throughout the study except for the fasting period prior to dosing and maintained in controlled environment room (45-60%R.H., temperature 63-71°F, 12-hour light/12-hour dark).

Dosing and observation: A single dose of undiluted test substance at a concentration of 125 mg/ml was administered to give a dose of 2500 mg/kg utilizing a ball tipped stainless steel gavage needle. After dosing the animals were returned to their cages and observed twice on the day of dosing and once a day thereafter. The animals were weighed on days 0, 7 and 14 after dosing. At the scheduled sacrifice, the animals were subjected to a gross necropsy.

RESULTS

No animals died during this study. One male had decreased feces production on day 12 and showed a slight body weight loss between day 7 and day 14. All the other animals displayed weight gain, no abnormal clinical signs and nothing of note with gross necropsy at scheduled sacrifice on day 14.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD RDS

STUDY TYPE:	*In vitro* Digestibility
MRID NO:	439995-03
CHEMICAL NO:	006430
TEST MATERIAL:	CryIA(c) & PAT Proteins
STUDY NO:	DGC-96-A22
SPONSOR:	Dekalb Genetics Corp., Mystic, CT
TESTING FACILITY:	Dekalb Genetics Corp., Mystic, CT
TITLE OF REPORT:	*In vitro* Digestibility of CryIA(c) and PAT Proteins
AUTHORS:	Donald S. Walters, Ph.D. & Whitney Adams
STUDY COMPLETED:	April 12, 1996
CONCLUSION:	Both the CryIA(c) and PAT proteins (as judged by absence of bands to comigrating standards) were degrade to the point they were no longer recognized by specific antibodies in a western blot assay. This degradation occurred at essentially the 0 timepoint in the full strength pepsin assays and by 5 minutes in the 0.01X concentration pepsin.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

Purified preparations of both the CryIA(c) and PAT were subjected to simulated gastric fluids and then assayed for recognition in a western blot assay.

TEST METHODS

Test substance: Leaf extract from DBT418 and microbially produced CryIA(c) and PAT proteins were used as test substances. Approximately one gram of fresh leaf tissue was collected from plants at pollen shed and subjected to an extraction procedure. The leaf material was frozen in liquid nitrogen and ground with a mortar and pestle then an extraction buffer (3 ml of 100mM NaCl, 13mM Na_2CO_3, 37mM $NaHCO_3$) was added. The mixture was centrifuged at 14,000xg at 4°C for 15 minutes. The supernatant was transferred to new tubes and recentrifuged to remove any remaining particulates. Leaf tissue from DK.DL non-transformed corn was also extracted and tested or spiked with microbially

produced CryIA(c) or PAT (at 500ng/5µl or 1900ng/5µl, respectively) and assayed.

Digestive Fluid Preparation: Two levels of gastric fluid were prepared using a base of 0.2 gm of NaCl and 0.7 ml of concentrated HCl in 100 ml total volume using distilled water to which was added either 32 mg or 0.32 mg of pepsin. The digestive fluid without pepsin was termed 0X, full strength pepsin was 1X and the lesser amount of pepsin was termed 0.01X.

Experimental procedure: To 55µl of gastric fluid containing pepsin, 5 µl of leaf extract or spiked leaf extract was added. The reaction was either immediately stopped or incubated for 2, 5, 15 or 30 minutes. The digestion reactions were stopped by the addition of 30 ml of 3X SDS-PAGE buffer to the 60 µl sample. The stopped samples were then heated to 90-100°C for 5 minutes prior to loading into lanes of an SDS-PAGE gel. The samples were subjected to electrophoresis and western blotting (described in MRID 439995-06, memorandum from D. Horne to M. Mendelsohn, Oct.16, 1996). The capture antibodies used in the western blot procedure were a mouse monoclonal for CryIA(c) and a polyclonal rabbit antibody for PAT. The detection antibodies for each assay were a horseradish peroxidase conjugate of an anti-mouse sheep antibody and a goat anti-rabbit conjugate of horseradish peroxidase for the PAT assay (MRID 439995-06)

RESULTS AND DISCUSSION

CryIA(c) Protein: The CryIA(c) protein was rapidly degraded in the 1X solution of gastric fluid. No detectable bands were seen at either the 0 or 2 minute assay point which were the only time points examined for this pepsin concentration. The banding pattern for either the spiked plant tissue extract or the microbially produced standard was unchanged after 0 or 2 minutes incubation in the digestive fluid solution without added pepsin. No immunoreactive bands were seen in non-spiked plant extract (DK.DL).

The banding pattern of the microbial standard CryIA(c) had less bands than that seen in the pepsin-free digestive fluid. While the major bands for CryIA(c) were evident and comigrating, there were also a series a lower molecular weight bands present in the pepsin-free digestive fluid suggesting a breakdown due to the high acidity of this solution. In the presence of one hundredth

the normal concentration of pepsin, the CryIA(c) degradation was slowed down such that at the 0, 2 and 15 minute samples showed immunoreactive banding at the CryIA(c) region. The banding at the 0 timepoint was less intense than found in the controls and showed the presence of lower molecular weight immunoreactive bands not seen in any other sample and suggestive of degradation products of the CryIA(c) protein. There was no immunoreactive band seen in the 5 minute incubation and a spot but not a true positive immunoreactive band seen in the 15 minute sample.

