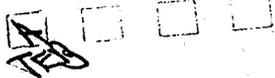


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DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist *MTW*

Secondary Reviewer: John L. Kough, Ph.D., Biologist *JLK*

STUDY TYPE:	Safety Assessment	REVISED
MRID NO:	447140-01	DEC 21 1999
TEST MATERIAL:	StarLink™ Genetically Modified Corn	
PROJECT NO:		
SPONSOR:	AgrEvo USA Company, Wilmington, DE	
TESTING FACILITY:	AgrEvo USA Company, Wilmington, DE	
TITLE OF REPORT:	Safety Assessment of Starlink™ Genetically Modified Corn Containing the Truncated <i>Bt</i> Insecticidal Protein Cry9C for Human Food Use	
AUTHOR(S):	Sally Van Wert, Ph.D.	
STUDY COMPLETED:	November 20, 1998	
CONCLUSION:	This data package provides a summary of data and information obtained by AgrEvo regarding the characterization and safety of the Cry9C protein, and the Cry9C-expressing StarLink™ corn. The data and other information provided does not provide a conclusive argument about the allergenicity potential of the Cry9C protein. The protein does share some characteristics with known allergens, however, it lacks others. Further data/justification should be provided as indicated in the review of each data package contained in this submission, and referenced in this review. Based upon the information provided in this summary, no definitive decision can be reached regarding the allergenicity potential of the Cry9C protein.	
CLASSIFICATION:	SUPPLEMENTAL. This report is a summary of data and other information submitted by AgrEvo and does not provide additional information not found in the data packages submitted in support of this application.	
GOOD LABORATORY PRACTICE:	This study is not subject to Good Laboratory Practice standards.	

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I. SUMMARY**Background:**

StarLink™ Corn has been genetically modified to express both the Cry9C and phosphinothricin acetyltransferase (PAT) proteins. The Cry9C protein is expressed via a modified *cry9C* gene isolated from *Bacillus thuringiensis* subsp. *tolworthi*. The PAT protein (confers resistance to glufosinate ammonium herbicides) is encoded for by the *bar* gene from *Streptomyces hygroscopicus*. The PAT protein has been previously tested and is not the primary concern of this report. The focus of this report is the safety of the Cry9C protein, as it is expressed in the StarLink™ corn plants.

The Cry9C protein has 1157 amino acids with the insecticidal fragment contained between amino acids 44 and 658. The truncated endotoxin consists of approximately 626 amino acids encoding for a crystal protein of approximately 70 kDa. In Cry9C expressing plants, the protein is not found in its normal crystalline form, but as a free protein in plant cells. The mode of action of this toxin, as with other Bt proteins, is mediated by receptor binding in the insect gut.

However, unlike other *Bt* proteins used in transgenic plants, Cry9C is significantly more stable. The protein was stable at temperatures up to 90° C and remained intact following 4 hours in simulated mammalian gastric juice (*in vitro*). It is these two characteristics which have raised concerns regarding the safety, specifically the food allergenicity of this protein if it was part of the human diet.

This submission consists of reviews of tests performed for or by AgrEvo on the Cry9C protein and/or the StarLink™ corn. In addition, there are literature citations of studies relating to these topics and comments/assessments made by scientists with knowledge in these subject areas. This safety assessment for the protein and plant line includes of:

1. Characterization of the parent corn
2. The transformation process
3. Characterization of the gene product (i.e. Cry9C protein)
4. Characterization of the genetically modified (GM) corn

Through these four subject areas, AgrEvo attempts to show that their StarLink™ Corn line is "substantially equivalent in all essential aspects" to its unmodified parent. The information and data provided includes a description of the protein and summaries of: human health effects testing; insecticidal activity; *in vitro* binding of the Cry9C protein; potential food allergenicity; protein stability; immune response to the Cry9C protein; and levels of expression of the protein in the CBH-351 line of corn.

Summary of Data:**A. Characterization of the Transformation Process for StarLink™ Corn (CBH-351):**

1. Source of *cry9C* and *bar* Genes - The CBH-351 transformation event contains three foreign genes: the *cry9C* gene modified from the insecticidal crystal gene of *B.*

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thuringiensis subsp. *tolworthi*; the *bar* gene from *Streptomyces hygroscopicus*; and the *bla* marker gene (confers resistance to ampicillin).

2. DNA Insert, Sequence, and Purity - C

] A series of Southern analysis have shown that one copy of the *cry9C* gene and four copies of the *bar* gene have been inserted at a single site in the corn genome.

3. Pleiotropic Effects - Expression of the *cry9C* gene occurs in all major tissues of the corn plant susceptible to European corn borer. This was accomplished by linking the gene to a constitutive and high expression promoter.B. Production and Safety Characterization of Cry9C Protein:1. Protein Structure - The bacterial *Cry9C* protein has 1157 amino acids, with the insecticidal fragment contained between amino acids 44 and 658, encoding for a protein of 70 kDa. The *Cry9C* protein *in planta* is free in the plant cells, not in crystalline form.2. Insecticidal Activity of Cry9C Protein in Mammals - Mode of Action - The mode of action of *Cry9C*, as with other *Bt* proteins, is mediated via receptor binding in the gut in insects. Further testing was performed to determine how this correlates with any *Cry9C* protein activity in mammals. This is discussed further in Section C below.3. In vivo Binding of Cry9C Protein to Insect GI-Tract Tissues - Biological Target Specificity - Previous studies with the European corn borer have shown that the crystal *Cry9C* protein recognizes a receptor, different from that recognized by the *Cry1Ab5* protein. It has also been shown that elimination of the trypsin cleavage site, which is responsible for the degradation from a toxic 70 kDa protein to the non-toxic 55 kDa protein (resistant to protease digestion), did not increase the toxicity in the target insect.C. In vitro Binding Potential of the Cry9C Protein to the GI-Tract Tissues of Mammals:1. In vitro - A study included in this same data package (MRID# 447343-01) investigated the binding of the *Cry9C* protein to the brush border membrane vesicles (BBMVs) from the midgut of European Corn borer and the BBMVs from mouse intestinal preparations. In this submission, AgrEvo indicates that they believe that addition of labelled *Cry9C* resulted in displacement of the labeled *Cry9C* in the insect BBMVs, but did not result in displacement of the labeled *Cry9C* in the mouse BBMVs. Therefore, according to AgrEvo, this would indicated that *Cry9C* binds specifically and saturably to the European Corn Borer BBMVs and not to the mouse intestinal BBMVs. However, the figures included in this submission are of very poor quality and it is not possible for EPA to reach the same conclusion. If adequate pictures are available, further justification for the belief by AgrEvo that displacement is occurring in this case should also be provided. Supplementary

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figures have been requested (as described in the review of MRID# 447343-01) for further analysis of these results.