PAT Protein: The western blots for the PAT protein were done at very high concentrations of PAT (1900ng) both as a standard and in the presence of the DK.DL control plant extract. In addition, the anti-PAT polyclonal antiserum seems to react with many components in the PAT enriched extract itself when tested at high concentrations. Only at 50 to 100 fold dilutions could a clear band for the PAT protein be discerned in these bands. Therefore, the quality of the western blots was compromised due to the high level of background or cross-reactivity from the immunological reagents.

Nevertheless, nearly all the immunoreactivity disappears when the PAT standards or spiked plant extracts are incubated with either 1X or 0.01X pepsin. At the 0 timepoint in the 1X pepsin, the general immunoreactivity cleared to allow visualization of the PAT band near the bottom of the gel and only a faint band comigrating with the PAT band was visible in the 2 minute incubation sample. For the 0.01X pepsin concentration digestion, the 0 timepoint showed a clearing of the intense banding pattern that by the 2 minute sample had cleared to a shadow with faint bands at high molecular weights and another comigrating with the PAT protein. All immunoreactive bands comigrating with the PAT protein standard were absent by 5 minutes incubation with the 0.01X pepsin. The higher molecular positive bands were not present in the 30 minute digestion sample.

An extract of the DBT418 tissue (expressing PAT protein) incubated with 0.01X pepsin showed positive bands somewhat below that expected for the PAT protein. This positive band was notably fainter to non-detectable by the 2 minute sampling time and not detectable whatsoever at the 15 minute incubation endpoint.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD

Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD

STUDY TYPE:	Product Characterization
MRID NO:	439995-04
CHEMICAL NO:	006430
TEST MATERIAL:	DNA from corn line DK.DL(DBT418)
STUDY NO:	DGC-95-A07
SPONSOR:	Dekalb Genetics Corp., Mystic, CT
TESTING FACILITY:	Dekalb Genetics Corp., Mystic, CT
TITLE OF REPORT:	Molecular Characterization of Transgene Content and Stability in Transgenic Corn Hybrid Line DK.DL(DBT418)
AUTHORS:	Michael Stephens, Ph.D., Emil M. Orozco, Ph.D. & David Walters
STUDY COMPLETED:	April 23, 1996
CONCLUSION:	Corn line DBT418 was transformed with three plasmids (pDPG165, pDPG320 and pDPG699) which potentially allowed for the incorporation and expression of the *bar*, *pinII* and *cryIA(c)* genes. The southern hybridization analyses of DBT418 indicate the presence of approximately 2 copies of the *cryIA(c)* and *bar* genes and a partial copy of the *pinII* gene. The results of probing the various genomic digests of DBT418 also indicated the single *pinII* gene present was an incomplete copy and one of the *bar* genes was rearranged.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

The molecular characteristics of the inserted traits as well as the genetic stability were examined using restriction endonucleases and gene probes.

TEST METHODS

Test substance: DNA isolated from corn line DK.DL(DBT418) is the test substance. DK.DL(DBT418) is hemizygous for the Bt transgene since only the DK female parent is homozygous for the trait. DNA from corn line DK.DL was used as a control substance since it has the same DL male parent and a non-transformed DK female parent to provide the genetic background of the corn line. Reference

substances were provided by the plasmids used for the initial transformation: pDPG699, pDPG165 and pDPG320 (plasmid maps are found in Appendix I to this review).

DNA isolation: Leaf tissue from 54 to 60 day old corn grown in Mystic, CT was taken from the youngest leaf with at least 12 inches of emerged foliage. These samples were frozen to -50°C and the tissue macerated with either a meat grinder or a glass rod. From 300 to 500 mg of this coarsely ground tissue was placed in a glass tube chilled in liquid nitrogen and extracted (500µl 7M urea, 0.3 M NaCl, 0.05M Tris, 0.02 M Na_2EDTA, 1% Sarkosyl followed by 500µl phenol/chloroform/isoamyl alcohol at 49.5:49.5:1). The tube was vortexed and allowed to stand at room temperature for the remaining extraction steps. The aqueous layer was separated by centrifugation and added to a tube with 50µl of 4.4M ammonium acetate, pH 5.2. The DNA was precipitated with 500µl of ice-cold 2-propanol, centrifuged and the alcohol drawn off. The DNA pellet was washed with 1 ml of 70% ethanol, centrifuged, alcohol withdrawn and the DNA pellet allowed to dry. The pellet was dissolved in 500µl TE buffer (10 mM Tris, 1 mM Na_2EDTA, pH 8.0) overnight at 1-9°C prior to a DNA concentration determination.

DNA concentration: DNA concentration was determined using a Hoefer TKO 100 Mini-fluorometer with calf thymus DNA used to generate the standard curve. A 2µl sample of DNA was added to 2ml of TNE (10 mM Tris, 1 mM Na_2EDTA, 200 mM sodium chloride, pH 7.4 with Hoechst dye #33258 @ 1µg/ml) and the samples were read.

DNA digestions: For Southern analysis, 5 to 10 µl samples of DNA were employed. For the genetic stability determinations, approximately 5 µg of genomic DNA based on fluorometric calculations were dried in a vacuum centrifuge and added to 25µl of autoclaved, deionized water. For copy number determinations, a volume equivalent to 10µg of DNA was used in each endonuclease digestion reaction. The restriction endonucleases were prepared in bulk to provide a solution for aliquoting 15 µl into each DNA sample. In each reaction mix, approximately 30 units of endonuclease activity with 4µl of the appropriate 10X digestion buffer, 0.1µM $MgCl_2$ and 1µg Rnase A. All digests were incubated at 35 to 39°C for 12 to 16 hours. The gels each included a negative control of digested genomic DNA from a non-transformed corn plant and a positive control which consisted of digested

genomic DNA from non-transformed corn mixed with a digest of plasmid DNA for the traits in question. The entire 40μl of digestion solution for each sample were added to lanes of an 0.8% agarose gel (prepared in 40mM Tris, 40mM acetic acid, 1mM Na_2EDTA, pH 8.0) after 4μl of tracking dye were added to the solution. The gels were subject to electrophoresis at approximately 100 V for 2.5 to 3.5 hours. The gels were removed, soaked in 1μg/ml of ethidium bromide and photographed under UV illumination next to a phosphorescent ruler.

Membrane blotting and probing: The gels were subjected to incubation in a series of solutions intended to fragment and denature the DNA (1st 0.25M HCl x2 for 15-30 minutes, then 0.5M NaOH, 1.5M NaCl x2 for 30-45 minutes and finally a single incubation for 30-120 minutes in 0.5M Tris, 1.5M NaCl, pH 7.4). The gels were then blotted onto Nytran membranes using 10X SSC (1.5M NaCl, 0.15M sodium citrate, pH 7.0) as buffer in the overnight capillary transfer. The DNA was crosslinked to the membrane by UV illumination and stabilized by baking at 90°C for 2-4 hours.
The radioactive probes were prepared by PCR amplification of plasmid DNA with the appropriate primers (Primer sequences in Appendix II). The PCR amplification employed 50pg of plasmid DNA and 30-35 cycles with Taq polymerase in a PCR heating block cycler. The PCR products (461bp for *cryIA(c)*, 279bp for *bar*, 541bp for *pinII*, 776bp for *bla* and 249 for the ColE1) were purified by agarose gel electrophoresis, ethidium bromide visualization and elution form the gel with a Elu-Quik® or QIAquick gel extraction kit.
The radiolabelling was accomplished by using a Random Primed Labelling Kit (Boehringer-Mannheim) with ^{32}P-labelled CTP and the PCR products as templates. The percent radiolabel incorporation was determined to be above 40% in all cases. The probes were separated from unincorporated nucleotides by chromatography on a G-50 Sephadex column.

Hybridization of probes: A three step preparation for the membranes involved a 5-10 minute incubation in 0.25X SSC with 0.2% SDS followed by a 1-3 minute wash in 0.1M NaOH, 0.2% SDS and finally a 20-30 minute wash in 0.2M Tris, 0.1X SSC and 0.2% SDS, pH 7.5. The membranes were then incubated for at least 2 hours at 60-65°C in hybridization solution (ExpressHyb from Clonetech). This prehybridization solution was discarded and replaced with

new solution and the radiolabelled probes were added after being denatured by boiling. The final hybridization of the probes with the membranes took place for 12-16 hours at 60-65°C. To remove the nonspecific probe the membranes were incubated with post-hybridization solution (0.25X SSC with 0.2% SDS for the genetic stability assays and 1X SSC with 0.2% SDS for the copy number determinations). The first washes occurred at room temperature for 5-10 minutes and the second washes for 30 minutes at either 65° (genetic stability) or 42°C (copy number). The final rinses were done at room temperature for several minutes. The washed membranes were wrapped in plastic and exposed to Kodak Xomat AR film at $\leq$-50°C.

PCR Analysis of the *pinII* gene: This assay was done after the original determinations to clarify the presence and integrity of the *pinII* gene. One to three inches of leaf tissue from 12-day-old seedlings of the corn genotype AX(AW.DBT418) was used as DNA source for the test material. The DNA was extracted by maceration of the leaf tissue with zirconia/silica beads in a 2.0ml test tube in the presence of 0.6 ml of a lysis buffer (Gentra Systems, Inc.) using a bead beater. The contents were centrifuged to pellet the contents, then 200 μl of protein precipitation solution (Gentra Systems, Inc.) were added and the tubes shaken vigorously. After a 20-30 minute incubation on ice, the tubes were spun again and 600μl of supernatant were transferred to a new microfuge tube containing 500μl of ice-cold 2-propanol. The contents of this tube were mixed by shaking then centrifuged to form a pellet containing the DNA. The supernatant was removed, the pellet washed with 750μl of 70% ethanol followed by a 5 minute centrifugation and removal of the ethanolic supernatant. The pellet was air dried at room temperature for one hour to allow evaporation of the ethanol and the pellet was resuspended in 500μl of TE buffer with RNase (10mM Tris, 1mM EDTA, 1μg/ml of RNase A).