2. In vivo - A 30-day mouse toxicity study was included in this submission under MRID# 447343-03.

D. Evaluation of the Potential for Mammalian Toxicity with the Cry9C Protein in Mice:

1. Acute Oral Toxicity - The acute oral toxicity of the Cry9C protein has been previously assessed under MRID# 4425810-07 (Kough to Mendelsohn, 1/6/98). The data from this study indicated that there were some clinical signs due to the dosing, but no deaths at a dose above the limit dose of 5000 mg/kg bodyweight.
2. Acute Intravenous Toxicity - An acute intravenous toxicity study was also included in this submission under MRID# 447343-02. The data in this study indicated that mice dosed at 0.3 mg/kg body weight did not produce any apparent adverse effects in the mice tested.
3. Subacute Oral Toxicity - A 30-day mouse toxicity study was included in this submission under MRID# 447343-03.

E. Potential for Allergenicity:

1. Molecular Weight - The Cry9C protein has a molecular weight of 68.7 kDa and AgrEvo claims is potentially glycosylated post-transcriptionally (although no data was provided, AgrEvo indicates in the submission that no glycosylation was measured in either the plant or bacterial produced Cry9C). According to AgrEvo, the majority of food allergens have a molecular weight between 10 and 40 kDa and are often glycosylated.
2. Sequence Homology - The minimal number of amino acids from an allergenic epitope needed to elicit an immunological response in a sensitized individuals serum appears to be between 8 and 12. No match between any 8 amino acid sequences in the Cry9C protein and any known allergenic proteins in the SWISS protein database, (previously reviewed: MRID# 443844-04 - Kough to Mendelsohn, 4/8/98) or the other available databases was found.
3. Stability to Digestion - Stability to digestion is another characterization common to known allergens. Purified Cry9C has been tested *in vitro* in simulated mammalian gastric fluid (previously reviewed: MRID# 442581-08 - Kough to Mendelsohn, 1/6/98). The samples of lyophilized Cry9C protein expressed in corn showed no signs of protein disintegration when subjected to simulated human gastric conditions. These digestions were done either with or without pepsin in low pH buffer and assayed by Western blot on samples taken up to 4 hours after exposure.
4. Stability to Heat and Processing - The Cry9C protein was shown to be stable at a temperature of 90° C for 10 minutes, without altering the toxicity to the target insect (previously reviewed: MRID# 442581-08 - Kough to Mendelsohn, 1/6/98).

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F. Immunological Response:

1. **Animal Model** - The Brown Norway Rat has been chosen by AgrEvo as a surrogate for testing the potential immune response in humans to Cry9C. This animal has been identified as a high IgE responder. The production of an IgE antibody response to a food antigen is part of the atopic syndrome in food allergies. This rat has been used in a study included in this submission (MRID# 447140-02).
2. **In Humans** - AgrEvo indicates that many workers (1990) have been involved in seed production, planting, harvesting, and processing the corn expressing Cry9C over a period of time (since 1996). No cases of sensitization or allergic response have been reported for these workers. In addition, as previously reviewed (MRID# 443844 - Kough to Mendelsohn, 4/8/98), 21 sera samples from suspected corn-sensitive individuals were screened with corn seed extracts from Bt Cry9C corn (CBH-351). The sera were assayed for specific IgE to aqueous wild-type or transgenic corn allergens by Radio Allergo Sorbent Test (RAST). All of the sera tested positive in the RAST assay by having a $\geq 3\%$ reactivity. The transgenic and wild-type aqueous extracts were not obviously different in responsiveness for individuals and a t-test of the RAST % reactivity did not reveal any significant differences.

G. Levels of Expression:

Included in this submission is a study which investigates the levels of Cry9C expression in corn, and various processed commodities (MRID# 447343-03). Unfortunately, the control corn line grown in the same location as the test line showed positive signals for both the Cry9C and PAT proteins. Because of this contamination, the results of this study are questionable. However, the study indicates here that the typical values of Cry9C in corn ears are about 0.5% of total protein.

H. Bioavailability:

The bioavailability of the Cry9C protein was examined (MRID# 447343-05) in a single dose gavage study in the rat, where blood samples were removed over an 8 hour period. Both ELISA and Western Blot analysis were used for detection of the Cry9C protein. The author of the study concluded that very small traces of Cry9C-like material was detected in the blood at the top dose. The report indicated that of the 298 mg/kg body weight dose, between 0.0002 and 0.0006% was absorbed (these values were either on or below the reported limit of detection of the assay). The identity of the Cry9C-like material could not be confirmed by Western Blot analysis.

Also included in this study were tests designed to confirm that the Cry9C protein is degraded in the intestinal system of the rat to a protein of 55 kDa and that gut bacteria may play a role in part of this degradation. AgrEvo indicates in this report that this result casts doubt over the importance of the *in vitro* (i.e., Cry9C stability *in vitro*) results and provides strong evidence for *in vivo* degradation of the Cry9C protein. However, BPPD does not necessarily agree with this interpretation. It may

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be true that the protein is broken down from a 68 kDa protein to a 55 kDa protein, but there is no additional evidence that the protein is further degraded. The apparent stability of the 55 kDa protein is not addressed, and therefore continues to pose an allergenicity concern.

I. Wholesomeness:

Poultry Feeding Study/Wholesomeness - An assessment of the wholesomeness of the GM corn versus control corn was conducted in chickens (S. Leeson, 1998). Chickens were separated into groups and received a diet of either the control corn or corn containing Cry9C at 65% (w/w). The chickens were weighed weekly and observed for clinical signs for up to 42 days. Results showed no adverse effects on feeding, growth, or clinical condition of the animals.

Irritancy - AgrEvo cites two studies (D.A. Douds, 1997; H.P.M. Noteborn, 1998) which indicated that mice in an acute oral study and a 30-day mouse repeat dose study did not show any evidence of irritation in their gastrointestinal tracts.