PCR detection of gene presence: 50μl of PCR reaction mixture consisted of 5μl of 10X PCR reaction mixture (Boehringer-Mannheim) 200μM each of deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 0.25μM of each of the primer sequences, 5μl of the DNA sample and sterile distilled water to yield 50μl.

DNA from corn line A14-01Y-A05 and A14-01Y-A03, transgenic for DBT418 and non-transformed, respectively, as well as AR02CS01, a

transgenic corn line with the *pinII* gene were used as samples of positive and negative plant DNA. Plasmid DNA containing the target sequence was included as a positive sample.

RESULTS AND DISCUSSION

Corn line DBT418 was transformed with three plasmids (pDPG165, pDPG320 and pDPG699) which potentially allowed for the incorporation and expression of the *bar*, *pinII* and *cryIA(c)* genes. The information provided by the southern hybridization analyses of DBT418 indicate the presence of approximately 2 copies of the *cryIA(c)* and *bar* genes and a partial copy of the *pinII* gene. Probing for other genes present on the plasmids (*bla*, resistance for ampicillin in bacteria and the ColE1 bacterial origin of replication) used to transform this corn line confirm that these genes are present at approximately 4 copies per genome as expected from the double copies of the *cryIA(c)* and *bar* gene would predict (Appendix III).

The results of probing the various genomic digests of DBT418 also indicated the single *pinII* gene present was an incomplete copy and one of the *bar* genes was rearranged. The *bar* rearrangement was indicated by the larger than expected size of one of the positive fragments. The *pinII* gene was also found on a larger than expected fragment indicating the loss of one endonuclease recognition site and spurred further analysis.

The further analysis involved both use of the *AdhI* intron I as a probe and a series of with the *pinII* gene PCR primers to detect what area of the gene was missing. The *AdhI* intronI probe indicated the presence of the native *Adh* gene on a high molecular weight fragment and another copy found only in transformed DBT418 and not in the non-transformed control DNA. The PCR priming experiment showed that only one of the PCR products was generated in DBT418 DNA and this was larger than expected for the section primed. These results indicate that there was a major loss within the *pinII* gene and that a functional product was not capable of being produced from this insert. (Appendix IV)

To demonstrate stability of the inserted *cryIA(c)* gene, DNA samples from 190 plants during the elite selection process. The DNA from these plants was extracted and probed for the presence of the *cryIA(c)* gene. 184 of the 188 plants (97.9% of valid samples or 96.8% of all sampled) that gave an acceptable DNA

extraction and digestion yielded fragments of the expected size providing data to support the contention that the *cryIA(c)* insert is stable through the breeding process. This percentage is stated as being an expected stability for a single trait encountered during the breeding process. (Appendix V)

It is important to note how Dekalb calculated the DNA amount attributable to the genome in the extracts and how corrections for mitochondrial and chloroplast DNA were made. Dekalb claimed that individual corn lines can vary as much as 37% for genome size and that 34% of the extracted DNA can be assumed to be from mitochondria and chloroplasts. Assuming that similar numbers are to be found in other crops, this emphasizes the wide variation to be assigned to gene copy determinations.

BPPD review dated 11/14/96

Page ____ is not included in this copy.

Pages 18 through 22 are not included.

The material not included contains the following type of information:

____ Identity of product inert ingredients.

____ Identity of product impurities.

____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

____ Sales or other commercial/financial information.

____ A draft product label.

____ The product confidential statement of formula.

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✓ FIFRA registration data.

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The information not included is generally considered confidential by product registrants. If you have any questions, please contact the individual who prepared the response to your request.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD

Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD

STUDY TYPE:	Product Characterization
MRID NO:	439995-05
CHEMICAL NO:	006430
TEST MATERIAL:	DNA from Corn Line DBT418
STUDY NO:	DGC-95-A14
SPONSOR:	Dekalb Genetics Corp., Mystic, CT
TESTING FACILITY:	Dekalb Genetics Corp., Mystic, CT
TITLE OF REPORT:	Demonstration of Stable Mendelian Inheritance of *cryIA(c)* and *bar* Genes in DBT418
AUTHOR:	David Walters
STUDY COMPLETED:	April 5, 1996
CONCLUSION:	The results of PCR testing for one early generation, three late generation and non-transformed genotypes indicate that the transgenes *bar* and *cryIA(c)* segregate as closely linked traits and in a 1:1 fashion when crossed with non-transformed lines. Such tight linkage stably maintained during several backcross generations indicate that the *cryIA(c)* and *bar* genes were probably inserted at the same location during the transformation process.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

The *cryIA(c)* and *bar* genes were examined by PCR in several backcross generations with elite lines to determine if the inheritance of these traits followed typical Mendelian patterns.

TEST METHODS

Test substance: DNA analysis was performed on the following corn genotypes resulting from crosses with line DBT418: AW/BC5/DBT418, BS/BC5/(AW.DBT418), DK/BC6/(AW.DBT418) and DBT418(AW)08(aBK). Genomic DNA from these lines was isolated and digested with the NcoI endonuclease and reacted with a *cryIA(c)* gene probe. Three plants from each genotype were tested. For the reference substance DNA from corn lines were selected by Dekalb. All lines were assessed for germination rate to determine stability and

rates ranged from ≥ 82% for the test crosses to ≥ 94% for the reference lines.