J. Consumer Safety:

AgrEvo indicates that there are no known reports of adverse incidents to man exposed to the Cry9C protein in seed, plant or bacterial form from occupational exposure. The company further attempts to calculate a Theoretical Maximum Daily Intake (TMDI) for the Cry9C protein. The calculation is based on the possible content of the Cry9C protein in various corn-derived commodities and on the maximum measured levels of Cry9C in processed grain. The global TMDI was 0.00293 and the European TMDI was 0.00193 mg/person/day. The acceptable daily intake or reference dose figure of 0.3 mg/kg/day was derived from the NOEL (i.e., 30 mg/kg/day) in the 30-day mouse study. Considering the calculated TMDI, there is a margin of exposure of approximately 3000.

II. DISCUSSION

The information provided in this submission is intended to support the belief by AgrEvo that the Cry9C protein, and the StarLink™ corn plants expressing this protein, do not pose a significant risk to human health. Some of the data and information provided by AgrEvo is compelling, and supportive of the belief of no significant risk. However, there is at least an equal amount of data and information that is either inconclusive, or indicates that Cry9C exhibits some characteristics similar to that of known food allergens.

Data which is supportive of the non-allergenic nature of the protein include its lack of amino acid sequence relatedness to other known allergenic proteins and its level of expression/titer in transgenic plants. Although there are no absolute characteristics which are definitive for allergenicity potential, taken by themselves, these characteristics do not necessarily raise concern about the protein. However,

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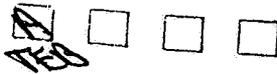
the stability of the protein at high heat (90° C) and in simulated gastric juice, do raise concerns about the protein.

Much of the remaining information provided in this submission is inconclusive. The data from the field trials (MRID# 447343-04), where the control samples showed the presence of the Cry9C and PAT proteins, makes this study supplemental at best. Because no explanation was provided for the problems with the control, it casts some doubt on the results of the test samples and the subsequent control samples used for the validation study. The protein binding study (MRID# 447343-01) is also supplemental because the figures included in the submission are not adequate to make a determination about the results of the study. The acute intravenous toxicity study (MRID# 447343-02) is an acceptable study, and indicates that there is no apparent toxicity from the protein dosed at 0.3 mg/kg body weight. However, this only provides further support to the conclusion reached in review of previous toxicity studies, that the protein is apparently not toxic.

Given the acceptable status of the intravenous study, and assuming the other two studies prove to be acceptable, they do not support a conclusive case that the Cry9C protein is not a potential allergen. There is not overwhelming evidence to indicate that Cry9C is a potential allergen, but there is not enough evidence to indicate that it is not. Included in this submission is: "An Expert Assessment of the Allergenic Potential of StarLink™ Corn", written by Dr. Andrew Cockburn. Dr. Cockburn concludes that, "StarLink™ corn is safe in all aspect as the unmodified corn used today". Further, a study by BIBRA International entitled: Development of New Methods of Safety Evaluation of Transgenic Food Crops, is also enclosed. The objective of this study is to investigate the potential immunogenicity and allergenicity of recombinant novel food proteins in transgenic tomatoes. The study indicates that the brown Norway rat, as used by AgrEvo for the Cry9C protein, is a suitable model for investigation of the allergenic components of foods. However, a letter also included in this submission, written by Dr. Samuel B. Lehrer indicates that he is skeptical about the applicability of this rat model in determining the allergenic potential of the Cry9C protein

As with much of the data and information submitted in this package, it is not possible to reach a definite conclusion regarding the allergenic potential of the Cry9C protein. Some of the data do not support the lack of allergenic potential, while other data indicates the possibility of properties similar to other allergens. Other data is basically supplementary, and does not directly address the protein, or its allergenic potential. As can be seen from the enclosed letter and reviews, even expert analysis of this protein differs. Based upon these factors, it is not possible for BPPD to determine that there is a lack of allergenic potential from Cry9C based upon the available information. Further data and clarification must be provided to aide in such a determination.

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DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD *JLK*
Secondary Reviewer: Doug Gurian-Sherman, Ph.D., Plant Pathologist, BPPD *DAS*

STUDY TYPE: Acute Oral Toxicity (152A-10) RECEIVED
 MRID NO: 442581-07 DEC 21 1997
 CHEMICAL NO: 006466
 TEST MATERIAL: Cry9C Protein from *Bacillus thuringiensis*
 STUDY NO: 3433.1
 SPONSOR: Plant Genetic Systems (America) Inc., Ghent, Belgium
 TEST FACILITY: Springborn Laboratories, Inc., Spencerville, OH
 TITLE OF REPORT: An Acute Oral Toxicity in Mice with Cry9C Protein as Purified
 from *Bacillus thuringiensis* Cry9C.PGS2
 AUTHOR: Deborah A. Douds, M.S.
 STUDY COMPLETED: January 11, 1997
 CONCLUSION:

There were no deaths in any test animals due to test material given above the limit dose of 5000mg/kg during the 14 day observation period. One male mouse displayed hair loss between days 2 and 5. One female displayed decreased activity on the day of dosing. Another female displayed decreased activity, wobbly gait, decreased feces and felt cool to the touch. A third female displayed decreased feces on day 1. All the male mice gained weight during the test period (except during the pre-dosing fast period). Two female mice failed to gain weight between day 0 (prefasted weight) and day 7 and three failed to gain weight between day 7 and 14. One female did not recover her pre-fasting body weight by day 14.

CLASSIFICATION: SUPPLEMENTAL. This study can be reclassified acceptable with submission of historical data demonstrating the correlation of the OD₂₈₀ and the ELISA Cry9C protein determinations or other data to verify the dose administration above 5000mg/kg to the test animals.

STUDY DESIGN

Two doses of purified lyophilized Cry9C protein were administered to mice by oral gavage within a 4 hour time span to give a final dose of 6500mg/kg bodyweight (5,525 mg Cry9C protein). The test animals were observed for clinical signs for 14 days prior to sacrifice and gross necropsy. This study has a GLP compliance statement.

TEST METHODS

Test Substance A tan powder was received from the sponsor and labeled as "Cry9C protein as purified from *Bacillus thuringiensis* Cry9C.PGS2 (purified lyophilized Cry9C bacterial extract)." The lot number was 9Ca-0196. The powder was described as at least 85% Cry9C protein. Prior

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to dosing the test material was mixed with 0.5% carboxymethyl cellulose to yield a 25%w/w final solution with a Cry9C concentration of 0.63gm/ml.

Attached as an addendum to the oral toxicity report is a study provided by PGS to characterize the test substance (study # 96QZM006). This report is also part of the "Test Substance Characterization" (MRID 442581-05) not examined in this review. The test substance is produced in a *Bacillus thuringiensis* strain 1715 cured of all native crystal plasmids and transformed with plasmid pGI9CK to allow expression of the Cry9C protein found in plants. The bacteria are grown, collected, pelleted by centrifugation then either stored as pellets at 4°C or freeze dried and stored at -20°C. Thirty grams of Cry9C bacterial powder is extracted for 4 hours at 4°C with stirring by solubilizing in 1.5mL alkaline buffer (0.05M Na₂CO₃-HCl, 0.01M DTT, 5mM EDTA, 0.1mM PMSF, pH 10) with 0.02% sodium azide. Undissolved material is removed by centrifugation (20 min at 12,000RPM in Sorvall SA-600) and the supernatant is dialyzed against 10L of 20mM TRIS/HCl, 25mM NaCl, 5mM EDTA, pH 8.6) using a 12 to 14,000 molecular weight cutoff Spectra/Por 4 tubing. The volume of the dialyzed solution is recorded and the protein concentration is determined by OD₂₈₀ reading and Bradford analysis. and an ELISA technique for Cry9C. The concentration is adjusted to 0.5mg protein/ml with dialysis buffer and the solution is subjected to a trypsin digest (0.05%w/v) for 1 hour at 37°C. The trypsin digest is stopped by addition of PMSF to a final concentration of 1mM. The volume of the digested solution is recorded and the protein concentration is determined by OD₂₈₀ reading and Bradford analysis. and an ELISA technique for Cry9C. Ammonium sulfate is then added to the pooled samples to a 77% final saturation (3M) and allowed to precipitate overnight at 4°C. The pellet is collected by centrifugation (20 min at 12,000RPM in Sorvall SA-600) then dissolved in 20mM ethanolamine/HCl (pH 9.5) and the protein concentration determined as mentioned above. The solution is dialyzed against 20mM ethanolamine twice, the protein concentration determined as mentioned above. The samples are then lyophilized, a final protein concentration is taken and the purified solution is also characterized by a Cry9C specific ELISA, SDS-PAGE, Western blot and an insect bioassay.

The results of the purification procedure described above for the bacterial powder yielded a lyophilized preparation which was 85% Cry9C by SDS-PAGE and western blot (data not presented). The LC₅₀ value was 72.9ng/ml (insect not specified, protocol not submitted) and Bradford protein was 0.89mg/ml. The ELISA (1.0mg/ml) and OD₂₈₀ (1.91mg/ml) readings for Cry9C protein concentration were not in agreement which was said to be unusual from a historical perspective comparing these assays. Due to this discrepancy, a subsample of the dosing material was passed through FPLC for further purification. The Cry9C concentration went from the original 1.0mg/ml to 1.3mg/ml which would suggest there was an interfering substance present and removed by FPLC. The dose calculation for the oral study was done using the OD₂₈₀ value rather than the original ELISA values for the non-FPLC purified material. However, there is still a discrepancy between the 1.3mg/ml calculated ELISA and the 1.91mg/ml OD₂₈₀ value which affects the final dose calculation. The historical data should be submitted to bolster using the OD₂₈₀ in lieu of the typical ELISA values.

Test Animals and Maintenance Young adult CD-1® (ICR) BR mice were received from Charles River Laboratories, Inc., Portage, MI. All animals were identified by metal ear tags and kept individually in suspended stainless steel wire cages with identifying cage cards. The animals were in a temperature controlled (69-71 °F) animal rooms with 12 hour light/12 hour dark cycles. The room was ventilated to produce 10-15 air changes per hour. The animals were provided Purina Mills certified Rodent Chow #5002 and reverse osmosis treated municipal water *ad libitum* except as noted for dosing. Five male and five female (nulliparous and nonpregnant) were selected at random from healthy stock for the test. No control, non-treated animals were included.

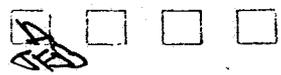
Test System On the day of dosing the test animals were weighed and fasted for 4 to 5 hours prior to dose administration. Just prior to dosing, the animals were reweighed then the test substance administered by oral gavage utilizing a ball tipped stainless steel gavage needle attached to a syringe. The dosing material was split and given to each animal in two doses within four hours at approximately 20ml/kg to insure that the amount of the Cry9C protein administered was greater than 5000mg/kg. The animals were observed three times after dosing on day 0 and twice daily thereafter until scheduled sacrifice on day 14. Animals were weighed on day 7 and 14. On day 14 the test animals were euthanized by inhalation of carbon dioxide and necropsied. The cranial, thoracic, pelvic and abdominal cavities were examined but no tissue samples were retained.

RESULTS AND DISCUSSION

There were no deaths in any test animals due to test material given above the limit dose of 5000mg/kg during the 14 day observation period. One male mouse displayed hair loss between days 2 and 5. One female displayed decreased activity on the day of dosing. Another female displayed decreased activity, wobbly gait, decreased feces and felt cool to the touch. A third female displayed decreased feces on day 1. All the male mice gained weight during the test period (except during the pre-dosing fast period). Two female mice failed to gain weight between day 0 (prefasted weight) and day 7 and three failed to gain weight between day 7 and 14. One female did not recover her pre-fasting body weight by day 14.

Since there were only ten animals in the study, no dose range for the test substance and no control animals with which to compare the treated animals, a clear determination on body weight gain effects is not possible. However, the weight loss in these animals did not appear significant and is similar to results seen before when large doses of protein were administered. Given the low amounts of Cry9C protein expressed in corn tissue (1.8 to 85µg Cry9C/mg soluble protein or 12 to 225µg Cry9C/gm fresh weight), there is little likelihood any adverse effects will be seen in dietary exposure.