Reference substance: In addition to the plant samples mentioned above, plasmids pDPG165 and pDPG699 containing the *cryIA(c)* and *bar* traits, respectively, were use as a source of the genes of interest. First, the plasmids were digested with the appropriate restriction enzymes to yield the fragments of choice: for pDRG165 a 2000 bp HinDIII/EcoRI fragment containing the *bar* gene with a 35S promoter and a Tr7 3' termination sequence; and for pDPG699 a 3200bp SalI/NotI fragment with the *cryIA(c)* gene with an AdhI intron VI and a pinII 3' termination sequence. These plasmids were used as a positive control to verify PCR functionality.

Test method: Seeds from 19 hybrid corn lines were grown to provide leaf tissue 12-13 days post-planting. The hybrids were chosen to provide materials from both earlier and later in the elite line development process. Leaf tissue was sampled from 48 seedlings from each line containing DBT418 and 24 seedlings from non-transformed lines. A one to three inch section was taken from the youngest leaf not showing signs of etiolation, wilting or other damage. These samples were collected and extracted for DNA as described (MRID 439995-04). The PCR reaction mixtures were prepared as described and the primer sequences used (BTSn64G/BTAsn506; *bar3/bar5* and AdhVI-U/AdhVI-L) were as listed (MRID 439995-04). PCR reaction mixtures were sampled and run on an agarose gel for the presence of a product of the expected length. A fragment of the expected length was deemed to be positive; no fragment was deemed to be a negative.

RESULTS AND DISCUSSION

The results of PCR testing for one early generation, three late generation and non-transformed genotypes indicate that the transgenes *bar* and *cryIA(c)* segregate as closely linked traits and in a 1:1 fashion when crossed with non-transformed lines. (Appendix I) These results from the four hybrid corn lines are those expected from traits inherited in a normal Mendelian manner. The PCR results from priming with the *Adh* intron indicate that the hybrid lines not found to generate PCR fragments to *bar* and *CryIA(c)* were still fully capable of being used in the PCR format. All the corn lines tested positive for the *Adh* intron. The transgene carriers gave the additional

positive results for testing with the *cryIA(c)* and *bar* indicating they possessed these genes.

There were six plants that gave equivocal PCR results indicating the *bar* and *cryIA(c)* genes were segregating. These plants were re-examined and five of the six indicated either the presence or lack of both genes in question. One plant with the DBT418(AW)08(aBK) genotype showed the *cryIA(c)* trait but lacked the *bar* gene. This was theorized to be a result of a rare recombination event during the breeding process. Such tight linkage stably maintained during several backcross generations indicate that the *cryIA(c)* and *bar* genes were probably inserted at the same location during the transformation process.

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DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD

Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD

STUDY TYPE:	Product Characterization
MRID NO:	439995-07
CHEMICAL NO:	006430
TEST MATERIAL:	Microbial and plant produced CryIA(c) protein
STUDY NO:	DGC-95-A19
SPONSOR:	Dekalb Genetics Corp., Mystic, CT
TESTING FACILITY:	Dekalb Genetics Corp., Mystic, CT
TITLE OF REPORT:	Characterization of the CryIA(c) Protein from Transgenic Plants and Demonstration of Equivalence to Microbially Produced CryIA(c)
AUTHORS:	Robert D. Millham, Clayton S. Vetsch & Donald S. Walters, Ph.D.
STUDY COMPLETED:	April 19, 1996
CONCLUSION:	Data from western blots indicates the plant expressed CryIA(c) comigrates and is recognized by specific antibodies to tryptic core of bacterial CryIA(c). N-terminal amino acid sequencing showed similar sequences between the bacterial tryptic core and DBT419 plant expressed CryIA(c) except that the plant sequence starts at glycine 26 rather than the expected methionine 1. No detectable glycosyl residues could be found in either the DBT418 or bacterial preparations even though other proteins did test positive for carbohydrates in the extracts. Bioassays establishing the similar activity of plant and microbial CryIA(c) were cited but no results could be confirmed.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

CryIA(c) protein isolated from DBT418 corn plants and *Bacillus thuringiensis* were purified and characterized for molecular weight, N-terminal amino acid sequence, immunorecognition, glycosylation and bioactivity to demonstrate the equivalence of the protein from the two sources.

TEST METHODS

Test and reference substances: Corn leaf tissue (line AW/BC2/DBT418.BS/BC1/DBT418(2Bt)) was used as source of the test substance. Corn leaf control substance was from line DK.DL, a non-transgenic line. The reference substance for CryIA(c) protein was obtained from a *B. thuringiensis* strain (ATCC 35866).