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DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD
Secondary Reviewer: Doug Gurian-Sherman, Ph.D., Plant Pathologist, BPPD

STUDY TYPE: *In Vitro* Digestibility
 MRID NO: 442581-08
 CHEMICAL NO: 006466
 TEST MATERIAL: Cry9C δ -endotoxin of *Bacillus thuringiensis*
 STUDY NO: none assigned
 SPONSOR: Plant Genetic Systems (America) Inc., Des Moines, IA
 TEST FACILITY: Plant Genetic Systems N.V., Ghent, Belgium
 TITLE OF REPORT: *In Vitro* Digestibility and Heat Stability of the Endotoxin Cry9C of *Bacillus thuringiensis*
 AUTHOR: Marnix Peferoen
 STUDY COMPLETED: March 28, 1997
 CONCLUSION: The samples of lyophilized Cry9C protein expressed in corn showed no signs of protein disintegration when subjected to *in vitro* digestion in simulated mammalian gastric fluid. These digestions were done either with or without pepsin in the low pH buffer and were assayed by western blot from samples taken at several time points from the mixing the reagents to after 4 hours exposure to the digestive fluids. The same 70kD double band seen in the original Cry9C protein in plant tissue at time 0 was also seen, undiminished, in all the subsequent incubation samples. No effect on Cry9C activity as determined by bioassay was seen after any heat treatment. The most stringent heat treatment was 90°C for 10 minutes.

CLASSIFICATION: ACCEPTABLE.

DEC 21 1999

STUDY DESIGN

Purified Cry9C δ -endotoxin as expressed in corn was examined for stability to heat and *in vitro* digestion in simulated gastric fluid. This study has a GLP statement indicating non-compliance but no reasons were given.

TEST METHODS

Test Substance: Cry9C protein purified from *Bacillus thuringiensis* and as found in lyophilized corn tissue was used as the test substance in these studies. The CryIA(b) endotoxin purified from fermentation of an *E. coli* strain containing the CryIA(b) gene was also used as a reference substance for other δ -endotoxins. The methods to prepare these substances is discussed below.

PREPARATION OF CRY9C PROTEIN IN *Bacillus thuringiensis*

A *B. thuringiensis* strain cured of its indigenous plasmids and transformed with plasmid pGI9CK was grown in fermentation to yield a culture that was pelleted by centrifugation. Half the pellets

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were lyophilized and stored at -20°C, the other half stored at 4°C. When needed, the bacterial powder was solubilized in alkaline buffer (0.04M Na₂CO₃-HCl; 0.01M DDT; 5mM EDTA; 0.1mM PMSF; pH 10) preserved with 0.02% NaN₃ and stirred for 4 hours at 4°C. The solution is clarified by centrifugation (20 minutes at 12,000 rpm in a Sorvall SA-600) then dialyzed in multiple fraction against a buffer (20mM TRIS/HCl; 25mM NaCl; 5mM EDTA, pH8.6). The dialysis tubing was a spectra-por #4 with a cutoff of 12-14kDa. The multiple fraction samples were then diluted with the dialysis buffer to 0.5 mg protein/ml and treated with trypsin (0.05%w/v) for 1 hour at 37°C. The trypsin digest was stopped by the addition of PMSF to yield a 1mM final concentration. All the samples, still separated, were then pooled, mixed and subjected to an ammonium sulfate precipitation (final concentration of 3M or 77% saturation) overnight at 4°C. The protein was pelleted out by centrifugation (20 minutes at 12,000 rpm in a Sorvall SA-600), dissolved in 20mM ethanolamine/HCl (pH9.5) and a protein determination done. The solution was again dialyzed in multiple fractions against 20mM ethanolamine with 2 changes, then against 10mM ethanolamine (pH9.5) with 2 changes. Finally the samples are lyophilized and the protein concentration of the powder determined.

PREPARATION OF CRY9C POWDER FROM CORN PLANTS

Seeds, marked CBH-351 from a segregating population of transformed plants, were grown for six weeks, leaf samples removed and bioassayed for activity against European corn borer. Four weeks later, the same plants were assayed for Cry9c by an ELISA technique (SOP PGS-9D/1, not provided). In the presence of conflicting results between the two assays, that particular corn plant was not used for further processing. Ten week old plants were harvested, the leaves and stalks were cut into 10cm sections for lyophilization. Roots were also harvested, washed and cut into 2-5cm fragments. The individual plant samples were lyophilized, blended to reduce particle size, transferred to plastic bottles and stored in the dark at room temperature. Prior to use a 50mg sample of the plant powder was removed and the proteins extracted (SOP PGS-9H/0, not provided). The protein concentration was determined (Bradford Microassay SOP PGS-9E/0, not provided) and presence of Cry9C determined by ELISA (SOP PGS9D/1, not provided) and bioassay against European corn borer on artificial diet (SOP PGS-9A/1, not provided).

PREPARATION OF CRY1Ab PROTEIN FROM *Escherichia coli*

Cry1Ab protein was recovered from a recombinant strain of *E. coli* containing the Cry1Ab gene. The purification process for the protoxin and activation to toxin are described in a cited article (Lambert, B.; Buysse, L.; Decock, C.; Jansens, S.; Piens, C.; Saey, B.; Seurinck, J.; van Audenhove, K.; van Rie, J.; van Vliet, A. & Peferoen, M. (1996) *A Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family Noctuidae. *Applied and Environmental Microbiology* 62, 80-86).

Test System

In vitro DIGESTION

Samples of tissue containing Cry9C protein or purified Cry9C protein were dissolved in simulated gastric fluid with pepsin (0.32% pepsin in gastric buffer (0.5gm NaCl, 1.75ml of 1M HCl in 250ml water, pH2.0)) and incubated at 37°C. Samples were removed for western blot

analysis at 0, 5, 15, 60 and 240 minutes of incubation. No pepsin inhibitor was added prior to preparation for SDS-PAGE.

HEAT STABILITY

Samples containing Cry9C or Cry1Ab were dissolved in TRIS buffer (20mM TRIS/HCl, 25mM NaCl, 5mM EDTA, pH8.6) and heated for 10 minutes in a water bath at 70, 80 or 90°C. The samples were then tested for insecticidal activity in a European corn borer bioassay (SOP PGS-9A/1, not provided). Two studies were done to examine the effect of heat treatment. The first was done with 500µg/ml of Cry9C and the bioassay was done at a single dose of 1250ng/cm² diet surface. The second assay was done with both Cry9C and Cry1Ab at 50µg/ml and tested at either 100ng/cm² or 1250ng/cm² diet surface.

INSECT BIOASSAYS

Bioassays of endotoxin activity after the various treatments described above were done on individual European corn borer larva in 24 well reading plates supplied with artificial diet (SOP PGS-9A/1, not provided). Details of the assay were not provided here.

RESULTS AND DISCUSSION

The samples of lyophilized Cry9C protein expressed in corn showed no signs of protein disintegration when subjected to *in vitro* digestion in simulated gastric fluid. Reproductions of the gels are seen in an appendix to this review. These digestions were done either with or without pepsin in the low pH buffer and were assayed by western blot from samples taken at several time points from ~~the~~ mixing the reagents to after 4 hours exposure to the digestive fluids.

The same 70kD double band seen in the original Cry9C protein in plant tissue at time 0 was also seen, undiminished, in all the subsequent incubation samples. Interestingly, the three bands from microbially produced Cry9C (either *E. coli* or *B. thuringiensis*) were different from the plant source Cry9C. One band in both microbial preps comigrated with the 70 kD doublet. The addition of pepsin resulted in the appearance of some lower molecular weight bands but the 70 kD double band did not decrease in intensity as in the normal digestion pattern. Typically, evidence of digestion in a western blot is the gradual reduction or disappearance of the high molecular weight bands with increasing amounts of lower weight bands appearing and/or disappearing over time.

The effects of heat treatments on Cry9C protein were tracked by the changes seen in European corn borer bioassays. Two studies were done to examine the effect of heat treatment. The first was done with 500µg/ml of Cry9C and the bioassay was done at a single dose of 1250ng/cm² diet surface. The second assay was done with both Cry9C and Cry1Ab at 50µg/ml and tested at either 100ng/cm² or 1250ng/cm² diet surface. The results of both assays are found in the attached appendix. Basically there was no effect on Cry9C activity by any heat treatment. The second series of assays showed a decline of % mortality of 88 to 79 % with the temperature increase from 70° to 80° or 90°C. This slight increase represents two more individual larva succumbing and is probably not a significant finding. The Cry1Ab showed a decrease in activity with

increased heat from 42% mortality to essentially background at 80°C. The stated LC₅₀ values are 37.9 and 128.7 for Cry9C and Cry1Ab, respectively were apparently not done with these samples. No units are stated for these values but they may be the same ng/cm² as the test system.

The results of these studies indicate the trypsin activated fragment of Cry9C is both stable to gastric digestion and heat to 90°C. These are two characteristics that are frequently found in proteins that are known to be the cause of food allergies. It is important to note that there are probably numerous proteins in food plants that are both heat stable and resistant to gastric digestion. These two biochemical characteristics are not by themselves the sole indicators that they are likely to be food allergens.

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DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD
Secondary Reviewer: Doug Gurian-Sherman, Ph.D., Plant Pathologist, BPPD

(JLP)

STUDY TYPE: Amino Acid Homology Comparison
MRID NO: 442581-09
CHEMICAL NO: 006466
TEST MATERIAL: Truncated Cry9C δ -endotoxin
STUDY NO: none assigned
SPONSOR: Plant Genetic Systems (America) Inc., Ghent, Belgium
TITLE OF REPORT: Amino Acid Sequence Homology Search with the Corn Expressed Truncated Cry9C Protein Sequence

DEC 21 1997

AUTHOR: Susan C. MacIntosh
STUDY COMPLETED: March 18, 1997
CONCLUSION: Three hundred sequences were listed as having regions of homology with the 626 amino acids of the Cry9C truncated toxin protein. The first 64 proteins in the list were all parasporal proteins from *Bacillus thuringiensis* otherwise known as δ -endotoxins. Other δ -endotoxins were found at 67, 76, 78, 79 and 80. These proteins had regions of homology that gave a "significant homology". The table of values indicated a matching score above 4 standard deviations would contain all the δ -endotoxins mentioned above. The algorithm for converting the matches and penalties into homology scores was not described although it was stated that "all other proteins (besides the δ -endotoxins referred to above) have less than 20% exact sequence matching and no major stretches of sequence homology could be detected, indicating that in these cases the sequence homology is not significant."

CLASSIFICATION: SUPPLEMENTAL. BPPD has not yet determined how to judge amino acid sequence homology for risk assessment purposes. No further studies need to be done at this time for analyzing homology.

STUDY DESIGN

The amino acid sequence of the Cry9C endotoxin was compared to a data base of amino acid sequences looking specifically for significant homologies with known toxins or allergens. This study has a GLP non-compliance statement.

TEST METHODS

Test Substance The 626 amino acid sequence of the truncated version of the Cry9C δ -endotoxin as expressed in corn was used to search the data base for significant areas of sequence homology.

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Test System The system used was the Intelligenetics Fast DB software providing a fast pairwise comparison of sequences. The databases searched included all the sequence entries in the PIR, Swiss Prot and HIV AA bases. The parameters for the search were described as not stringent and based on exact matching although how these parameters are defined for the search were not described. It appears from the values presented that a positive match is given a value of +1. The homology determinations were corrected by giving a penalty value of -1 for each mismatch or gap. Gaps were also weighted for their size with an additional factor of (0.05 x gap size) being added to the penalty.

RESULTS AND DISCUSSION

There were 300 sequences listed as having regions of homology with the 626 amino acids of the Cry9C truncated toxin protein. The first 64 proteins in the list were all parasporal proteins from *Bacillus thuringiensis* otherwise known as δ -endotoxins. Other δ -endotoxins were found at 67, 76, 78, 79 and 80. These proteins had regions of homology that gave a "significant homology".

The table of values indicated a matching score above 4 standard deviations would contain all the δ -endotoxins mentioned above. The algorithm for converting the matches and penalties into homology scores was not described although it was stated that "all other proteins (besides the δ -endotoxins referred to above) have less than 20% exact sequence matching and no major stretches of sequence homology could be detected, indicating that in these cases the sequence homology is not significant."

Other parasporal proteins (δ -endotoxins) from *Bacillus thuringiensis* subspecies are found at 105 and 138 in the list. Other proteins listed within 5 standard deviations along with the first 64 δ -endotoxins and having regions of significant amino acid homology include a serine carboxypeptidase from rice; dynein, a cytosolic protein involved in chromosome movement and maintenance in *Aspergillus*; a transcription repair coupling factor from *Haemophilus*; a putative cystathionine β -lyase (EC 4.4.1.8) from *Saccharomyces*; a transmembrane protein from *Saccharomyces*; a virB4.1 protein from *Agrobacterium*; a glucoamylase S1/S2 precursor (EC3.2.1.3) from *Saccharomyces* and a transcription initiation protein SPT6 from *Saccharomyces*.