Extraction and enrichment: Approximately 10kg of leaf tissue was frozen and extracted in 30L of carbonate buffer (50mM carbonate, 1.5% polyvinylpolypyrrolidine, 1mM phenylmethylsulfonylfluoride, 5μg/ml leupeptin, 5mM dithiothreitol, pH 9.5). The leaf extraction process was done in small batches by homogenization of 1kg leaf material with 3L of buffer. The extract was filtered through Miracloth and double cheesecloth and centrifuged to remove gross cellular debris. The supernatant was treated with polyethylenimine (0.15%), stirred 10-30 minutes then centrifuged for 20 minutes at 12,000g. Ammoniun sulfate was added to the supernatant until 35% saturation was reached and kept at 4°C from 2 hours to overnight. The protein enriched precipitate was pelleted by centrifugation at 12,000g for 30 minutes and stored at -10°C until needed.

Further purification for AA sequencing: The protein enriched pellet from the above process was further purified to obtain a protein pure enough for sequence determination. The pellet was resuspended in carbonate buffer (as described above with 14mM mercaptoethanol replacing the DTT) followed by centrifugation to remove the insoluble material. The supernatant was dialyzed overnight against phosphate buffered saline (pH 7.4) then analyzed for protein (BCA assay) and CryIA(c) content (ELISA). The extract was further purified by reacting the mixture with an anti-CryIA(c) monoclonal IgG antibody then Protein-G sepharose beads (1μl beads/3μg IgG). This suspension was then centrifuged to remove the beads and the process repeated two to four times again with the addition of more IgG antibody depending on the amount of CryIA(c) left in the solution (as determined by ELISA). The protein-G pellets were washed three times in PBS followed by the addition of 50-75μl of 100mM glycine (pH 2.5) to elute the CryIA(c)/antibody complexes. The beads were removed by centrifugation, the final Ag/Ab complex was concentrated by Spin-X 0.22μm filters and amount of CryIA(c) determined by western blot. The complexes were dialyzed against CAPS (pH 10.5), frozen and concentrated to <10μl using a Speed-Vac. Pooled eluates were boiled in 3X SDS sample buffer without BME and subjected to an 11% PAGE. The gels were electroblotted to a PVDF membrane and

stained to visualize all protein bands. Bands corresponding to the reference standard CryIA(c) were cut out with a razor blade, destained and washed with deionized water. The bands were then dried and submitted for Aa sequencing.

Amino acid composition and N-terminal sequencing: Sample tubes for composition analysis were pyrolyzed at 520°C overnight before receiving the membrane bound sample (~10% of total sample). The samples were dried, 200µl of 6N HCl/0.1% phenol were added and the vials purged with argon prior to hydrolysis for 1 hour at 150°C. Excess HCl was removed by vacuum evaporation and the PVDF membrane extracted with two washes 0.1M HCl/20% methanol. The extract was evaporated, redissolved in 0.5% EDTA and loaded onto a derivitizer (Perkin Elmer PE/ABD 420A). The AA derivatives were analyzed on a Shimadzu CR4A Chromatopac system. For AA sequence analysis, the samples were subjected to an automated Edman degradation process in the Perkin Elmer PE/ABD 470A protein sequencer. The reaction chamber was washed with methanol, charged with the PVDF membrane and run on gas phase chemistry to generate the phenylthiohydantoin amino acid derivatives.

Glycosylation determination: The CryIA(c) proteins were analyzed by an Amersham ECL™ Glycoprotein Detection kit. This system uses a periodate oxidation reaction to produce a reactive aldehyde residue in any carbohydrate residues present which are subsequently reacted with biotin hydrazide. The biotinylated glycoproteins are separated on SDS-PAGE and the biotin present is detected by the binding of a streptavidin-HRP conjugate and visualized with the ECL system. Modifications to this system for use with the DBT418 produced CryIA(c) include the use of the immunoprecipitated form of the protein. This was pooled and dialyzed against a 100mM acetate buffer. After dialysis the amount of CryIA(c) was quantified by western blot then concentrated and reacted with the periodate reagents and analyzed for the presence of glycosyl residues on the a PVDF membrane after SDS-PAGE. The blots were split after running and one half treated for glycoprotien determination the other half treated to a typical western blot for CryIA(c).

Biological activity: The microbially produced CryIA(c) protein (CH-95-03) was assayed for insecticidal activity against tobacco hornworm (*Manduca sexta*). The neonate larvae were raised on artificial diet with five different concentrations of CryIA(c) applied to the diet surface in 24 well plates. The exposure

levels ranges from 2.8ng/1.88cm^2 to 45.0ng/1.88cm^2 and thirty individual 1-2 day old larvae were exposed to each dose level over five days. The results were plotted by probit analysis using a POLO-PC program for an LC_{50} determination.

RESULTS AND DISCUSSION

The expected molecular weight of the CryIA(c) protein expressed in DBT418 corn was about 66kD from AA sequence estimation. This form of the CryIA(c) protein represents the trypsin resistant core with an additional 28 amino acids at the N-terminal trypsin recognition site and apparently ending at glutamic acid 613 rather than the full-length 130 kD CryIA(c) protoxin. The results of western blot assays show positively stained bands migrating with a molecular weight of slightly less than the 66.2kD molecular weight standard. The bands from both the DBT418 plant preparations and CryIA(c) from *B. thuringiensis* (CH-95-03) comigrated indicating they were the same molecular weight and immunoreactivity.