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DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD *JLK*
Secondary Reviewer: Doug Gurian-Sherman, Ph.D., Plant Pathologist, BPPD *DAS*

STUDY TYPE: Food Allergenicity- Amino Acid Sequence Homology

MRID NO: 443844-04

CHEMICAL NO: 006466- *Bacillus thuringiensis* subsp. *tolworthi* Cry9C toxin DEC 21 1999

TEST MATERIAL: Presumed amino acid sequence of Cry9C

STUDY NO: none assigned

SPONSOR: Plant Genetic Systems (America) Inc., Des Moines, IA

TEST FACILITY: Plant Genetic Systems NV, Ghent, Belgium

TITLE OF REPORT: Cry9C *Bacillus thuringiensis* Insecticidal Protein Identification of Sequence Homology with Allergenicity by Searching Protein Databanks

AUTHOR: Marnix Peferoen

STUDY COMPLETED: September 12, 1997

CONCLUSION: Sequence identity for any of the eight amino acid regions in Cry9C was found only to other Bt crystal proteins. No match between any 8 amino acid sequence in Cry9C and any of the allergenic proteins known in the SWISS protein database was found. This lack of homology at a finer level of examination is further evidence that Cry9C is not related to known allergens using a structural consensus approach.

CLASSIFICATION: SUPPLEMENTAL. BPPD has not yet determined how to judge amino acid homology for risk assessment purposes. No further studies need to be done at this time for analyzing homology.

STUDY DESIGN A homology comparison was done utilizing a sequential series of 8 amino acids of the Cry9C protein against the sequences from known proteins to assess the similarity of any given 8 amino acids in Cry9c to known allergens. No laboratory work is included and the study is not considered applicable to GLP standards.

TEST METHODS

Test Substance: DNA sequence of Cry9C analyzed as a stepwise series of overlapping 8 amino acids.

Test System: The FASTDB software from European Bio-informatics Institute was used to screen the SWISS protein databank.

RESULTS AND DISCUSSION

Similar to the previous homology analysis, sequence identity for any of the eight amino acid regions in Cry9C was found only to other Bt crystal proteins. No match between any 8 amino acid sequence in Cry9C and any of the allergenic proteins known in the SWISS protein database was found. This lack of homology at a finer level of examination is further evidence that Cry9C is not related to known allergens using a structural consensus approach.

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23/APP # PF-867

APP

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD
Secondary Reviewer: Doug Gurian-Sherman, Ph.D., Plant Pathologist, BPPD

STUDY TYPE: Food Allergenicity- Sera Reactivity

MRID NO: 443844-05

CHEMICAL NO: 006466- *Bacillus thuringiensis* subsp. *tolworthi* Cry9C toxin

TEST MATERIAL: Wild type and CBH-351 corn kernels expressing Cry9C

STUDY NO: AgrEvo Study Number Tox-97002

SPONSOR: Plant Genetic Systems (America) Inc., Des Moines, IA

TEST FACILITY: Tulane University School of Medicine, New Orleans, LA

TITLE OF REPORT: Investigation of Allergens in Wild-Type and Transgenic Corn

AUTHOR: Samuel Lehrer, Ph.D.

STUDY COMPLETED: August 19, 1997

CONCLUSION: The 21 sera samples from suspected corn-sensitive individuals all tested positive in the RAST assay by having $\geq 3\%$ reactivity. The transgenic and wild-type aqueous corn extracts were not obviously different in responsiveness for individuals and a t-test of the RAST % reactivity did not reveal any significant differences. The RAST inhibition assay gave results indicating that both wild type and transgenic corn extracts gave substantial inhibition of the wild type corn RAST. Statistical analysis of the inhibition curves generated for RAST inhibition from wild type versus transgenic corn extracts did not indicate significantly different 50% inhibition values, slopes or y-intercepts. The type of extract, aqueous or alcoholic, utilized in the inhibition assays was never specified. Both the wild type and transgenic aqueous corn extracts gave higher levels of reactivity in the immunoblot assay than the alcoholic extracts. A comparison of the IgE reactions for specific corn atopic individuals indicated that there were similar reactive banding patterns in both transgenic and wild type corn. In some individuals there were a greater number of reactive bands ranging in molecular weight whereas in others there were only one or two bands, generally of lower molecular weight, which had very significant staining. There was no identification of individuals in the SDS-PAGE lanes so no correlation between the intensity of the % reactivity in RAST and the number or intensity of staining in the immunoblot assay could be made. A two-fold dilution series with a pool of 10 RAST positive corn atopic sera was tested against the wild type and transgenic corn extracts. The pattern of reactivity was very similar between the transgenic and wild type extracts with the intensity of the reaction again being higher for the aqueous versus alcoholic extracts. There were some unique bands present in either the wild

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type or transgenic extracts but since these bands did not show detectable effects on the serum reactivity kinetics in the RAST or RAST inhibition assays it is difficult to judge the importance of their presence.

CLASSIFICATION:

SUPPLEMENTAL. This study does not address the potential for inducing food allergy from a novel protein lacking a history of dietary exposure. An additional control testing purified Cry9C protein against corn atopic sera should have been included to establish the negative reactivity background. The study does establish a baseline of corn allergen reactivity for subsequent comparisons if such an allergic response does occur over time.

STUDY DESIGN

A comparison of the reactivity of corn sensitive individuals' serum to extracts of Cry9C containing corn hybrids and their "wild-type" corn ancestors was done to establish if an alteration in the corn reactivity had occurred. This study was not done according to GLP standards.

TEST METHODS

Test Substance: Extract of Cry9C and wild-type corn kernels was done by grinding 40 gm of corn kernels to a fine meal texture and extracting the meal in two solutions. The first extraction was done by adding a 4°C phosphate buffered saline solution (0.5M NaCl, 10µM EDTA, pH 7.8), extracting the meal for 1 hour with stirring followed by a 20,000g centrifugation and collecting the aqueous supernatant which was stored at -20°C. The pellet was subsequently extracted with 4°C 55% isopropanol containing 0.5% 2-mercaptoethanol, 0.5% sodium acetate for 1 hour with stirring. The slurry was centrifuged at 13,000 g with the alcoholic supernatant retained and stored at -20°C. The dry weight yields of these extracts was determined to be 3.0 to 3.7% for the aqueous extracts and 2.0 to 2.7% for the alcoholic extracts.