The AA composition results were not reported except to say they matched close enough to proceed with the AA sequencing. (Sequencing results found in Appendix I) The results from two separate corn transformations (DBT418 & DBT419) with the same plasmid are presented. Both results show sequences that start at amino acid 26 rather than amino acid 1. The sequence results are also equivocal for DBT418, most probably due to significant contamination by the monoclonal antibody used during CryIA(c) purification. The analysis for line DBT419 shows exact correlation to the microbial sequence (starting at amino acid 26 and continuing to amino acid 33). There were no differences between the extraction processes used for the two corn lines except for the inclusion of a trypsin digest in the DBT419 preparation prior to the immunoprecipitation step.

Two points can be made for this study:
1) use of a trypsin digest during purification greatly reduces contamination by other proteins;
2) the CryIA(c) expressed in corn by this plasmid appears to start at amino acid glycine 26. This is probably due the action of a inherent plant protease after toxin synthesis but this possibility cannot be distinguished from some result of the purification process at this time. The results from the microbial CryIA(c) protein show that the species of protein found

in plant preparations is not due to trypsin cleavage since the expected trypsin cleavage product is formed in the microbial preparation.

The CryIA(c) protein does not display a positive staining reaction for the presence of glycosyl residues. There were no positive bands in the blot probed for the presence of carbohydrate that comigrated with the CryIA(c) protein band from an immunoprobed blot. There were, however, bands that were positive in both the bacterial and plant CryIA(c) protein extracts. Some of these positive glycoprotein bands comigrated with the mouse IgG bands from the plant purified CryIA(c) as would be expected. The positively stained bands in the bacterial extracts did not comigrate with any detectable protein bands from Ponceau S stained SDS-PAGE preparations. The non-oxidized preparations showed no positive carbohydrate bands in the expected regions indicating the positive results were indeed from carbohydrate recognition.

The results of the bioassay against tobacco hornworm (*Heliothis virescens*) were not presented. The company claims probit analysis of the results of CryIA(c) from DBT418 corn lines gave a significant slope on the dose response and the LC_{50} value for the two assays combined was 2.4 ng/cm^2, identical to the published values for this toxin. The data from these assays were presented in MRID 439995-10 (reviewed in memorandum from D. Horne to M. Mendelsohn, 9/12/96) and it is difficult to confirm the company's assertions from the data presented. Only one dose level of CryIA(c) was employed in these tests, no positive CryIA(c) controls were included and conversion from diet incorporation of lyophilized leaf tissue to the cited surface contamination values is not evident.

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DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JLK

Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD RDS

STUDY TYPE:	Product Characterization
MRID NO:	439995-08
CHEMICAL NO:	006430
TEST MATERIAL:	Microbial and plant produced PAT protein
STUDY NO:	DGC-95-A20
SPONSOR:	Dekalb Genetics Corp., Mystic, CT
TESTING FACILITY:	Dekalb Genetics Corp., Mystic, CT
TITLE OF REPORT:	Characterization of the phosphinothricin Acetyltransferase (PAT) Protein from Transgenic Plants and Demonstration of Equivalence to Microbially Produced Phosphinothricin Acetyltransferase
AUTHORS:	Lucille B. Laccetti, Whitney R. Adams, Judith E. Nutkis, Robert D. Millham & Donald S. Walters, Ph.D.
STUDY COMPLETED:	April 19, 1996
CONCLUSION:	PAT derived from DBT418 plants, from *E. coli* as a histidine-TAG PAT form and from *E. coli* as a histidine-TAG PAT digested with thrombin migrate with similar, but not exact, mobilities and are all immunoreactive with anti-PAT rabbit antibodies. The histidine-TAG PAT has only 88% of the enzymatic activity of the plant extracted form. The amino acid analysis of the three forms yielded the expected sequence for each form save the initial methionine in the plant sequence.
CLASSIFICATION:	Acceptable.

STUDY DESIGN

Biochemical and other tests were used to demonstrate the similarity of *E. coli* and plant produced phosphinothricin acetyltransferase (PAT) proteins. These tests include immunoreactivity, presence of carbohydrate residues, amino acid sequence, molecular weight and enzymatic activity.

TEST METHODS

Test substance: PAT was isolated from corn line DBT418 using the method described in MRID 439995-07 up to the 35% ammonium sulfate precipitation step. At this point the CryIA(c) protein had been precipitated and the supernatant still contained the PAT protein. PAT was further purified from this supernatant as described later in the test substance purification. The reference substance (CH-95-06) was PAT purified from an *E. coli* production system. This form of the enzyme was generated as a "histidine-TAG" labelled PAT protein from plasmid pDPG675. The extra 20 amino acids at the amino-terminal end of the PAT protein, including six consecutive histidine residues, was added to facilitate purification. The microbial product could be readily removed with a nickel resin in an affinity column. The PAT protein itself would be released from the histidine-TAG by the action of thrombin protease (Novagen pET system).