Test Sera: Sera from 21 purported corn sensitive individuals were collected from three sera banks with cooperating allergists: Tulane University (3), Kaiser-Permanente, Los Angeles, CA (8) and Plasma Lab International, Everett, WA (10). The corn allergy of the individuals was determined by having any two of the following: a history of corn allergy or food allergy compatible with corn reactivity; a positive skin prick test to corn extract and/or a positive Radio Allergo Sorbent Test (RAST) that was at least three fold greater than negative control sera. No sera from people who have been confirmed by the positive double-blind placebo controlled food challenge is available at this time. Control sera was obtained from five non-atopic individuals who can tolerate corn.

Test System: The sera were confirmed by RAST using aqueous extracts bound to paper discs. The coupling to the Whatman #50 paper discs was done by cyanogen bromide activation using 1mg dry weight of aqueous corn extract. Twenty-five µl of serum was diluted with 75µl of 0.001M PBS (pH 7.2), added to the discs, incubated overnight at room temperature and washed three times with 0.9% saline to remove unbound serum. The discs were reacted with equine anti-

human IgE labelled with I^{125} (15,000 cpm; Sanofi Diagnostics Pasteur, Inc.) and washed three times with 0.9% saline to remove unbound equine reagent. The discs were then read in a Beckman 5500 gamma counter to determine bound I^{125} . The assays were performed in duplicate and expressed as a percent of the added I^{125} with a value of $\geq 3\%$ considered positive.

Another RAST format was used to judge the response utilizing a pooled sample of 17 RAST positive sera. These sera were tested against the bound antigen in a RAST inhibition format wherein the pooled sera are exposed to both a RAST disc with corn allergens and a 50 μ l sample of corn allergen solution. The corn allergen solution consisted of either wild type or transgenic corn allergen extract as described above in a dilution series (0.1 μ g to 1mg in 10-fold dilutions). The experiment was repeated three times. The results of the inhibition assay were analyzed by linear regression after a logit-log transformation to determine if there was a difference in the response between the two extracts.

The extracts were also assayed by an immunoblot format whereby the constituent proteins of the corn extracts were separated by SDS-PAGE, blotted and bound to a cyanogen activated nitrocellulose membrane (0.45 μ m pore size, BAS85, Schleicher & Schüll), then reacted with sera. The SDS-PAGE was a typical 6cm high 12.5% separating gel. The samples were denatured by boiling for 5 minutes prior to loading onto the gel. The SDS-PAGE gels were transferred by semi-dry blotting. For detection the blots were reacted overnight with either individual sera or a pooled sample of 10 RAST positive sera. The sera were diluted 1:2 with TBS-Tween (100mM Tris-HCl; 100mM NaCl; 2.5mM $MgCl_2$; 0.2% w/v NaN_3 ; 0.05% v/v Tween 20, pH 7.4). This same buffer solution was used for dilution and washing steps throughout the immunoblot tests. After reaction with the sera and washing, the binding was visualized by the binding of a 1:1,000 dilution of an alkaline phosphatase conjugated anti-human IgE monoclonal antibody (Southern Biotechnology Associates, Birmingham, AL). The blot was then washed with freshly made assay buffer (100mM diethanolamine/HCl; 1.0mM $MgCl_2$, pH 10.0), reacted for five minutes with a 1:20 dilution of Nitroblock® chemiluminescence enhancer (Tropix, Bedford, MA), washed with buffer and then incubated for 5 minutes with 250 μ M CSPD (disodium 3-(4-methoxyspiro{dioxetane-3,2'-(5'-chloro)tricyclo{3.3.1.1.^{3,7}}decan}-4-yl)phenyl phosphate; Tropix, Bedford, MA). Excessive liquid was drained and the strips were exposed to autoradiography film for 15, 30, 60 or 120 seconds.

The strips were also visually assessed, after washing with TBS-Tween and TSB-AP (100mM Tris/HCl; 100mM NaCl, 5mM $MgCl_2$, pH 9.5), by a colorimetric assay employing 450 μ M BCIP (5-bromo-4-chloro-indolyl-phosphate disodium salt, SIGMA, St. Louis, MO) and 400 μ M NBT (nitroblue tetrazolium chloride, SIGMA, St. Louis, MO) dissolved in TBS-AP and incubated at 37°C.

RESULTS AND DISCUSSION

The 21 sera samples from suspected corn-sensitive individuals all tested positive in the RAST assay by having $\geq 3\%$ reactivity. The transgenic and wild-type aqueous corn extracts were not obviously different in responsiveness for individuals and a t-test of the RAST % reactivity did

not reveal any significant differences. However, the % reactivity ranged from 3.91 & 4.40% to 39.39% & 39.90% for individuals against wild type and transgenic corn extracts, respectively. Ten of the sera had above 20% reactivity, a level considered substantial sensitivity.

The RAST inhibition assay was run three times and gave results indicating that both wild type and transgenic corn extracts gave substantial inhibition of the wild type corn RAST. Statistical analysis of the inhibition curves generated for RAST inhibition from wild type versus transgenic corn extracts did not indicate significantly different 50% inhibition values or y-intercepts. The first two analyses indicated that there was a significantly different slope between the transgenic and wild type corn extracts. However, a repeat RAST inhibition assay including four replicates for each data point rather than two used for the first two assays indicated that the slopes were not significantly different between the extracts. The type of extract, aqueous or alcoholic, utilized in the inhibition assays was never specified.

Both the wild type and transgenic aqueous corn extracts gave higher levels of reactivity in the immunoblot assay than the alcoholic extracts. A comparison of the IgE reactions for specific corn atopic individuals indicated that there were similar reactive banding patterns in both transgenic and wild type corn. In some individuals there were a greater number of reactive bands ranging in molecular weight whereas in others there were only one or two bands, generally of lower molecular weight, which had very significant staining. There was no identification of individuals in the SDS-PAGE lanes so no correlation between the intensity of the % reactivity in RAST and the number or intensity of staining in the immunoblot assay could be made.

A two-fold dilution series with a pool of 10 RAST positive corn atopic sera was tested against the wild type and transgenic corn extracts. The pattern of reactivity was very similar between the transgenic and wild type extracts with the intensity of the reaction again being higher for the aqueous versus alcoholic extracts. There were some unique bands present in either the wild type or transgenic extracts but since these bands did not show detectable effects on the serum reactivity kinetics in the RAST or RAST inhibition assays it is difficult to judge the importance of their presence. Overall, this study does not address the potential for inducing food allergy from a novel protein lacking a wide history of dietary exposure. The study does establish a baseline of corn allergen reactivity for subsequent comparisons if such an allergic response does occur over time.