Purification of PAT from DBT418 corn plants: After the CryIA(c)-containing pellet was removed, additional ammonium sulfate was added to the supernatant to yield 40% saturation. This solution was centrifuged, the pellet discarded and the supernatant having additional ammonium sulfate until 65% ammonium sulfate saturation was obtained. The 40-65% pellet from centrifugation was retained frozen until further fractionation was needed. The first steps of further purification were dissolution and dialysis against 20mM Tris-HCl, pH 8.0 followed by fractionation over a Q-sepharose HP column (0-1000mM KCl gradient over 460 ml) into 4ml volumes. The 4ml fractions were desalted using Centriprep-10 concentrators and portions removed to characterize the fraction by SDS-PAGE. Duplicate fractions were run, blotted and one blot probed with anti-PAT rabbit polyclonal antibody, the other blot stained with Ponceau S for general protein levels. Examination of the fractions indicated that fraction 52 at approximately 336mM KCl had the highest anti-PAT antibody binding and least interference from other proteins. The blot for this fraction was cut out, washed and sent for microanalysis.

Immunoreactivity and molecular weight: Western blots were done with PAT derived from DBT418 plant extracts, fraction 52 described above and the histidine-TAG labelled form produced by *E.coli*. Molecular weight standards and a non-transgenic plant control extract along with the samples were run on the SDS-PAGE then electroblotted onto nitrocellulose membranes. The membranes were blocked with PBS/Tween 20/powdered non-fat dry milk then

reacted with the following reagents: anti-PAT rabbit polyclonal antiserum, goat anti-rabbit conjugate with horseradish peroxidase followed by an ECL detection system.

Amino acid composition, N-terminal sequencing and glycosylation: The processes used to analyze is the same described for the sequencing of CryIA(c) found in MRID 439995-07.

PAT activity determinations: The enzymatic activity was monitored by the catalysis of ^{14}C-acetyl CoA and phosphinothricin to yield a ^{14}C-acetylated PAT. Plant extracts (using 50mM Tris HCl, 2mM EDTA, 0.15mg/ml leupeptin and PMSF, pH 7.5) were prepared, centrifuged and the supernatant used for activity determinations. The reaction mixture (25µl total) consisted of a diluted plant or microbial PAT prep (in 50mM Tris, 2mM EDTA, 0.1mg/ml BSA, pH 7.5) with 1.0µg of unlabelled acetyl-CoA, 0.792µg phosphinothricin and 60nCi of ^{14}C acetyl-CoA (1nMole). The reaction was started with the addition of radiolabelled acetyl-CoA, continued for 5 minutes at 35°C, then a 20µl sample was spotted onto a silica gel TLC plate. The reaction products were separated using a ammonium hydroxide/1-propanol (2:3) solvent then the plates were air-dried and exposed at least 2 hours to X-ray film for autoradiography. The films were evaluated by scanning densitometry. An evaluation was made comparing the ^{14}C disintegrations to the scanning results to generate a standard curve. PAT activity was also analyzed for the preparations used for sequencing and microbially-produced PAT. The PAT protein amounts were determined from western blot analyses.

RESULTS AND DISCUSSION

Western blot analysis indicates that the major immunoreactive protein in DBT418 corn extracts comigrates with histidine-TAG PAT and histidine-TAG PAT digested with thrombin and all have a molecular weight greater than 22 kD but less than 30 kD. The expected molecular weight of PAT as expressed in DBT418 is 23 kD with the additional amino acids due to the histidine-TAG adding 1.9 kD and the product of thrombin digestion being approximately 23.4 kD. The banding pattern and relative weights of the protein samples tested confirm these predictions. The non-transgenic plant extract showed no immunoreactive bands. An additional immunoreactive band (~50kD) was present in the DBT418 extracts and the thrombin digested histidine-TAG PAT preparations.

Enzymatic activity assays showed that the PAT found in DBT418 plant extracts and that purified from microbial production had similar levels of activity. This similarity is based on the intensity of ^{14}C labelled phosphinothricin product found in autoradiographs. The company states that in the absence of either substrate no radioactive product is formed. However, there is a low level of background activity (~4%) that could be attributed to the action of general plant acetyltransferases. Additionally, while no data was presented the company claims microbial histidine-TAG PAT has less activity (~88%) than PAT isolated from plants.

The amino acid composition data was not presented but it was claimed to be similar enough to the expected to proceed with the sequencing analysis. The sequence data presented indicates the amino acids obtained for the histidine-TAG PAT, the thrombin digest of histidine-TAG PAT and the DBT418 plant extracts were as expected (appendix attached). The DBT418 PAT does not show the original methionine expected for the sequence introduced. This is a common phenomenon in plants where the initial methionine is cleaved after translation.

The glycoprotein detection assay did not show any carbohydrate positive band present where the PAT protein migrated. The spectrum of glycoproteins present in the DBT418 plant extracts was different from that found in the CryIA(c) extracts (seen in MRID 439995-07). This difference was probably due to the increased proportion of inherent plant glycoproteins in these extracts compared to the amount among the higher weight proteins in the CryIA(c) extracts. None of the non-oxidized preparations showed any positive carbohydrate staining as would be expected since the carbohydrate residues require periodate oxidation to react with subsequent reagents.

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